Effects of Turning Duration During Incubation on Embryo Growth, Utilization of Albumen, and Stress Regulation

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ABSTRACT Eggs from Cobb broiler breeders were incubated for 18 d. Eggs were not turned (T0) or were turned until 9 (T9), 12 (T12), 15 (T15), or 18 (T18) d. First, the effects of turning on embryo and albumen weights were studied. Samples of eggs were opened at d 9, 12, 15, and 18 for embryo and albumen weighing. The results show that embryos from unturned eggs had lower weights and higher remaining albumen weights than those from turned eggs. At d 18, albumen utilization was completed in the T12, T15, and T18 groups only. Also, further turning until d 15 and 18 increased embryo weights. The responsiveness of the embryo after adrenocorticotropic hormone (ACTH) injection was studied to test stress control in embryos. Blood samples were collected from embryos at 60 and 150 min after injection at d 12, 15, and 18 and were analyzed for corticosterone concentrations. The results showed that basal corticosterone levels increased with embryo age. At 60 min after ACTH injection, corticosterone levels were lower at d 12 than at d 15 and 18. At 150 min after ACTH injection, corticosterone levels followed different trends according to incubation stage and turning duration. The highest basal corticosterone levels were obtained with the T15 group at d 15 and 18. Also at d 18, corticosterone levels in the T15 group were the highest at 150 min after ACTH injection. We concluded that egg turning was required during incubation until d 12, at least, and should not be stopped until after d 15.

(Key words: albumen use, egg turning, embryo growth, stress level)

INTRODUCTION

Egg turning requirements during incubation involve parameters such as frequency, axis of setting and turning, turning angle, planes of rotation, and stage of incubation requiring turning (Wilson, 1991). Some of the benefits of egg turning during incubation include reduction in malpositioning of the embryo (Robertson, 1961), prevention of abnormal adhesion of the embryo or embryonic membranes to the shell membrane (New, 1957), and the complete and timely closure of chorioallantois at the small end of the egg (Deeming, 1989a, b). These benefits have been related to influences on embryo physiology such as protein accumulation in amniotic fluid, growth rate of the area vasculosa, gas exchange (Deeming, 1989a,b; Wilson, 1991; Tona et al., 2003), and thyroid hormone levels (Tona et al., 2003). The influence of turning on these parameters seems to depend on the duration and frequency of turning (Wilson, 1991; Elibol et al., 2003; Tona et al., 2003) and the stage of incubation or development of the embryo. Currently, it is a common practice to turn eggs once per hour, at an angle of 90°, until the 18th day of incubation. We have been studying the effects of turning chicken eggs during the last stages (d 12 to 18) of incubation on physiological and physical parameters of the embryo and the hatched chicks (Tona et al., 2001, 2003). These studies demonstrated that turning and duration of egg turning influence physiological factors such as plasma triiodothyronine and thyroxine and air chamber partial pressure of CO2 and O2 at internal pipping stage and also have some bearing on incubation duration, hatchability, chick quality, and chick growth potential.

There is some evidence that the strain and age of the breeder chicken and the duration of storage of the egg may also interact with the effect of turning on these parameters (Wilson, 1991; Tona et al., 2001). The underlying cause of these differences between strains or quality of the eggs is still unclear. It is also necessary to understand more clearly how turning exerts its effect at different stages of incubation to benefit embryo growth, hatchability, and chick quality. Even though egg turning has its benefits, it has always been thought of as a stress factor to the developing embryo (Vince et al., 1979), which may imply that the inability to cope with the stress of turning at certain times during

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Abbreviation Key: ACTH = adrenocorticotropic hormone; HPA = hypothalamo-pituitary-adrenal; T0 = no turning; T9, T12, T15, and T18 = turning until 9, 12, 15, and 18 d of incubation, respectively.
development of the embryo may result in poor performance of the embryo. Also, the effects of egg turning on chick juvenile growth (Tona et al., 2003) may be linked to embryo growth up to hatch. Therefore, the aim of this study was to investigate the effects of egg turning until d 9, 12, 15, or 18 as well as the effects of no turning during incubation on the use of albumen by the developing embryo and the growth of the embryo. The study also investigated the effect of turning on the development of the hypothalamic-pituitary-adrenal (HPA) axis and stress control by the developing embryo during incubation.

**MATERIAL AND METHODS**

**Experiment 1: Effect of Turning Duration on Use of Egg Albumen and Embryo Weight**

We studied 900 incubating eggs produced by Cobb broiler breeders. The eggs were collected between 1000 and 1100 h and were stored for an average of 7 d at 15°C and 70% relative humidity. The eggs were set for incubation in forced-draft incubators at a dry bulb temperature of 37.6°C and a wet bulb temperature of 29°C with turning once an hour at an angle of 90°. Two replications of 150 eggs per turning treatment per incubation setting were studied. Following our previous study (Tona et al., 2003), eggs were not turned (T0) or were turned until 9 (T9), 12 (T12), 15 (T15), or 18 (T18) d of incubation. When turning was stopped before d 18 of incubation, the eggs stayed in turning trays and were kept horizontal until transfer.

At d 9, 12, 15, and 18 of incubation, samples of 30 eggs per replication per turning treatment were broken to determine weights of the growing embryos and the amount of remaining albumen. After breaking each egg, its embryo was removed carefully and separated from all attachments such as yolk sac and chorioallantoic membrane. The embryo was then wiped with absorbent paper before weighing. Also for each egg, the remaining thick albumen was separated from the embryonic fluid before weighing.

**Experiment 2: Effect of Turning Duration on Stress Level in Embryos and Responsiveness to Adrenocorticotropic Hormone Injection**

It is generally accepted that changes in some physiological parameters such as corticosterone are indications of stress level. It has also been shown that corticosterone can be detected in chick embryo blood before the 10th day of incubation, but the pituitary control of adrenal function becomes important at about d 14 of incubation (Wise and Fry, 1973; Kalliecharan and Hall, 1976; Iqbal, 1989). This experiment determined changes in corticosterone levels as a function of turning duration or without turning. Because turning may not be the only source of stress to the embryo during incubation (e.g., increasing partial pressure of CO₂ levels), we determined the effect of turning duration on the responsiveness of the embryos after injection of adrenocorticotropic hormone (ACTH).

In the second experiment, 3 replications of 150 eggs from unturned or turning treatments were studied as described in experiment 1. Blood samples were collected at d 12, 15, and 18 of incubation from 12 embryos per replication per turning treatment (T0, T9, T12, T15, and T18) for measurement of corticosterone levels in plasma. Also, in relation to turning treatments, another 16 eggs per replication were used for determining the effect of ACTH or saline solutions injection on corticosterone level in embryo turned for 9, 12, 15, or 18 d or in unturned eggs. Eggs were injected on d 12, 15, or 18 but not on d 9 of incubation because of the difficulty of injection at that embryonic stage. One nanogram of ACTH (rat fragment 1–2) in 0.1 mL of saline solution or 0.1 mL of saline solution was injected into an allantoic vessel situated close to the shell membrane as described by Decuypere et al. (1982). The choice of dose was based on the observations of Decuypere et al. (1989) who showed previously that 1 ng of ACTH is a minimal effective dose for evoking stress in chicken embryos. The injection was made possible by candling for blood vessel localization and cutting a triangle around the vessel through the shell without damaging the shell membrane. After injection, the injection area on the egg was sealed with adhesive tape and replaced in the incubator without turning until the time for blood collection.

Sixty minutes after injection blood samples were collected from half of the embryos injected; for the second half blood samples were taken after 150 min to determine if turning duration affected stress level induced by ACTH in the embryos. Corticosterone concentrations in plasma samples were measured using a commercially available double antibody RIA-kit (Decuypere et al., 1983; Meeuwis et al., 1989). All samples were run in the same assay to avoid interassay variation.

**Statistical Analysis**

The data were processed with a statistical software package. The effects of turning duration and developmental stage on embryo weights, remaining albumen weights, and corticosterone concentrations were analyzed using a 2-way, fixed effects ANOVA model (Neter et al., 1996). A probability value of 0.05 was retained as the degree of significance. The model was as follows:

\[
Y_{ijk} = \mu + \alpha_i + \tau_j + (\alpha\tau)_{ij} + e_{ijk}
\]

where \(Y_{ijk}\) = embryo weight or albumen weight or corticosterone concentration of egg k from turning duration i and developmental stage j, \(\mu\) = overall mean, \(\alpha_i\) = main effect of turning duration i, \(\tau_j\) = main effect of developmental...
**RESULTS**

**Experiment 1: Effects of Turning Treatments on Embryonic Growth and Albumen Use**

Embryo and albumen weights relative to incubation period and turning were shown in Figure 1 (A and B). Irrespective of turning treatment, embryo weights (Figure 1A) increased from d 9 to 18 of incubation ($P < 0.001$). From d 12 of incubation onward, embryos from unturned eggs had lower weights than those from turned eggs ($P < 0.01$). Figure 1B shows clearly that incubation and turning durations had significant effects on remaining albumen weights in the eggs. Albumen weights between turned and unturned eggs were similar at d 9 of incubation. At d 15, a significant decrease in albumen weight was recorded for turned and unturned eggs, but there was a greater decrease in turned eggs. At d 18, a further decrease in albumen weights was observed in both groups, but the level of decrease was greater in unturned eggs. By d 18 of incubation, albumen weight was at the lowest level in turned eggs, but a significant amount was still present in unturned eggs.

Data on embryo and remaining albumen weights in relation to turning treatments of T0, T9, T12, T15, and T18 are presented in Figure 2. The changes in albumen and embryo weights according to turning treatment are illustrated in Figure 2 (A, B, and C), corresponding to termination of incubation at 12, 15, and 18 d, respectively. Incubation and turning durations had significant effects on embryo weights and the amount of remaining albumen. Total albumen use was completed at d 18 of incubation in eggs turned until d 12 onward. From d 12 of incubation, further turning until d 15 and 18 increased embryo weights.

In general, there was a negative linear relationship between embryo and albumen weights irrespective of turning treatment. This is clearly illustrated by relationship between albumen and embryo weights at d 15 of incubation (Figure 3).

**Experiment 2: Effects of Incubation Stage and Turning Treatments on Embryo Corticosterone Levels**

Plasma corticosterone concentrations in embryos from control eggs (uninjected eggs) and from eggs injected with ACTH at d 12, 15, and 18 of incubation according to turning treatments are shown in Figure 4. Overall, basal corticosterone levels increased with developmental stages ($P < 0.001$) irrespective of turning treatment (see controls). However, the level of increase in corticosterone at d 15 and 18 of incubation varied among turning treatments. At d 15, the levels were ordered as follows: $T_0 < T_9 = T_{12} < T_{15}$ (Figure 4B) and at d 18 were $T_0 = T_9 = T_{18} < T_{12} = T_{15}$ (Figure 4C). At d 15 and 18, eggs for which turning had stopped at d 15 had the highest basal corticosterone levels.

Saline injection had no effect on the levels of corticosterone at d 12, 15, or 18 of incubation. Also, levels were similar between uninjected and saline-injected embryos irrespective of turning duration or developmental stage (data not shown). Sixty minutes after ACTH injection, corticosterone levels increased significantly in embryos on d 12, 15, and 18 of incubation, irrespective of turning duration. However, responsiveness to ACTH was lower at d 12 (Figure 4A) than at d 15 (Figure 4B) and 18 (Figure 4C), both of which showed similar responsiveness. Turning treatments had no effect on responsiveness to ACTH at 60 min after injection.

At 150 min after ACTH injection, corticosterone levels were lower than those recorded at 60 min after injection. However, the levels of reduction in corticosterone levels depended on the stage of incubation and turning treatments. At d 12 (Figure 4A) or 15 (Figure 4B) of incubation,
FIGURE 2. Changes in albumen and embryo weights in relation to turning treatments at d 12 (A), 15 (B), or 18 (C) of incubation. Among turning treatments, embryo weights or albumen weights sharing no common letter are different (P < 0.05).

FIGURE 3. Relationship between embryo weights and albumen weights at d 15 of incubation.

Corticosterone levels in embryos were still higher than those of eggs without injection. However, at 150 min after injection, corticosterone levels followed different trends according to incubation stage: d 15 > d 12 = d 18 of incubation. Corticosterone levels were not affected by turning treatment at d 12 or 15 of incubation. At d 18 of incubation (Figure 4C), the levels of corticosterone in embryos at 150 min after injection were statistically comparable with levels in the embryos from noninjected eggs except in the embryos of T15 group. Levels of corticosterone in embryos of T0, T9, and T12 groups were significantly lower than those corresponding to levels at d 15 of incubation. Corticosterone levels in eggs of T15 group remained elevated above other turning treatments after 150 min of ACTH injection.

DISCUSSION

The results from this study demonstrate the importance of egg turning during incubation. Albumen use by the growing embryo and growth rate were influenced by turning treatments. Turning treatments also influenced adrenal function through changes in corticosterone secretion. Our data also suggest that turning may influence HPA regulation of stress levels in embryos. The effects of turning however were related to the stages of the development of the embryo. Turning treatments showed advantages in enhancing albumen use and growth reflecting advanced development of embryos from turned eggs compared with unturned ones. Interactions between turning treatments and incubation stages suggest that there are periods during incubation when turning may be more beneficial.

This study demonstrates that whether or not eggs are turned, there is a natural process of albumen use that may be related to the growth of the embryo and increasing corticosterone level, which may indicate a logical consequence of the developing adrenal HPA. During incubation, albumen proteins move into the amniotic fluid and are retracted by the embryo. Thus, turning seems to be an additional impetus to increase the timely use of the albumen. Our results on this aspect are consistent with those
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FIGURE 4. Corticosterone levels at d 12 (A), 15 (B), and 18 (C) of incubation in relation to turning treatments and incubation period. *Within each incubation period, values sharing no common letter are different (P < 0.05). T0 = no turning; 9, T12, T15, and T18 = turning until 9, 12, 15, and 18 d of incubation, respectively; ACTH = adrenocorticotropic hormone.

Lack of egg turning (T0) or turning until d 9 (T9) showed a copious amount of remaining albumen at d 18 of incubation and, therefore, lower body weights of embryos. Similarly, termination of incubation at d 12 or 15 after turning until these days also resulted in significant amount of remaining albumen, and lower body weights of embryos, suggesting that lower use of albumen may be the cause of lower hatchability and viability of embryos as previously observed by Tona et al. (2001) when eggs were transferred to hatcher baskets at 15 to 17 d of incubation. The large difference in remaining albumen or embryo weights at d 12, 15, and 18 of incubation among eggs of the T12, T15, and T18 groups attest to the quality of chick that may emerge from these eggs and may influence their posthatch performance. Even though eggs were all incubated until d 18 with turning treatments of T12, T15, or T18 and albumen use completed, embryo weights were still different among turning groups (Figure 4C), suggesting that egg turning and its duration influence other internal factors besides albumen use. Hence, a turning treatment of 12 d was required for complete use of the albumen, but for optimum growth of the embryo turning should continue until d 18 (T18). It is therefore hypothesized that turning on its own may have a stimulatory effect on embryo growth rate, which effect may be inhibited as soon as turning is stopped.

Increasing levels of corticosterone with the advancing age of the embryo found in this study are consistent with those reported previously and may be due to the increasing functionality of the HPA axis (Wise and Fry 1973; Freeman and Flack 1980; Decuypere et al., 1989). At d 18 of incubation, corticosterone levels obtained, irrespective of turning duration, were in the range of those from the results of Decuypere et al. (1989).

Although the HPA axis is functional at the 13th day of incubation (Freeman, 1974), the presence of glucocorticoids in the blood of chick embryos is detectable between the 10th and 15th days of incubation. Then the amounts of these glucocorticoids increase in blood plasma between the 15th and 19th days of incubation (Avrutina and Kisljuk, 1982; Avrutina et al., 1985). Because corticosterone levels at 60 or 150 min after injection of saline solution were similar to corticosterone levels in uninjected embryos, higher corticosterone levels following ACTH injection, especially 60 min after injection, indicated that chicken embryos were stimulated by ATCh as previously demonstrated by Avrutina et al. (1984). Our data suggest a rapid increase in corticosterone response to ACTH to a peak level followed by a decrease to basal levels within a certain period of time. The pattern of responsiveness in our study is consistent with that reported previously by Decuypere et al. (1989). Thus, overall lower corticosterone levels at d 12 of incubation might have been due to incomplete development and lower activity of the HPA but also indicated that there was already a responsiveness to exogenous ACTH even before the actual functional HPA axis was established. The comparable corticosterone levels at 60 min after ACTH injection at d 15 and 18 of incubation confirm the hypothesis that the HPA was completely established and functional in 15- to 18-d-old embryos. However, after the peak response to ACTH, it was intriguing that the return of corticosterone to normal levels differed between d 15 and 18 treatments. It is tempting to suggest that a dose response can be translated into a longer sustained peak but not to a higher peak (Decuypere et al., 1989). Therefore, because a similar amount of ACTH was injected into the embryos at d 15 and 18, it can be hypothesized that the dose at d 15 was higher than the dose at d 18 due to weight differences between embryos at d 18 and 15. In contrast, at d 18 of incubation, the effect of turning treatments on corticosterone levels was apparent. Indeed, stopping egg turning at d 15 of incubation inhibited the down-regulation of corticosterone. These observations demonstrate clearly
that egg turning duration influences the regulation of stress in growing embryo and the functioning of the HPA. The higher level of corticosterone of the T15 group on d 18 of incubation at 150 min after ACTH injection matched those observed in the control group, suggesting that this was a natural phenomenon in T15 embryos. This result suggested that the embryos from the T18 group coped better with stress factors. It can be hypothesized that, after establishment of the HPA, turning events enabled the development of feedback control of corticosterone levels to cope with stress. Also, if eggs are turned toward the end of the establishment of the HPA, turning should continue for a few more days to allow embryo to cope with stress, which may optimize the activity of the HPA. Discontinuation of turning while the HPA axis is being established may present an additional stressor that can lead to physiological imprinting and altered responsiveness. This may be a basis for explaining our previous report in which we showed that eggs turned until d 15 of incubation had lower chick quality and posthatch performance. The lower performance may be related to these effects.

We concluded that egg turning during incubation allowed a rational use of albumen by the developing embryo and, therefore, influenced embryonic growth. The duration of turning also showed some relationship to stress control in the embryo and maturation of the HPA. Lower corticosterone ratios at 60 to 150 min after ACTH injection indicated that it is not advisable to stop turning around d 15 of incubation. Considering together the effects of turning on albumen use and the control of corticosterone levels, it is recommended to turn eggs during incubation at least until d 12 or 18 but not stopping turning at d 15 of incubation.

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