Effects of Storage Time on Incubating Egg Gas Pressure, Thyroid Hormones, and Corticosterone Levels in Embryos and on Their Hatching Parameters

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ABSTRACT Incubating eggs (1,800 total) produced by a commercial flock of Cobb broiler breeders were used to determine the effects of storage duration (3 and 18 d) on gas partial pressure, thyroid hormones, and hatching parameters. Partial pressure of oxygen (pO2) and carbon dioxide (pCO2) were measured on d 18 and at internal pipping (IP) during incubation. Blood samples were collected for determination of triiodothyronine (T3), thyroxine (T4), and corticosterone concentrations in the embryos at IP and in newly hatched chicks. From 464 to 510 h of incubation, eggs were checked individually every 2 h to determine the timing and duration of IP, external pipping (EP), and total hatching time. At 18 d of incubation and at IP, pCO2 was greater in air cell of eggs stored for 3 d compared to those stored for 18 d (P < 0.05), but pO2 was greater in eggs stored for 18 d. At IP, T3 and corticosterone levels were higher in plasma of the embryos of eggs stored for 3 d compared to those stored for 18 d, but it was the reverse in newly hatched chicks (P < 0.05). Embryos from eggs stored for 18 d required more time to complete IP compared to embryos of eggs stored for only 3 d (P < 0.05), whereas the duration of EP was not affected by storage. The overall longer incubation was, however, not only due to prolonged IP but also to later occurrence of IP. It was concluded that prolonged IP as a result of long storage may be related to the late increase in corticosterone level, which may be a necessary stimulus for higher T3/T4 ratio, late increase in pCO2 level, and decrease in pO2. The effect of long storage was a delay in hatching and a continuous increase in T3 due to higher corticosterone levels between IP and hatching, which may be an indication of the more stressful event of hatching of embryos from eggs stored longer. Differences in pCO2, pO2, T3, T4, and corticosterone levels in the incubating eggs may be manifestations of these changes culminating in altered hatching parameters and consequently differences in chick quality and growth potentials.

(Key words: storage time, gas pressure, hatching parameter, thyroid hormone, corticosterone)

INTRODUCTION

Oxygen and CO2 exchanges and hormonal balance especially of triiodothyronine (T3), thyroxine (T4), and corticosterone are of fundamental importance for embryonic development during incubation and may affect survival of the embryo (Decuypere et al., 1979; Tullett, 1990). Oxygen uptake by the egg increases slowly as the embryo grows during the first 2 wk of incubation (Tazawa, 1980). However, increased metabolic demand and the development of lung respiration toward the end of incubation lead to increased O2 supply. CO2 supply also acts as a stimulant for early embryonic development (Tullett, 1990).

The hatching process of the eggs is dependent on physical (George, 1978) and physiological factors such as CO2 level and hormonal concentrations, especially T4 and T3 (Visschedijk, 1968; Decuypere et al., 1979; Buys et al., 1998). Gas exchanges depend on ventilation rates during incubation and characteristics of incubating egg such as eggshell conductance and albumen viscosity (Ancel and Visschedijk, 1993; McLoughlin and Gous, 1999). Less-than- optimum incubation temperature from d 17 onward delays pipping and hatching, as well as decreases serum levels of T3 and T4 during the perinatal period (Decuypere et al., 1981). On the other hand, Ockleford et al. (1983) showed that advancing hatching time increased T3 concentrations in chicks but found no differences in thyroid hormone concentrations between stimulated and non-

Abbreviation Key: EP = external pipping; IP = internal pipping; pCO2 = carbon dioxide partial pressure; pO2 = oxygen partial pressure; T3 = triiodothyronine; T4 = thyroxine.
stimulated chicks at equivalent physiological stages of development. This observation indicates that the developmental stage is a major determinant of T3 and T4 concentrations. Nevertheless, lower T3 and T4 values were found in newly hatched chicks that hatched 8 to 10 h later than the first hatchlings (Iqbal et al., 1989). Changes in T3 and T4 levels illustrate individual variability as well as an interaction between chronological age and developmental stage in the determination of the concentrations of thyroid hormones at a given time (Decuypere et al., 1990).

In the chick embryo, although corticosterone can be detected in the circulation by d 10 of incubation (Wise and Fry, 1973; Kalliecharan and Hall, 1974, 1976) pituitary control of adrenal function becomes important at about d 14 of incubation (Wise and Fry, 1973; Freeman, 1974). Corticosterone concentration is related to embryonic development (Karnofsky et al., 1951). Because egg storage before setting for incubation results in low rate of embryonic development, it is not clear whether incubating egg storage duration affects corticosterone levels during perinatal stages. It was shown in a previous report that the duration of the storage of incubating eggs has an impact on incubation duration, chick qualitative aspects, and chick postnatal juvenile growth (Decuypere et al., 2002).

It is the aim of this work to study the probable causes of the observed effects of increasing storage time of incubating eggs on hatching parameters. Gas pressure; T3, T4, and corticosterone levels; and physical hatching parameters [such as the timing of internal pipping (IP), external pipping (EP), and hatching] were determined during the last days of incubation of eggs stored for 3 or 18 d before setting. Also, the duration of some critical stages during incubation was determined in order to ascertain when and where storage may affect this process and relate it to the physiological measurements taken.

**MATERIALS AND METHODS**

**Incubation Management**

Incubating eggs (1,800) produced by commercial flocks of Cobb broiler breeders were studied. The eggs were collected between 1000 and 1100 h and were stored for 3 or 18 d at 15°C and 75% relative humidity. All the eggs were numbered and weighed individually prior to the beginning of incubation.

Six replications of 150 eggs per group were followed. The eggs were set for incubation in forced-draft incubators at a dry-bulb temperature of 37.6°C and wet-bulb temperature of 29°C. On d 18 of incubation, the eggs were candled, and those with evidence of living embryos were transferred from turning trays to hatcher baskets.

From 464 to 510 h of incubation, the transferred eggs were checked individually every 2 h for pipping and hatching. During this period, the time of IP, EP, and emergence from individual eggs were recorded. These data were used to calculate the duration of IP (duration between IP and EP), duration of EP (duration between EP and emergence), and hatching time (duration between IP and emergence). Total incubation was then calculated as the time between setting and emergence. These measurements allowed us to determine which physiological stage variation in the duration of egg storage had an impact on the final stages of chick embryo development.

**O2 and CO2 Measurements**

Within each replication, 12 eggs with evidence of living embryos per storage were used to measure the gas partial pressure (pO2 and pCO2) in the air cell on d 18 of incubation. The same measurement was taken at IP by using samples of 18 eggs per storage time. These measurements were made directly in the air cells of the eggs by means of a blood gas analyzer for the measurement of pCO2 and pO2. The direct measurement in eggs was done by using a small hole of 0.9 mm with a needle just above the air cell, and the needle of the blood gas analyzer was immediately introduced into the hole as previously described (Dewil et al., 1996; Buys et al., 1998).

**T3, T4, and Corticosterone Analysis**

For T3, T4, and corticosterone measurements, blood samples were collected from embryos at IP and from chicks immediately at hatch. Within each replication, sampled eggs (18 eggs per storage duration) utilized at IP to measure gas pressure were used in the determination of T3, T4, and corticosterone levels. Blood samples were also taken from samples of 35 newly hatched chicks from each replication of eggs stored for 3 or 18 d throughout hatching to determine T3, T4, and corticosterone concentrations. T3 and T4 concentrations were measured in plasma samples by RIA as described previously (Huybrechts et al., 1989; Darras et al., 1992). Intra-assay coefficients of variation were 4.5 and 5.4% for T3 and T4, respectively. Antisera and T3 and T4 standards were purchased.4 Corticosterone was measured using a commercially available double antibody RIA kit from IDS Ltd.5 All samples were run in the same assay in order to avoid inter-assay variability (Decuypere et al., 1983; Meeuwis et al., 1989).

**Statistical Analysis**

The data were processed with a statistical software package.6 The general linear models procedure was used to analyze pCO2; plasma T3, T4, and corticosterone concentrations; IP, and EP duration; and hatching time in relation to storage time. The model was as follows:

\[ Y_i = \mu + \alpha_i + \varepsilon_i \]
where, \( Y_i = pCO_2, pO_2, T_3, T_4, \) ratio of \( T_3/T_4, \) corticosterone, IP and EP duration or hatching time of egg from storage time \( i, \mu = \) overall mean, \( \alpha_i = \) main effect of storage time \( i, \) and \( \varepsilon_i = \) random error term from storage.

**RESULTS**

**Effects of Storage Duration on Partial Gas Pressures**

Figure 1 shows the CO\(_2\) and O\(_2\) gas pressures in the air cell of eggs at d 18 of incubation and at IP. At d 18 of incubation as well as at IP, pCO\(_2\) was higher \((P < 0.05)\) and pO\(_2\) was lower \((P < 0.001)\) in the air cell of eggs stored for 3 d compared to those stored for 18 d. In both groups of eggs, pCO\(_2\) was higher and pO\(_2\) was lower at IP than that at d 18 of incubation \((P < 0.05)\).

**Effects of Storage Duration on T\(_3\), T\(_4\), and Corticosterone Concentrations**

Blood plasma levels of T\(_3\) and T\(_4\) at IP and in newly hatched chicks (day old) are shown in Table 1. At IP, T\(_3\) and corticosterone levels were higher in the plasma of embryos of eggs stored for 3 d compared to those stored for 18 d. The ratio of T\(_3)/T_4\) was lower in embryos stored for 18 d \((P < 0.001)\).

In newly hatched chicks, however, the levels of T\(_3\), T\(_4\), and corticosterone were higher \((P < 0.05)\) in those from eggs stored for 18 d. The ratio of T\(_3)/T_4\) at this stage was still lower in chicks from eggs stored for 18 d \((P < 0.05)\). It is worthy of note that, compared with levels at IP, there was a significant decrease in T\(_4\) levels but little change in T\(_3\) in chicks from eggs stored for 3 d, thus increasing T\(_3)/T_4\) ratio significantly. However, in eggs stored for 18 d, T\(_3\) levels were increased in chicks at hatch but without change in T\(_4\) resulting equally in an increased T\(_3)/T_4\) ratio.

Corticosterone levels at IP were similar to those of newly hatched chicks from eggs stored for 3 d. However, in the embryos from eggs stored for 18 d there was an increase in corticosterone levels of almost 5 ng/mL in newly hatched chicks compared with those in embryos at IP \((P < 0.001)\).

**Effects of Storage Time on Hatching Parameters**

Figure 2 shows the distribution of hatching times for the eggs stored for 3 or 18 d before setting for incubation. The hatching curve shows that the majority of eggs stored for 3 d hatched between 478 and 494 h of incubation reaching a peak at 486 h, whereas for eggs stored 18 d most hatching only started to increase at 484 h to reach a peak at 504 h. The figure thus shows a delay in the hatching of eggs stored for 18 d compared that for eggs stored for 3 d.

Table 2 shows the duration of specific incubation hatching activity during embryonic development. The total duration of incubation was greater for eggs stored for 18 d. Split into the different times spent at similar stage of embryo, the data show that embryos of eggs stored for
18 d were at the IP stage longer than embryos of eggs stored for only 3 d \( (P < 0.001) \). Furthermore, IP occurred much earlier in eggs stored for 3 d \( (P < 0.001) \). The duration of EP was not different between embryos from both groups of storage. Thus, the hatching process was prolonged by about 7 h in embryos of eggs stored for 18 d mainly because of prolonged IP. Moreover, the start of IP was about 9 h later in egg stored for 18 d compared to those stored for only 3 d, as shown by the data of duration from setting to IP (Table 2).

**DISCUSSION**

The duration of egg storage before incubation is a phenomenon that is prejudicial for embryonic development during incubation. The effects of storage on gas exchange, thyroid hormone levels, and physical hatching parameters during the last days of incubation have been investigated in this study. Prolonged incubation for eggs stored for long periods has been reported previously by Mather and Laughlin (1976) and Muambi et al. (1981). Our results show that longer storage prolonged incubation duration by at least 15 h in eggs stored for 18 d compared with eggs stored for 3 d. This prolongation is the result of a delayed start of IP as well as an increase in IP duration but not EP duration.

The \( O_2 \) and \( CO_2 \) exchanges are of fundamental importance for embryonic development during incubation (Tullett, 1990), especially for metabolism (Rahn et al., 1979). Lower p\( CO_2 \) at d 18 of incubation was associated with longer storage and possibly indicated a younger developmental stage of longer-stored eggs at the same chronological incubation time or day. However, at presumed identical physiological stage or age of IP, lower p\( CO_2 \) levels (and higher p\( O_2 \)) were also found for embryos of eggs stored 18 d. At this stage of IP, T\(_3\) concentration was also lower for eggs stored for 18 d. It is generally assumed that T\(_3\) is the calorigenically active form of thyroid hormones. The negative relationship between T\(_3\) concentration and the p\( O_2 \)/p\( CO_2 \) ratio may indicate that T\(_3\) is indeed related to the metabolic rate of the embryo. Its increase from the onset of lung respiration up to hatching coincides with the progressive increase in \( O_2 \) consumption and with the onset of homeothermic response in chicks during this period (Decuyper et al., 1978, 1979).

The lower T\(_3\) level in embryos of eggs stored for 18 d may therefore indicate that embryos at this stage were weaker compared to those in the eggs stored for 3 d, because both are at a presumed similar developmental stage. T\(_3\) was still increasing between IP and emergence in the embryos of eggs stored for 18 d. This delay in the increase of T\(_3\) may be a major factor in the prolongation of hatching time in eggs stored for long periods before setting. It may also explain the slightly wider spread of hatch of eggs stored for 18 d compared to 3 d storage eggs. Considered together, short storage seems to favor higher T\(_3\)/T\(_4\) ratio. At the same time, increased T\(_3\)/T\(_4\) ratio was coincidental with higher corticosterone level. Both acting together may favor early pipping and hatching.

At hatch, T\(_3\) levels in chicks from eggs stored for 18 d were somewhat higher, as if they had to compensate for the delay during IP. This higher T\(_3\) at hatch compared with levels at IP seems aberrant because Decuyper et al. (1979) demonstrated that T\(_3\) and T\(_4\) levels were always maximum on the day of IP, as observed in chicks from eggs stored for 3 d in the current experiment. Moreover, Ockleford et al. (1983) pointed out that advancing the hatching time results in an increase of T\(_3\) concentration in chicks.

Unlike the relationship between T\(_3\) levels and hatching time, Scott et al. (1981) pointed out that corticosterone levels are not affected by incubation duration. Our results however indicate that corticosterone levels alter with duration of incubation. Corticosterone levels were lower at

**TABLE 1. Corticosterone, triiodothyronine (T\(_3\)), thyroxine (T\(_4\)), levels and T\(_3\)/T\(_4\) ratio in embryos at internal pipping stage and in newly hatched chicks in relation to the storage time**

<table>
<thead>
<tr>
<th>Developmental stage</th>
<th>Storage</th>
<th>Corticosterone</th>
<th>( T_3 )</th>
<th>( T_4 )</th>
<th>( T_3/T_4 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Internal pipping</td>
<td>3</td>
<td>16.00 ( \pm ) 1.18</td>
<td>3.98 ( \pm ) 0.19</td>
<td>8.92 ( \pm ) 0.73</td>
<td>0.74 ( \pm ) 0.08</td>
</tr>
<tr>
<td></td>
<td>18</td>
<td>13.62 ( \pm ) 1.19</td>
<td>3.07 ( \pm ) 0.20</td>
<td>7.85 ( \pm ) 0.56</td>
<td>0.59 ( \pm ) 0.05</td>
</tr>
<tr>
<td>Newly hatched chicks</td>
<td>3</td>
<td>15.95 ( \pm ) 1.02</td>
<td>3.57 ( \pm ) 0.08</td>
<td>6.09 ( \pm ) 0.27</td>
<td>0.92 ( \pm ) 0.05</td>
</tr>
<tr>
<td></td>
<td>18</td>
<td>18.14 ( \pm ) 1.31</td>
<td>3.95 ( \pm ) 0.10</td>
<td>7.70 ( \pm ) 0.39</td>
<td>0.76 ( \pm ) 0.05</td>
</tr>
</tbody>
</table>

\( ^a,b \)Values sharing no common letters are significantly different according to the storage time \( (P < 0.05) \).

**TABLE 2. Hatching parameters in relation to the storage time**

<table>
<thead>
<tr>
<th>Incubation parameters</th>
<th>Storage time</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3 d</td>
</tr>
<tr>
<td>Total incubation duration of 50% hatched chicks</td>
<td>486 ( \pm ) 0.22</td>
</tr>
<tr>
<td>Duration of internal pipping (IP)</td>
<td>5.33 ( \pm ) 0.44</td>
</tr>
<tr>
<td>Duration of external pipping (EP)</td>
<td>12.5 \pm 0.45</td>
</tr>
<tr>
<td>Duration from IP to emergence</td>
<td>17.83 ( \pm ) 0.37</td>
</tr>
<tr>
<td>Duration from setting to IP</td>
<td>468.17 ( \pm ) 0.20</td>
</tr>
</tbody>
</table>

\( ^a,b \)Values sharing no common letters are significantly different according to the storage time \( (P < 0.05) \).
IP in eggs with longer incubation and also higher in newly hatched chicks of these eggs. In eggs stored for 18 d for which incubation was prolonged, corticosterone levels at IP were lower. The lower level of corticosterone at this stage may explain the reduced concentration of T4 also at this stage, which possibly resulted in prolonged IP.

Decuyper et al. (1983) and Meeuvis et al. (1989) demonstrated that corticosterone is required for the peripheral conversion of T4 to T3 during prenatal life. Thus, the higher corticosterone level in short storage eggs might have served to boost the shift of T3/T4 ratio. Longer storage of eggs seemed to reduce the capability of embryos to produce corticosterone at IP. With late increase in corticosterone levels before hatch, levels of T3, T3/T4 ratio and corticosterone increased significantly in newly hatched chicks beyond those found in chicks of shorter incubations (i.e., chicks from eggs stored for 3 d). Moreover, because corticosterone is involved in maintaining homeostasis, metabolism, and stress, its levels in embryos or in newly hatched chicks can influence chick postnatal growth. In chicks from eggs stored for only 3 d, corticosterone levels remain unchanged, and T4 levels were well down such that T4 levels remain unchanged. Therefore, the significantly higher T3 and corticosterone levels in chicks hatched from eggs stored for 18 d compared to chicks from eggs stored for 3 d suggest a continued peripheral conversion of T4 to T3 when T4 levels should have been significantly reduced to the boost T3/T4 ratio. This finding may be an indication for stress and, therefore, result in subsequent higher mortality and reduced growth speed.

Mather and Laughlin (1976) and Muambi et al. (1981) suggested that prolonged incubation of stored eggs could be due to eggshell composition, which may influence conductance. The conductance of shells from a number of bird species has been shown to increase with time of storage (Simons and Wiertz, 1966), but evidence from chicken eggs is contradictory. Sparks and Boards (1984) and Deeming (1987) found that the conductance of chicken and Muscovy duck eggshells was independent of the shell quality (cuticle). An increase in conductance could decrease pCO2, hence the trigger for IP and EP. Duration of IP but not duration of EP, when the embryos are already with beak in O2-rich atmospheric air, could strengthen this hypothesis of change in conductance with storage time. However, the lack of differences in EP duration of embryos versus storage time suggests that eggshell quality may not be at play in determining incubation duration at this stage. These differences are probably due to the differences in initial quality of eggs stored for different periods. What exactly changes physico-chemical properties of eggs during storage requires further investigation.

In conclusion, we have shown that prolonged total incubation due to longer egg storage is manifested before and during IP but not during EP. The lack of storage effect during EP suggested that effects may be due to internal egg qualities that were altered during storage but not due to the eggshell qualities. Differences in pCO2, pO2, and T3, T4, and corticosterone levels in incubating eggs may be manifestations of these changes culminating in altered hatching parameters and consequently differences in chick quality and growth potentials.

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