

# Effect of increased CO<sub>2</sub> in the second half of incubation on embryonic growth and on the hatching process

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In this study, the effect of increasing CO<sub>2</sub> concentrations from embryonic day (ED) 10 onwards up to 4% from ED12 till ED 18 was investigated on embryonic development, the hatching process and the acid-base balance in embryonic blood. Embryonic growth was not influenced by high CO<sub>2</sub> in the incubator, compared to the control group. 4% of CO<sub>2</sub> was not detrimental for the hatching process (hatching hour, hatching percentage). During normal development, embryos developed respiratory acidosis. During the first days of increased CO<sub>2</sub>, a higher pCO<sub>2</sub> and lower pO<sub>2</sub> (only in first experiment) was observed in the air cell of the CO<sub>2</sub> incubated group. Blood parameters of the CO<sub>2</sub> group showed a higher pCO<sub>2</sub>, lower pO<sub>2</sub> (only in the first experiment), higher HCO<sub>3</sub><sup>-</sup> and a higher pH. This difference disappeared towards ED18. We hypothesize that the embryo reacts on the high CO<sub>2</sub> by adaptation of its renal function and/or by a higher release of calcium carbonate of the shell, which might result in overcompensation to buffer the pH.

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**Keywords:** incubation; carbon dioxide; embryonic development; acid-base balance

## Introduction

High CO<sub>2</sub> levels (>8%; Taylor and Kreutziger, 1969) are shown to be detrimental for the development and hatching process of the chick embryo although certain threshold levels are needed to stimulate the hatching process (Visschedijk, 1968a; Buys *et al.*, 1998; Hassanzadeh *et al.*, 2002). However, there is little information on the physiological effects induced by these higher CO<sub>2</sub> levels during incubation. In this study, the effect of gradually increasing CO<sub>2</sub> concentrations from embryonic day (ED) 10 onwards up to 4% from ED12 until ED18 were investigated on embryonic growth and the hatching process. In addition, much emphasis was put on how these environmental higher CO<sub>2</sub> concentrations could affect the acid-base system in the blood of the embryo since it is known that CO<sub>2</sub> can affect this system during adult phases by inducing respiratory acidosis.

## Materials and methods

### Incubation and experimental design

Two experiments were conducted using Cobb eggs from flocks (different flock per experiment) of 39 weeks old. Before the start of incubation, all eggs were weighed and numbered. During the first ten days, a normal incubation (T 37,6°C, wet bulb temperature of 29°C, turning of 90°/h) (incubator Pasreform, Zeddam, the Netherlands), was performed. On ED10, the experimental group was put in a closed incubator to allow CO<sub>2</sub> to gradually rise till 2% CO<sub>2</sub> on the 11<sup>th</sup> day. CO<sub>2</sub> levels continued to rise to reach 4% at ED12 and this high CO<sub>2</sub> level was continued till day 18. The incubation of the control eggs was done in the normal ventilated incubator. The humidity in both incubators was matched in order to prevent differences in egg weight loss. On the 18<sup>th</sup> day, eggs were transferred to hatching baskets under normal ventilation.

In experiment 1, 450 Cobb eggs were set under normal incubation conditions. On the tenth incubation day, 300 eggs were put in a closed incubator where CO<sub>2</sub> was risen gradually till 4% reached by the 12<sup>th</sup> incubation day. 150 eggs served as control eggs and had continuously a normal incubation. Gas pressure samples from the air cell and embryo weight of 12 eggs was taken on the 11<sup>th</sup>, 12<sup>th</sup>, 13<sup>th</sup>, 14<sup>th</sup>, 18<sup>th</sup> incubation day and at internal pipping (IP) stage. Blood samples from the chorioallantois membrane (CAM) were collected from those eggs on the 12<sup>th</sup>, 13<sup>th</sup> and 14<sup>th</sup> day.

In experiment 2, 750 Cobb eggs were incubated under normal conditions during the first ten days. On the tenth day all eggs were equally divided into two groups: the experimental and the control group. Gases, blood and embryo samples of 15 living embryos were taken daily from the tenth day till the 18<sup>th</sup> day and on IP, external pipping (EP) and hatch stage.

#### Sampling

On several days, samples of eggs from living embryos were taken from the experimental and control group. Eggs were weighed to calculate egg weight loss. The gas partial pressures (pO<sub>2</sub> and pCO<sub>2</sub>) in the air cell were measured by using a blood gas analyzer (Synthesis 10, Instrumentation Laboratories, Lexington). Blood was taken from an O<sub>2</sub>-rich capillary from the CAM. Lithium heparinized blood samples were used for gas pressure and pH measurement by means of a blood gas analyzer (GEM 3000, Instrumentation Laboratories, Lexington), which calculated the concentration of [HCO<sub>3</sub><sup>-</sup>] based on the pH and pCO<sub>2</sub>. Embryos were weighed and the initial egg weight was used to calculate relative embryo weight.

#### Hatching process

Between the 456<sup>th</sup> and 504<sup>th</sup> hour of incubation, eggs were checked every two hours for hatching event occurrences. At hatch, chicks were weighed and hatching percentage (as percent of number of fertile eggs) was calculated.

#### Statistical analysis

The data were processed using the statistical software package SAS version 8.2. Means of the measured parameters were compared by Tukey's test. Significance was based on P < 0,05.

## Results

### Experiment 1

The relative embryo weight did not differ on any sample day. The hatching process was significantly accelerated in the CO<sub>2</sub> incubated group (483±0,43 h) compared to the normal incubated group (488±0,54 h). Hatching percentage was 96% in the CO<sub>2</sub> group and 95% in the control group. Hatched chicks had equal body weights. Table 1 shows the mean gas partial pressures of the air cell, the pH, pCO<sub>2</sub>, [HCO<sub>3</sub><sup>-</sup>] and pO<sub>2</sub> from the blood of embryos of the first experiment. In both groups, air cell pCO<sub>2</sub> increased and pO<sub>2</sub> decreased with embryonic development. The same trend was followed in blood gas pressures (ED12-ED14). [HCO<sub>3</sub><sup>-</sup>] increased with age, pH remained constant between ED12 and ED14.

**Table 1. Average ± st error air cell and blood parameters of the first experiment, per embryonic day (ED)**

		ED11	ED12	ED13	ED14	ED18	IP
pCO <sub>2</sub> air cell (mm Hg)	control	7,1±0,40 <sup>a</sup>	10,02±0,60 <sup>a</sup>	13,55±0,85 <sup>a</sup>	21,46±0,95 <sup>a</sup>	33,58±1,17	40,17±1,00
	CO <sub>2</sub>	8,26±0,29 <sup>b</sup>	14,66±0,70 <sup>b</sup>	21,28±1,47 <sup>b</sup>	26,66±1,30 <sup>b</sup>	32,47±1,77	44,04±1,96
pO <sub>2</sub> air cell (mm Hg)	control	135,25±0,35	133,30±0,61 <sup>a</sup>	131±0,82 <sup>a</sup>	124±0,98	116,2±1,06	95,28±2,50
	CO <sub>2</sub>	133,41±0,41	131,41±0,52 <sup>b</sup>	128,5±0,80 <sup>b</sup>	124,7±0,68	119,1±1,96	91,9±3,41
pH blood	control		7,65±0,0065	7,64±0,010 <sup>a</sup>	7,64±0,014 <sup>a</sup>		
	CO <sub>2</sub>		7,71±0,034	7,70±0,014 <sup>b</sup>	7,70±0,010 <sup>b</sup>		
pCO <sub>2</sub> blood (mm Hg)	control		17,5±0,88	20,4±0,81 <sup>a</sup>	22,88±1,53 <sup>a</sup>		
	CO <sub>2</sub>		17,8±0,37	24,2±1,37 <sup>b</sup>	27,5±1,36 <sup>b</sup>		
pO <sub>2</sub> blood (mm Hg)	control		90,66±2,74 <sup>a</sup>	86,3±1,52 <sup>a</sup>	110,3±9,48 <sup>a</sup>		
	CO <sub>2</sub>		79,6±2,29 <sup>b</sup>	77,2±2,18 <sup>b</sup>	67,1±1,31 <sup>b</sup>		
[HCO <sub>3</sub> <sup>-</sup> ] blood (mmol/l)	control		19,28±0,73	22,09±0,84 <sup>a</sup>	24,36±0,91 <sup>a</sup>		
	CO <sub>2</sub>		21,85±1,70	26±1,099 <sup>b</sup>	34,04±1,23 <sup>b</sup>		

Differences between the control and CO<sub>2</sub> group per ED are indicated with a, b (p<0,05)

#### Experiment 2

The relative embryo weight differed only on the 16<sup>th</sup> incubation day, when the relative embryo weight of the control group was significantly higher than that of the CO<sub>2</sub> group. The average hatching time was not different between the experimental (486±0,60 h) and control group (485±0,50 h). Hatching percentage was 87% and 89% in the CO<sub>2</sub> group and control group respectively. The weight of the hatched chicks did not differ between the two groups. Table 2 shows the mean pCO<sub>2</sub> and pO<sub>2</sub> of the air cell, the pH, pCO<sub>2</sub>, [HCO<sub>3</sub><sup>-</sup>] and pO<sub>2</sub> from the blood of embryos of the second experiment. The air cell pCO<sub>2</sub> increased while the pO<sub>2</sub> decreased in both groups with age. The blood pCO<sub>2</sub> and pO<sub>2</sub> showed a similar trend as the air cell partial pressure gases during embryonic development, with a rise in pO<sub>2</sub> and decrease in pCO<sub>2</sub> from the moment the chick is in the EP stage and has switched to pulmonary respiration. The pH stays, in both groups, relatively constant but becomes less alkali towards the end of incubation.

Table 2. Average  $\pm$  st error air cell and blood parameters of the second experiment, per embryonic day (ED)

		ED10	ED11	ED12	ED13	ED14	ED15
pCO <sub>2</sub> air cell (mm Hg)	control	7,12 $\pm$ 0,68	9,99 $\pm$ 0,35 <sup>a</sup>	13,40 $\pm$ 0,60 <sup>a</sup>	18,22 $\pm$ 0,70	23,16 $\pm$ 1,02	29,48 $\pm$ 0,86
	CO <sub>2</sub>	7,12 $\pm$ 0,68	11,70 $\pm$ 0,55 <sup>b</sup>	15,96 $\pm$ 0,64 <sup>b</sup>	19,68 $\pm$ 0,74	24,52 $\pm$ 0,79	29,74 $\pm$ 1,50
pO <sub>2</sub> air cell (mm Hg)	control	144 $\pm$ 0,86	142,14 $\pm$ 0,45	137,73 $\pm$ 0,62	132,73 $\pm$ 0,73	126,93 $\pm$ 1,09	120,80 $\pm$ 0,77
	CO <sub>2</sub>	144 $\pm$ 0,86	141,80 $\pm$ 0,78	137,50 $\pm$ 0,58	132,43 $\pm$ 0,69	127,27 $\pm$ 0,88	122,50 $\pm$ 1,62
pH blood	control	7,67 $\pm$ 0,0056	7,64 $\pm$ 0,0091 <sup>a</sup>	7,60 $\pm$ 0,0081 <sup>a</sup>	7,60 $\pm$ 0,0063 <sup>a</sup>	7,56 $\pm$ 0,0079 <sup>a</sup>	7,60 $\pm$ 0,019 <sup>a</sup>
	CO <sub>2</sub>	7,67 $\pm$ 0,0056	7,70 $\pm$ 0,018 <sup>b</sup>	7,71 $\pm$ 0,013 <sup>b</sup>	7,69 $\pm$ 0,012 <sup>b</sup>	7,67 $\pm$ 0,017 <sup>b</sup>	7,66 $\pm$ 0,016 <sup>b</sup>
pCO <sub>2</sub> blood (mm Hg)	control	10,38 $\pm$ 0,47	15,84 $\pm$ 0,47	20,53 $\pm$ 0,68	25,86 $\pm$ 0,67	30,6 $\pm$ 1,21	33 $\pm$ 1,97
	CO <sub>2</sub>	10,38 $\pm$ 0,47	15,21 $\pm$ 0,96	20,84 $\pm$ 0,97	26,2 $\pm$ 1,32	31,07 $\pm$ 1,22	34,13 $\pm$ 1,83
pO <sub>2</sub> blood (mm Hg)	control	104,55 $\pm$ 3,57	99,84 $\pm$ 1,54	94,26 $\pm$ 1,74	90,26 $\pm$ 0,88	80,6 $\pm$ 2,21	75,4 $\pm$ 3,92
	CO <sub>2</sub>	104,55 $\pm$ 3,57	95,64 $\pm$ 2,64	88,84 $\pm$ 2,28	80,2 $\pm$ 0,93	77,42 $\pm$ 2,34	69,66 $\pm$ 1,40
[HCO <sub>3</sub> <sup>-</sup> ] blood (mmol/l)	control	15,50 $\pm$ 0,44	17,08 $\pm$ 0,35	20,34 $\pm$ 0,57 <sup>a</sup>	25,45 $\pm$ 0,52 <sup>a</sup>	27,39 $\pm$ 0,93 <sup>a</sup>	31,94 $\pm$ 1,04 <sup>a</sup>
	CO <sub>2</sub>	15,50 $\pm$ 0,44	18,78 $\pm$ 0,88	26,69 $\pm$ 1,14 <sup>b</sup>	31,38 $\pm$ 1,12 <sup>b</sup>	35,70 $\pm$ 0,79 <sup>b</sup>	38,85 $\pm$ 1,16 <sup>b</sup>
		ED16	ED17	ED18	IP	EP	hatch
pCO <sub>2</sub> air cell (mm Hg)	control	33,43 $\pm$ 0,98	35,08 $\pm$ 1,43	37,49 $\pm$ 1,98	41,29 $\pm$ 3,19		
	CO <sub>2</sub>	35,82 $\pm$ 1,67	34,46 $\pm$ 1,82	35,59 $\pm$ 0,84	37,09 $\pm$ 2,71		
pO <sub>2</sub> air cell (mm Hg)	control	117,20 $\pm$ 0,68	115,20 $\pm$ 1,01	112,43 $\pm$ 1,78 <sup>a</sup>	99,33 $\pm$ 5,47		
	CO <sub>2</sub>	117,93 $\pm$ 2,27	118,46 $\pm$ 1,76	117,87 $\pm$ 1,04 <sup>b</sup>	100,67 $\pm$ 4,61		
pH blood	control	7,60 $\pm$ 0,022 <sup>a</sup>	7,64 $\pm$ 0,026	7,50 $\pm$ 0,01	7,44 $\pm$ 0,0118	7,49 $\pm$ 0,0159	7,50 $\pm$ 0,018
	CO <sub>2</sub>	7,68 $\pm$ 0,019 <sup>b</sup>	7,73 $\pm$ 0,017	7,55 $\pm$ 0,018	7,46 $\pm$ 0,009	7,50 $\pm$ 0,0102	7,48 $\pm$ 0,018
pCO <sub>2</sub> blood (mm Hg)	control	34,76 $\pm$ 1,50	30,6 $\pm$ 2,48	48,75 $\pm$ 1,74	48,83 $\pm$ 1,42	42,57 $\pm$ 2,07	33,18 $\pm$ 0,98
	CO <sub>2</sub>	32,91 $\pm$ 2,35	29,3 $\pm$ 1,43	46,2 $\pm$ 1,67	47,83 $\pm$ 2,04	40,44 $\pm$ 2,29	32,33 $\pm$ 1,03
pO <sub>2</sub> blood (mm Hg)	control	66,07 $\pm$ 2,62	61,7 $\pm$ 5,16	16,25 $\pm$ 1,03	18,58 $\pm$ 0,74	22,5 $\pm$ 2,32	32,90 $\pm$ 1,97
	CO <sub>2</sub>	64,16 $\pm$ 2,63	71,5 $\pm$ 4,58	16,2 $\pm$ 0,90	19,25 $\pm$ 1,03	21,77 $\pm$ 1,46	35,41 $\pm$ 2,27
[HCO <sub>3</sub> <sup>-</sup> ] blood (mmol/l)	control	34,37 $\pm$ 0,57 <sup>a</sup>	33,83 $\pm$ 1,12 <sup>a</sup>	38,3 $\pm$ 1,31	33,66 $\pm$ 1,00	32,05 $\pm$ 1,03	25,99 $\pm$ 0,57
	CO <sub>2</sub>	38,06 $\pm$ 1,43 <sup>b</sup>	38,46 $\pm$ 1,53 <sup>b</sup>	40,96 $\pm$ 1,07	34,5 $\pm$ 1,06	31,96 $\pm$ 1,36	24,56 $\pm$ 1,06

Differences between the control and CO<sub>2</sub> group per ED are indicated with a, b (p<0,05)

## Discussion

Four percent of CO<sub>2</sub> in the incubator was not detrimental for embryos in their development and hatching, as Taylor and Kreutziger (1969) and Taylor *et al.* (1971) already observed. From the 13<sup>th</sup> till the 16<sup>th</sup> day of incubation, the threshold for optimal hatching percentage appeared to be 8% of CO<sub>2</sub>, during the last days of incubation the threshold was 7% CO<sub>2</sub> (Taylor and Kreutziger (1969) and Taylor *et al.* (1971)). In the first experiment, the CO<sub>2</sub> incubated group hatched significantly earlier, which was not seen in the second experiment. Partial pressure of CO<sub>2</sub> in the air cell did not differ between the groups. Visschedijk (1968 a, b) and De Smit *et al.* (2006) found an accelerated time of IP and hatch due to higher pCO<sub>2</sub> and lower pO<sub>2</sub> in the air cell.

However, our results on the partial pressures of gases in the air cell can not explain the earlier hatching time found in the CO<sub>2</sub> group of the first experiment.

Our results on partial pressure of gases in the air cell are in accordance with the results of Romijn and Roos (1938), De Smit *et al.* (2006), Bamelis (2003), showing a rise in pCO<sub>2</sub> and a decline in pO<sub>2</sub> in the air cell. These changes are due to the increasing metabolism of the embryo during this stage of development. During the first days of exposure to high CO<sub>2</sub>, a higher pCO<sub>2</sub> in the air cell could be observed in the experimental group. This could be the result of a lowered CO<sub>2</sub> diffusion due to the high percent of CO<sub>2</sub> in the surrounding air. This higher pCO<sub>2</sub> in the air cell might inhibit oxygen to enter the egg, as can be seen in a lower pO<sub>2</sub> during some of the first experimental days in the air cell and in the blood. Embryonic growth however was not enhanced in the CO<sub>2</sub> group, which otherwise could partially explain the lower pO<sub>2</sub>.

Our results on blood pH, pO<sub>2</sub>, pCO<sub>2</sub> and [HCO<sub>3</sub><sup>-</sup>] show similar patterns during normal embryonic development as Tazawa *et al.* (1971) and Girard (1971), who found that pH and pO<sub>2</sub> decrease, while [HCO<sub>3</sub><sup>-</sup>] and pCO<sub>2</sub> increase during embryonic development. Since there is diffusion resistance due to the shell and membranes, and since the metabolism of the embryo increases during development, pCO<sub>2</sub> rises and pO<sub>2</sub> in the blood decreases. The carbon dioxide dissolves in the blood, resulting in the release of bicarbonate and H<sup>+</sup>, which causes acidification throughout development. Girard (1971) concluded that the embryonic chick develops a relative respiratory acidosis, because blood pH and pO<sub>2</sub> decreased and blood pCO<sub>2</sub> increased during development. Dawes and Simkiss (1969) and Boutilier *et al.* (1977) found another, relatively constant, pH pattern of the embryonic blood through development. As blood pCO<sub>2</sub> levels progressively increased during the incubation period, the pH was compensated for by an elevation of [HCO<sub>3</sub><sup>-</sup>]. A significant decline of pH occurred between days 12 and 14, which was correlated with a lag in the increase of blood [HCO<sub>3</sub><sup>-</sup>]. Dawes and Simkiss (1969) concluded that the changes in blood bicarbonate levels are largely due to an influx of extra 'non-respiratory' bicarbonate. They suggested the bicarbonate originate from the resorption of the eggshell, the activity of the kidney, or both. From the foregoing, we would expect that if embryos are exposed to high CO<sub>2</sub> concentrations, an even more pronounced acidification would occur above the natural developmental acidification. However, our data do not confirm this hypothesis. A higher blood pCO<sub>2</sub> and higher [HCO<sub>3</sub><sup>-</sup>] and lower blood pO<sub>2</sub> was observed in embryos exposed to 4% CO<sub>2</sub> than normal incubated embryos, but also a more alkaline blood pH. We hypothesize that the embryo reacts on this high CO<sub>2</sub> by adaptation which could exist on two levels. Firstly, renal function might be stimulated, which might result in overcompensation in order to react to the severe respiratory acidification to buffer the pH. Secondly, the significantly higher [HCO<sub>3</sub><sup>-</sup>] could be the result of extra resorption of bicarbonate from the eggshell with the purpose to buffer the protons resulting from the acidification by CO<sub>2</sub>.

It can be concluded that embryos show physiological adaptations to high CO<sub>2</sub> levels in the incubator. More research however is needed to explain their adaptations, investigate endocrine parameters and post-hatch performance.

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