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Higher levels of CO_2 during late incubation alter the hatch time of chicken embryos

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Abstract

1. It has been reported that the increasing CO_2 tension triggers the embryo to pip the air cell and emerge from the egg. However, the mechanism by which higher CO_2 concentrations during the last few days of incubation affect chick physiology and the hatching process is unclear. This study investigates the effect of CO_2 concentrations up to 1% during pipping, on the onset and length of the hatch window and chick quality.
2. Four batches of Ross 308 broiler eggs (600 eggs per batch) were incubated in two small scale custom built incubators (Petersime NV). During the final three days of incubation, control eggs were exposed to a lower CO_2 concentration (0.3%), while the test eggs experienced a higher CO_2 concentration program (peak of 1%).
3. There were no significant differences found in blood values, select organ weight and body weight. There was also no difference in hatchability between control and test groups. However, a small increase in the chick weight and the percentage of first class chicks was found in the test groups. Furthermore, plasma corticosterone profiles during hatching were altered in embryos exposed to higher CO_2, however they dropped to normal levels at day 21. Importantly, the hatching process was delayed and synchronised in the test group, resulting in a narrowed hatch window (HW) which was 2.7 hours shorter and 5.3 hours later than the control group.
4. These results showed that exposing chicks to 1% CO_2 concentration during pipping did not have negative impact on physiological status of newly hatched chicks. In addition, it may have significant impact on the physiological mechanisms of controlling hatching and have benefits for health and welfare of chickens by reducing the waiting time after hatching.

Introduction

There is a large variation in eggshell conductance within a batch of chicken eggs, resulting in a large variation in gas exchange, and this creates differences in hatching time which can be further increased by differences in storage time, egg size, breeder flock age, and incubation conditions. The variation in hatching time within a batch of eggs is expressed as the hatch window. An elongated spread of hatch window results in poor uniformity within the batch of chicks and impairs post-hatch growth (Careghi et al., 2005; van de Ven et al., 2009; Willemsen et al., 2010). A number of events are known to be required to initiate the hatching process in chickens. One of these is a change in the levels of O_2 and CO_2.With increasing metabolism and limited conductance of the eggshell (Hamidu et al., 2007), in a natural nest the CO_2 level increases from 0.05 to 0.90% (Boutilier et al., 1977; Buys et al., 1998),while the O_2 concentration declines from 20.9 to 20.3% (Walsberg, 1980). In the air cell of the egg, the O_2 concentration decreases to approximately 14.2%, and CO_2 concentration increases to 5.6% (Visschedijk, 1968). At pipping, the embryo adopts convective gas exchange by the lungs with subsequent progress towards hatching (Khandoker et al., 2003; Tazawa et al., 1983). In artificial incubation, 0.30 % of CO_2 is widely used throughout incubation. Higher CO_2 profiles from the time of internal pipping are sometimes used by industry to delay and narrow the hatch window (Tona et al., 2013). However, the alteration of CO_2 levels during late incubation has not been well investigated in relation to hatching. Furthermore, it is questionable how it affects hatchability and chick quality. Despite the tolerance of embryos for high ambient levels of CO_2 increasing with embryonic age (Molenaar et al., 2010), higher ambient CO_2 levels could still exert stress on the embryo and represent a hazard for respiration gas transport, acid-base balance and overall physiological status of the newly hatched chicks. However, the effect of altered CO_2 concentrations compared to normal ($\leq 0.3\%$) during the late stages of incubation when conductive gas exchange is established on chick quality and subsequent performance is unclear. The aim of this

study was to investigate the effects of late exposure to higher CO_2 (up to 1%) on the physiological stats of chicks during the final days of incubation. Hatch window, hatchability, chick score, body weight, organ weight, blood parameters and plasma corticosterone levels were compared between the higher CO_2 group and control group to identify possible effects of higher CO_2 on timing of hatching and chick quality.

MATERIALS AND METHODS

Experimental design

Four batches of fertilised Ross 308 eggs (600 eggs per batch) were obtained from a local supplier (Henry Stewart & Co. Ltd, Lincolnshire, UK). The eggs were weighed, numbered and randomly placed in two small custom-built "BioStreamer" incubators (Petersime NV, Zulte, Belgium). Each incubator was able to set 300 eggs in 2 trays.

Incubation conditions (machine temperature, humidity, CO_2 concentration and ventilation rate) were continuously monitored and controlled by the incubator controller (BIO-IRIS, PetersimeTM). The patterns of CO_2 levels in the control incubator and test incubator were programmed and achieved by adjusting ventilation. Two incubators were swapped for control group and test group. All parameters were identical in the two groups up to day 18. From day 18, the test group experienced higher CO_2 levels, up to 1% at day 19. In the control group, CO_2 concentration was maintained at 0.3%. The internal pipping (IP) and Hatch were detected and recorded by the incubator controller (Petersime BIO-IRIS^{TM}) which indicates the start and the end of hatching process. Hatch window (HW) in this study is defined as the duration between IP and Hatch.

All eggs were candled at day 18 and those with evidence of a living embryo were transferred from the turning trays to hatching baskets. Both machines were stopped after 512h (21 days and 8 hours) of

incubation and chicks were scored for quality using a standard method (Tona et al., 2003) at take-off. This method assessed chick quality based on several physical conditions within a total scale of 100 according to their importance (activity, feather, eye, leg, comb, navel area and remaining yolk). Chicks with full score (100%) are first class chicks. Hatchability (the percentage of fertile eggs that hatch) was determined based on breakout results.

Chick and physiological parameters

Samples from four incubation stages were collected to investigate the impact of higher CO_2 on blood values and plasma corticosterone: embryos at 18 days and 6 hours of incubation (plasma samples at day 18 was only collected for hormone measure), external pipping embryos (EP) at day 19 from the test group when CO_2 reached 1% and from the control group at the same time, newly hatched chick (H0) at day 20 when chicks just emerge from the shell, and chicks (d21) from both groups at take-off . Eggs or chicks were randomly collected through a porthole fitted at the side of the incubator without interrupting the incubation conditions. Chicks were euthanised through cervical dislocation. Relative heart weight (RHW), relative liver weight (RLW) and relative stomach weight (RSW) were calculated by dividing organ weight by chick weight.

Arterialised blood was collected from allantoic veins of embryos and the left ventricle of chicks using heparin coated syringes. 200ul whole blood was immediately analysed (epoc Portable Blood Gas Electrolyte and Critical Care Analyser, Woodley Equipment Company Ltd, UK) to get the blood values including pH, partial pressure of carbon dioxide (pCO2; mmHg), bicarbonate (HCO3; mmol/L), total carbon dioxide (TCO2; mmol/L), sodium (Na; mmol/L), potassium (K; mmol/L), ionized calcium (iCa; mmol/L), glucose (Glu; mmol/L), lactate (Lac; mmol/L), hematocrit (Hct; %) and hemoglobin (Hb; g/dl). The remaining blood was centrifuged at 3,000 rpm for 10 min. The plasma was decanted into 1.5 ml tubes and frozen at -20°C for corticosterone (CORT) analysis. Plasma CORT was measured using a

commercially available double antibody RIA-kit (IDS Ltd, Boldon, England) (Tona et al., 2007). Animal experiments were performed with ethics approval from the Royal Veterinary College Animal Ethics Committee.

Statistical analysis

Data was analysed using SPSS (PASW statistics 20) and presented as means ± standard error of the mean (SEM). A linear mixed model was used to analyse the effect of CO_2 treatments (control and test) on hatchability, HW and chick quality:

$Y=\mu + CO_2$ treatment +incubator +batch + ε

Second linear mixed model was used to analyse the effect of CO_2 treatments and incubation stage (d18, EP, H0 and d21) on embryonic parameters, blood values and corticosterone concentrations. The model was: $Y=\mu + CO_2$ treatment + incubation stage + interaction (treatment ×incubation stage) +incubator +batch + ε

CO_2 treatment, incubation stage, interaction, incubator were fixed effects; batch was random effect. The interaction was removed from the original model when it is not significant. When the effect of incubation stage was statistically different ($p<0.05$), the means were further compared using Least Significant Difference (LSD) test.

RESULTS

Hatch performance

Hatchability, chick scores, the time of IP and hatch window were analysed. Mixed effects model showed that there was no significant effect from incubator. No difference in overall hatchability and chick scores were observed between control group and test group, however the percentage of first class chicks was 3.05% higher in test groups than the control (Figure 1), but not statistically significant. The test group had a delayed IP of 5.3 hours compared to control groups. Furthermore, the duration between IP and H was influenced by the CO_2 concentration in the hatcher. On average, the test group had a HW that was 2.7 hours shorter than the control group (Table 1).

Chick and organ weight

There were no effects of incubator and batch on embryo and chick weights from EP to day21. Moreover, at any incubation time there was no difference in absolute and relative heart weight, liver weight and stomach weight between control group and test group (Table 2). However chicks in the test group were, on average, heavier than the control chicks at d 21, but not significant.

Blood values

There were no significant effects of CO_2 treatment, incubator and batch on blood values. However, differences of gas partial pressures (pCO_2) and acid-base status (pH and HCO_3^-) during the final three days of incubation were observed and present in Figure 2. In the test group of embryos, the levels of pCO_2 increased slightly between EP and H0 before returning to the baseline level of approx 25mmHg at d21. In contrast the control group of embryos did not experience this increase, rather maintaining a constant pCO_2 throughout. Chicks hatched under higher CO_2 incubation had slightly higher pCO_2 at H0 compared to control chicks.

Blood pH maintained around 7.5 and decreased slightly from EP until day 21. A consistently higher trend of HCO_3^- concentrations were observed in the test group chicks blood throughout. HCO_3^- concentrations between H0 and d21 was significantly different in both test group and control group ($P<0.05$). Additionally, no effect of CO_2 treatments on other blood values were found between control and test group (Na+, K+, Ca++, Glu, Lac, Hct, Hgb and TCO_2; data was not shown).

Plasma corticosterone concentration

Chick plasma CORT levels were analysed and there were no effect of CO_2 treatment, batch and incubator. However, some changes in CORT profile from day 18 to day 21 of incubation time between the control group and the test group were found (Figure 3). The CORT levels increased significantly from about 5.0ng/ml at day 18 to about 10.0 ng/ml at EP which doubled when embryos started pipping in both control and test groups ($P<0.01$). In control chicks, plasma CORT dropped to a lower level at H0 and then increased again at day 21. However, a different pattern of changes was observed in the test group, with an increase at H0 before dropping to become equal to that seen in controls.

158 **DISCUSSION**

159 Physiological parameters and endocrine (thyroid hormones and corticosteroids) are known to undergo
160 dramatic changes during the last developmental days and some of these changes have been causally
161 linked with the transition from chorioallantoic to lung ventilation, piping and hatching process. Impaired
162 respiratory function results in CO_2 retention in the body leading to an elevated body fluid pCO_2 and
163 resulting in respiratory acidosis or primary hypercapnia (Boutilier et al., 1977; Ferner & Mortola, 2009).
164 It has been shown in previous studies (Bruggeman et al., 2007; Buys et al., 1998; Everaert et al., 2008)
165 that air cell and blood gas pressures are altered by exposing embryos to high CO_2 during the first and
166 second weeks of incubation. In general, environmental hypercapnia results in an increased blood pCO_2,
167 blood pH and HCO_3^- concentration in avian embryos (Bruggeman et al., 2007; Everaert et al., 2008;
168 Everaert et al., 2011).However, the significant increase of pCO_2 by higher environmental CO_2
169 concentrations were not found in this study. Tazawa et al (1983) has reported that during normal chicken
170 embryonic development arterialised blood pCO_2 reach a maximum value of about 40mmHg at internal
171 pipping before falling to about 25 mmHg and kept steady during pipping and hatching; our study
172 confirms that blood pCO2 maintained at a stable level of 25mmHg from EP to day 21 of incubation.
173 Moreover, our results show numerically higher pH and HCO_3^- concentration when CO_2 level increased
174 to 1%. The respiratory compensatory response for hypercapnia is a rise in the bicarbonate level along
175 with the increased arterial pCO2 to return the pH towards normal. But the blunted chemosensitity was
176 only occurred by adult that was experienced prenatal high levels of CO2. Ventilatory chemosensitivity
177 and thermogenesis of the chicken hatchling after embryonic hypercapnia.
178
179 However, blood pH and HCO_3^- were between 7.5-7.65 and 19.5-26 mmol/L which are in the normal
180 range of chicken embryo according to previous studies (Everaert et al., 2011; Tazawa et al., 1983) which

reflects the regulatory capacities of chick embryos to cope with ambient hypercapnia. This is probably due to the tolerance of embryos for ambient high CO_2 increase with embryonic age (Molenaar et al., 2010); another reason could be the sampling time which was from external pipping when chicks had already accessed to air. The other blood parameters were similar in newly hatched chicks between the control group and the test group. This indicates that the embryo can cope with up to 1% environmental CO_2 at pipping and hatching without affecting the acid-base balance.

It has been reported that a general stimulation of the hypothalamo – hypophyseal axis seems to occur in preparation for the hatching process and CORT is a critical hormone which is involved in hatching process (Decuypere et al., 1991). The CORT values measured at day18 and external pippping show a dramatic increase in both groups. It is consistent with previous research which reported that plasma CORT remains relatively stable through day 18 with a significant increasing trend and reaching a peak that occurs at the onset and during hatching and declines again after hatch (Kalliecharan & Hall, 1974, 1976; Scott et al., 1981). This may be due to the initiation of pipping and the shift from chorioallantoic to pulmonary gas exchange. Furthermore, an altered profile of chick plasma CORT in the external pipping and newly hatched chicks, accompanied by changes in the hatching process which were seen in chicks exposed to the CO_2 concentrations up to 1% during the final days of incubation. In the newly hatch chicks, CORT decreased in the control groups while increased continuously in the test groups which might be triggered by higher ambient CO_2 concentrations. However, in test groups CORT levels dropped so that they align with controls at day21. This profile may explain the shift in timing of hatch and the shorter hatch window. The peaks of CORT concentration and the onset of hatching occurred between day 19 to 20 in control groups and at or after day 20 in test groups. Therefore, the majority of chicks from control groups were hatched earlier and had lighter body weight at take-off than the test

groups. This is accordance with the results of previous studies. To achieve a delay and short hatch window, increasing CO_2 during incubation can stimulate corticosterone secretion (Blacker et al., 2004). In commercial practice, the CO_2 concentration is sometimes increased to 2% at the onset of pipping to stimulate the chickens to hatch (French, 2010). Exposure to CO_2 concentrations up to 1% from the onset of pipping had a similar affect across the whole batch of embryos. It might accelerate the embryos emerging from the shell in order to get enough oxygen thus narrow the hatch window of the test cohort. Critically, the hatch window was narrowed without significantly affecting embryo development, the majority of physiological parameters, hatchability and quality of newly hatched chicks.

The CO_2 and CORT levels in incubating eggs may be manifestations of these changes culminating in altered hatching parameters; and consequently, differences in chick quality and growth potentials. This study demonstrated that incubation under higher CO_2 concentrations up to 1% during pipping and hatch did not affect blood physiological parameters and quality of newly hatched chicks, but may be beneficial in terms of hatching synchronicity when compared to normal CO_2 levels (0.3%). However, it cannot exclude that embryonic hypercapnia altered some structural components of the respiratory pump, as it can happen with hypercapnia in the postnatal period (Rezzonico et al., 1990), limiting muscle force, respiratory compliance or airway conductance. A delay of the normal developmental process or a long-lasting and permanent condition cannot be answered by the current data. Therefore, the precise mechanisms that connect environmental CO_2, hatching and epigenetic effects warrant further investigation.

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296 **Table 1.** *Data of IP and HW of four CO_2 experiments*

Group	IP[a]	HW (h)
Control	465.0±0.7	28.5±1.8
Test	470.3±3.0	25.8±1.3
P-value	0.18	0.27

297 [a] *hours of incubation time*

298 **Table 2.** *Embryo, chick and organ weight (g) and relative heart, liver and stomach weight (% of*

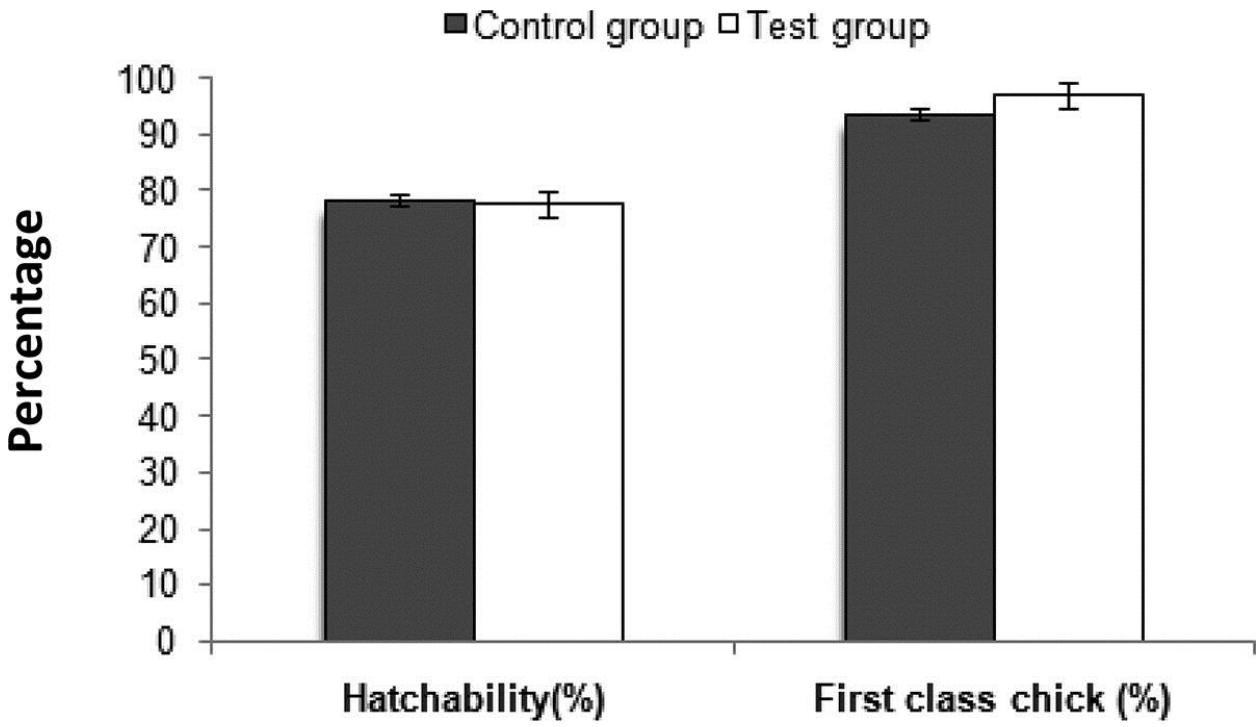
299 *embryo or chick weight) from EP to day 21*

Incubation stages	Group	Chick weigh (g)	Heart weight (g)	Relative heart weight (%)	Liver weight (g)	Relative liver weight (%)	Stomach weight (g)	Relative stomach weight (%)
EP	Control	43.04±0.95	0.30±0.01	0.70±0.03	0.72±0.02	1.69±0.07	2.22±0.09	5.17±0.19
	Test	42.23±0.73	0.29±0.01	0.68±0.02	0.72±0.03	1.69±0.06	2.22±0.07	5.27±0.17
H0	Control	42.18±0.83	0.36±0.01	0.86±0.03	0.88±0.02	2.09±0.07	2.59±0.06	6.19±0.24
	Test	42.16±1.15	0.34±0.01	0.81±0.03	0.91±0.02	2.19±0.07	2.44±0.07	5.80±0.11
d21	Control	40.63±0.76	0.35±0.01	0.88±0.02	0.95±0.02	2.35±0.05	2.63±0.06	6.48±0.15
	Test	41.76±0.86	0.37±0.01	0.88±0.03	0.94±0.03	2.26±0.08	2.61±0.08	6.28±0.17

300 Data are presented as mean ± SEM (n= 13 to 23); EP, external pipping; H0, newly hatched

301 chick; d21, the end of incubation

Figure 1. *Mean and standard error (SE) of hatchability and first class chicks in the control and test groups.*



306 **Figure 2.** *The arterialised blood pCO_2, pH and bicarbonate concentration (HCO_3^-) in control*
307 *group and test group. Mean values ± SEM (indicated by bars, n=7 to 17 per group per day); the*
308 *asterisks indicate that the difference between adjacent two mean values is significant at*
309 *P<0.05(*); EP, external pipping chick at day 19; H0, newly hatched chick at day 20; d21, chick*
310 *at take-off.*

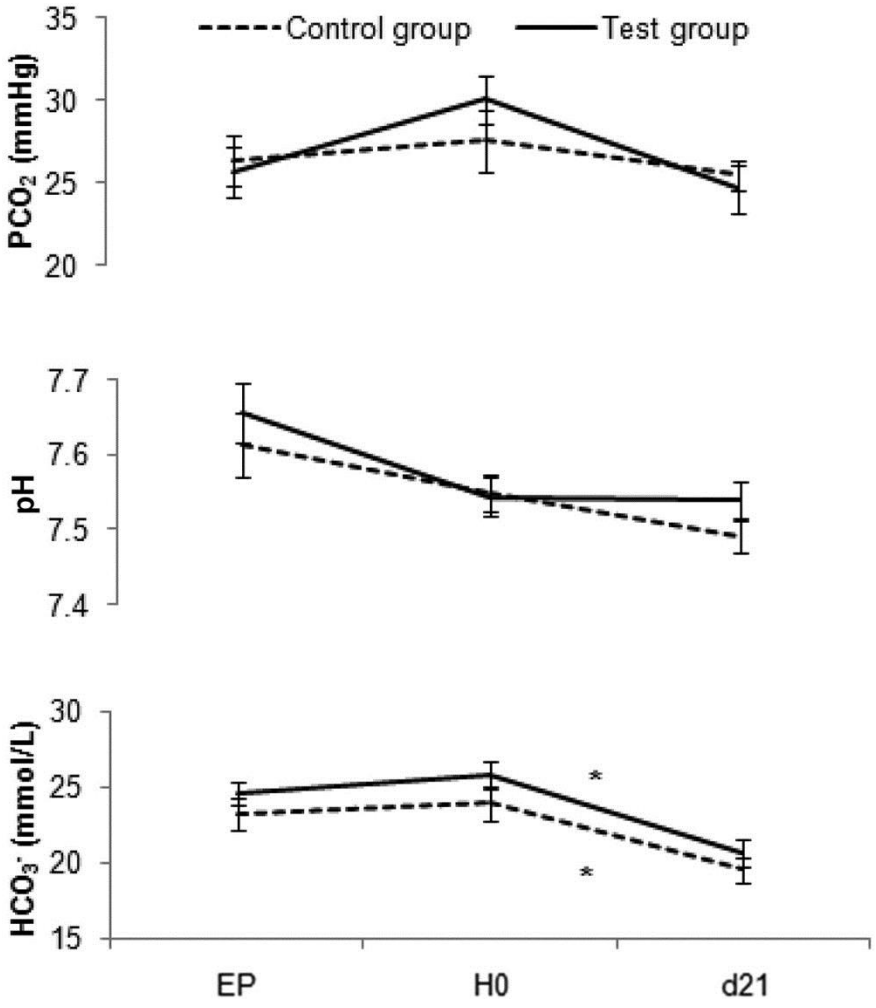


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Figure 3. *Plasma corticosterone levels from developing chick embryo during the late stage of incubation. Mean values ± SEM (indicated by bars, n=12 to 22 per group per day); the asterisks indicate that the difference between adjacent two mean values is significant at P<0.01(**); d18, chick at day 18 of incubation time; EP, external pipping chick at day 19; H0, newly hatched chick at day 20; d21, chick at take-off.*