

Glucocorticoid Administration During Incubation: Embryo Mortality and Posthatch Growth in Chickens

R. Heiblum,^{1,*} E. Arnon,^{*} G. Chazan,[†] B. Robinzon,^{*} G. Gvoryahu,^{*} and N. Snapir^{*}

**Department of Animal Sciences, Faculty of Agricultural, Food and Environmental Quality Sciences, The Hebrew University of Jerusalem, P.O. Box 12, Rehovot 76100, Israel; and †Hafez-Hayim Poultry Integration, Kibbutz Hafez-Hayim 76817, Israel*

ABSTRACT The effects of glucocorticoids (GC) on embryonic mortality and posthatch BW were studied. Cortisol hemisuccinate or corticosterone in 0.1-mL vehicles were injected into the albumen of 7-d-old White Leghorn chicken embryos. Embryonic mortality rates and the age after injection at which death occurred were determined. When 0.02 to 20 μg cortisol per egg were injected in saline, total embryonic mortality rate increased in a dose-response manner, with a median lethal dose (LD₅₀) at 10 μg . Saline injection alone caused a similar mortality rate to that caused by injection of 2 μg cortisol (around 35%). However, whereas mortality among the cortisol-treated embryos was greatest on Days 16 to 18, most of the saline-treated embryos died around the time of injection. The

lethal effect of corticosterone, which is endogenous GC in adult chickens, was compared to that of cortisol by injecting both in the same vehicle (a saline:ethanol mixture) and was found to be similar. However, when 2, 10, or 20 μg of corticosterone was injected in oil, mortality rates were lower than those caused by the matching doses of cortisol in saline, probably due to the lower diffusion rate of the steroid out of the oil carrier. Hatch weight was significantly lower in chicks treated with 10 and 20 μg cortisol, and BW of the latter was lower compared with control throughout the 3-mo observation. In conclusion, cortisol and corticosterone are equally active in causing embryonic mortality. Posthatch BW is affected only by GC doses that are equal to or greater than the LD₅₀.

(Key words: glucocorticoid, chicken, embryo, mortality, body weight)

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INTRODUCTION

The presence of glucocorticoids (GC) is essential for tissue differentiation and maturation (Fisher, 1992). However, at high doses, GC may inhibit cell proliferation and growth (Orth et al., 1992) and, consequently, could be detrimental to development. In Japanese quail (Kaltner et al., 1993) and chicken embryos (Mashaly, 1991), GC administered during the first week of incubation impaired development, retarded growth, and increased mortality. In the present study, we examined the effect of a wide range of doses of cortisol, administered into the albumen, on embryonic mortality and posthatch growth. The advantage of cortisol over corticosterone, which is the major endogenous GC in adult chickens (Harvey et al., 1986), is that it can be provided in a water-soluble form (cortisol hemisuccinate; sodium salt), which ensures a faster diffusion of the steroid than if the GC is administered in oil as a vehicle.

Cortisol was found to be active in studies on the differentiation of retinal cells in the chicken embryo (Ben-Dror

et al., 1993). However, in adult chickens, corticosterone had greater affinity to the GC receptor in the oviduct (Groyer et al., 1985) and was more potent in suppressing adrenocorticotrophic hormone-induced steroidogenesis in adrenal cells (Carsia et al., 1983) than cortisol. Thus, the effects of cortisol and corticosterone on mortality were compared by injecting both in a mixture of saline and ethanol. In addition, the influence of the vehicle in which the GC was administered, whether in oil or saline, on embryonic mortality rates was determined. Different doses of cortisol in saline as a vehicle were injected, and embryonic mortality was compared to that caused by matching doses of corticosterone in oil.

MATERIALS AND METHODS

Birds and Injection Procedure

White Leghorn chicken embryos were used in the five experiments presented. They were incubated in a commercial forced-air rotating incubator at 37.5 C and 55 to 57%

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¹To whom correspondence should be addressed: heiblum@agri.huji.ac.il.

Abbreviation Key: CBG = corticosteroid-binding globulin; CORT = cortisol; CORTIC = corticosterone; GC = glucocorticoid; GLM = general linear model; LD₅₀ = median lethal dose; X-INCUB = a brief interruption in incubation.

humidity. Trays were positioned at a 45° angle, and eggs were rotated every hour by 90° along the short axis. GC solutions and vehicles were filter sterilized and injected on the 7th d of incubation, when differentiated adrenal cortex cells are already present (Romanoff, 1960) and after endogenous corticosterone could be detected (Siegel and Gould, 1976). Trays of 150 eggs each were removed from incubation, one at a time, and candled in a room at 25 C and 62% humidity, unless otherwise stated. Unfertilized eggs and dead embryos were replaced with viable ones, and trays were transferred to an adjacent room at 21 C and 62% humidity. Eggs were placed small end up; their tops were sterilized with 70% ethanol, and a hole, less than 1 mm, was drilled in the shell with a dental drill. Solution (0.1 mL) was injected into the albumen with a 23-ga needle. Holes were sealed with dental wax, and eggs were inverted and returned to incubation. The injection procedure lasted 20 to 30 min per tray. Eggs were transferred into hatching baskets on Day 19 of incubation. Temperature was gradually lowered from 37.3 to 36.6 C, and humidity was increased from 67 to 87%, with increasing airflow. At the end of a 21-d incubation, eggs that did not hatch were opened and examined. Age of mortality was determined according to Hamburger-Hamilton criteria (Hamburger and Hamilton, 1951). The number of unfertilized eggs and eggs with embryos that died before the time of injection (0 to 6 d) was deducted from the total number of eggs to obtain the number of viable embryos at the time of injection. Eggs with a cloudy, foul-smelling content were considered contaminated. Gross abnormalities were recorded. Surviving chicks were sorted by sex (using sex-linked feather phenotype) and only males were kept for rearing. Birds were wing-tagged and reared in cages until 3 mo of age, as previously described (Heiblum et al., 2000). Chicks were fed a commercial chicken feed ad libitum (3,050 kcal/kg, 21.5% protein; Product 1163²).

Experiment 1. Effect of Cortisol on Embryo Mortality. Cortisol-21-hemisuccinate-sodium salt (Product H-4881³) was dissolved in saline (0.85% NaCl). Seventy-five embryos per group were injected with vehicle (saline) or with doses of cortisol hemisuccinate in saline that provided 0.02, 0.2, 2, or 20 µg cortisol per egg (CORT-0.02, CORT-0.2, CORT-2, and CORT-20, respectively). A group of 136 embryos was removed from incubation into the same environmental conditions as those that were injected for 30 min, but this group was not drilled or injected (X-INCUB). One hundred fifty eggs that were not injected and their incubation was not interrupted served as controls. The age of embryonic mortality was determined for the eggs that did not hatch. With the exception of X-INCUB and CORT-0.02, chicks were weighed weekly, from Day 3 post-hatch (Day 1 being hatch day) until the age of 88 d. CORT-20 chicks were weighed for the first time on Day 10, due to their very poor condition at hatch.

Experiment 2. Effect of Low Dose of Cortisol Versus Corticosterone on Embryo Mortality. Solutions of 2 µg/mL cortisol (as hemisuccinate) or corticosterone³ were prepared in saline containing 0.1% ethanol. One hundred fifty eggs per group were injected with 0.2 µg/egg cortisol or corticosterone (CORT-0.2 and CORTIC-0.2, respectively). A group of additional 150 eggs was injected with the vehicle—saline + 0.1% ethanol (saline1). The age of embryonic death was determined. Chicks were weighed on day of hatch only. As the purpose of the experiment was to compare the effect of the two GC on embryonic mortality, control and X-INCUB groups were not included.

Experiment 3. Effect of High Doses of Cortisol Versus Corticosterone on Embryo Mortality. Solutions of 200 µg/mL cortisol or corticosterone were prepared in saline containing 4.3% ethanol. Groups of 75 eggs each were injected with 20 µg/egg cortisol or corticosterone (CORT-20 and CORTIC-20, respectively). The vehicle (saline + 4.3% ethanol) was injected into 50 eggs (saline2). X-INCUB and control groups were included for the purpose of monitoring their posthatch growth. The age of embryonic death was determined. Chicks were weighed at 3, 35, 55 and 87 d of age.

Experiment 4. Effect of Cortisol in Saline Versus Corticosterone in Oil. Corticosterone was dissolved in corn oil, and cortisol hemisuccinate was dissolved in saline. Eggs were injected with 2 µg GC for CORT-2 (n = 74) and CORTIC-2 (n = 75), with 10 µg GC for CORT-10 (n = 90) and CORTIC-10 (n = 75), or with 20 µg GC for CORT-20 (n = 192) and CORTIC-20 (n = 162). Fifty eggs were injected with saline, and 50 others were injected with oil. Eggs were not candled prior to injection. The age of embryonic death was determined. The growth of the chicks after hatch was not monitored.

Experiment 5. Effect of 2 and 10 µg per Cortisol Egg on Posthatch Growth. Groups of 150 eggs each were injected with saline or 2 µg cortisol per egg, and 300 eggs were injected with 10 µg cortisol in saline per egg. BW of CORT-2, CORT-10, saline, and control chicks were measured at 2, 13, 26, and 54 d of age.

Statistical Analysis

Data were analyzed using the SAS program (SAS, 1994). Embryonic mortality rates were analyzed using chi-squared analysis (SAS, 1994) followed by modified Bonferroni correction (Aickin and Gensler, 1996). Fisher's exact test results were used when chi-squared was not a valid test. Individual growth curves were constructed using a polynomial model, and groups were compared using general linear model analysis (GLM, SAS, 1994), followed by a pair-wise *t*-test on predicted values for a midpoint on the growth curve with modified Bonferroni correction. GLM followed by Student Neuman Keuls test were used to compare BW of different groups at hatch (SAS, 1994).

RESULTS

Effect of Different Doses of Cortisol on Embryo Mortality

Figure 1 shows the mortality rates at different embryonic ages (Days 7 to 9, Days 10 to 15, Days 16 to 18, and

²Matmor, LTD, Ad-Halom, 79258, Israel.

³Sigma-Aldrich, LTD, Rehovot, 76100, Israel.



FIGURE 1. Percentage and age of mortality of embryos injected with 0.1 mL saline (SALINE) or 0.02, 0.2, 2, or 20 μg cortisol (CORT) in saline on Day 7 of incubation. CONTROL = uninjected-uninterrupted controls; X-INCUB = uninjected with incubation interrupted for 30 min; N = the number of viable embryos at the time of injection. Significant differences compared with CONTROL, X-INCUB, and SALINE are marked with *, # and +, respectively ($P < 0.05$ in a chi-squared test with modified Bonferroni correction).

Days 19 to 21) and total mortality rate after injection of cortisol in Experiment 1. All groups were compared to control, X-INCUB, or saline in pair-wise chi-squared tests. The probabilities for each set of comparisons were adjusted using the modified Bonferroni correction, with only the comparisons with $P \leq 0.05$ considered significant, and were marked as such in Figure 1. Mortality rates increased with increasing doses of cortisol and were significantly higher than in controls at all the doses assayed ($P < 0.01$). Total mortality rate was also significantly higher in X-INCUB and saline than in the control ($P < 0.03$ and $P < 0.01$, respectively). In fact, more than one-third of the viable embryos at the time of injection died as a result of saline injection alone (almost as high as in CORT-2). Most of the deaths among the saline group occurred around injection time (between 7 to 9 d of incubation), whereas most of embryos injected with cortisol died 10 d after injection (on Days 16 to 18). Interestingly, mortality rates on Days 7 to 9 for CORT-0.02 and CORT-2 were significantly lower than for saline ($P < 0.02$).

Effect of Cortisol Versus Corticosterone

When the same doses of cortisol and corticosterone were injected in the same vehicle (saline + ethanol), they caused similar rates of mortality across the different embryonic ages (Experiments 2 and 3, Figure 2). However, when cortisol was injected in saline and corticosterone in oil, total mortality rates as a result of the former were higher at all the doses assayed and were significantly higher at 10 and 20 μg ($P < 0.02$ and $P < 0.001$ respectively, Experiment 4, Figure 3). The greater lethality of cortisol in saline was manifested at 10 to 15 d of incubation, when mortality in CORT-20 was significantly higher than in CORTIC-20 ($P < 0.001$). Also, at the embryonic age of 16 to 18 d, when

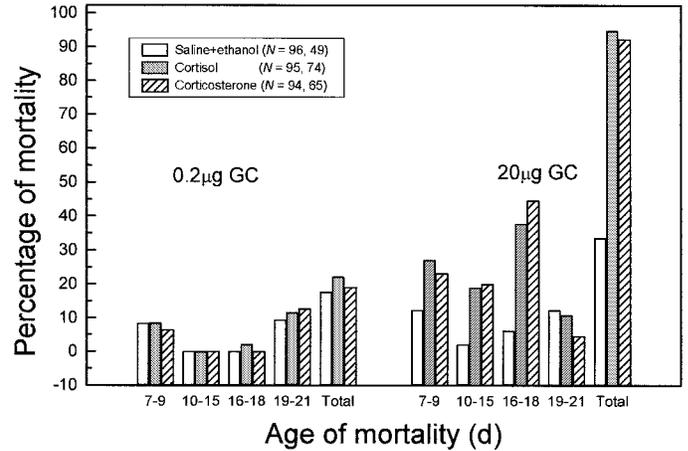


FIGURE 2. Percentage and age of mortality of embryos injected with cortisol or corticosterone in saline + ethanol on Day 7 of incubation (Experiments 2 and 3). A dose of 0.2 μg glucocorticoid (GC) per egg was injected in 0.1 mL saline + 0.1% ethanol (SALINE1) and 20 μg GC per egg were injected in 0.1 mL saline + 4.3% ethanol (SALINE2). N = the number of viable embryos at the time of injection. The two numbers inside the parentheses indicate the values of N for SALINE1 and SALINE2 (saline + ethanol) or for 0.2 and 20 μg GC, respectively.

mortality rates in CORT-2, CORT-10, and CORT-20 were significantly higher than in the groups injected with matching doses of corticosterone ($P < 0.03$, $P < 0.02$ and $P < 0.001$, respectively, for the matching corticosterone-cortisol comparisons). However, mortality rate soon after injection, was significantly higher in embryos injected with 20 μg corticosterone than in those injected with same dose of cortisol ($P < 0.001$). Total mortality rates caused by either of the two vehicles alone were similar (Figure 3 insert).

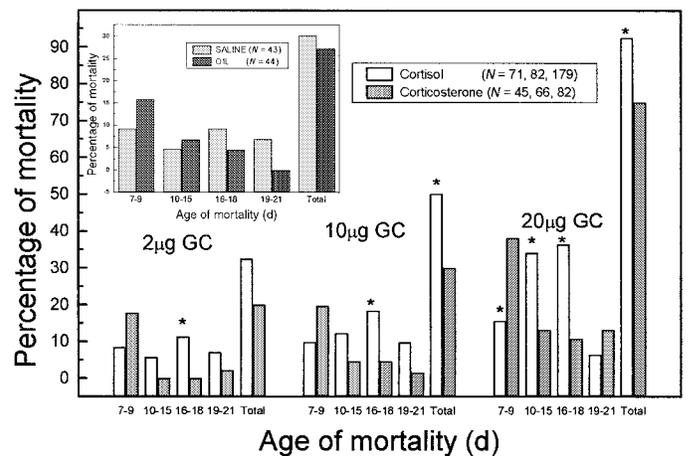


FIGURE 3. Percentage and age of mortality of embryos injected with 2, 10, or 20 μg cortisol in saline or corticosterone in corn oil on Day 7 of incubation (Experiment 4). Insert: percentage and age of mortality of embryos injected with 0.1 mL saline (SALINE) or oil (OIL) vehicles. N = the number of viable embryos at the time of injection. The three numbers inside the parentheses indicate the values of N for embryos injected with 2, 10, or 20 μg glucocorticoid (GC) per egg, respectively. *Indicates significant differences between the two GC at the same dose ($P < 0.05$ in a chi-squared test).

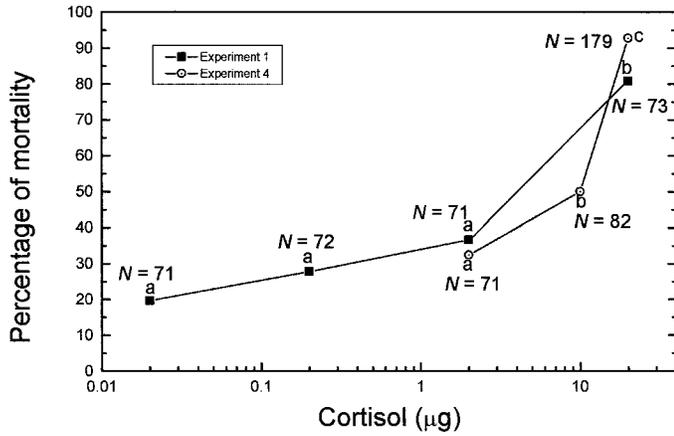


FIGURE 4. Percentage of total dead embryos out of viable embryos at the time of injection, after treatment on Day 7 of incubation with 0.02 to 20 µg cortisol per egg. *N* = the number of viable embryos at the time of injection (data from Experiments 1 and 4). Different letters indicate significantly different values ($P < 0.05$ in a chi-squared test with modified Bonferroni correction).

Dose Response of Embryo Mortality

Total embryonic mortality rates after treatment with different doses of cortisol in Experiments 1 and 4 are shown in Figure 4. In Experiment 1, mortality rate increased at a low constant rate between 0.02 and 2 µg and then rose significantly at 20 µg per egg (an increase of about 45%). The calculated median lethal dose (LD_{50}) from the line connecting the 2 and 20 µg points in that curve was 4 µg per egg. However, according to the results of Experiment 4, the LD_{50} was 10 µg per egg.

Microorganism contamination was found in very few of the dead embryos. In Experiment 1, for example, none of the embryos that died due to saline injection were contaminated. High concentrations of the two GC injected with same vehicle (Experiment 3) caused hemorrhages in allantois, body, and brain. Of the dead embryos in CORT-20 and CORTIC-20 treatments (Figure 3), 40 and 53.5%, respectively, had hemorrhages compared with only 12.5% of the dead embryos in the vehicle-injected group. Deformities such as unclosed skulls and enlarged brains were also observed in embryos that were treated with a high dose of GC. Incomplete yolk-sac retraction was typical in CORT-20 and CORTIC-20 surviving chicks. They all had lighter and matted looking feathers. Abnormal feathering was observed also in CORT-2 and CORT-10 chicks. Few cases of chicks whose leg were spread apart were observed in all injected chicks, including ones injected with saline. These chicks had great difficulty moving and did not survive past the first few days.

Posthatch BW

Growth curves of male chicks that did hatch in Experiment 1 are presented in Figure 5. Embryonic injection of saline or 0.2 µg, or 2 µg cortisol did not affect BW, as compared with control. However, embryonic treatment with 20 µg cortisol significantly inhibited growth through-

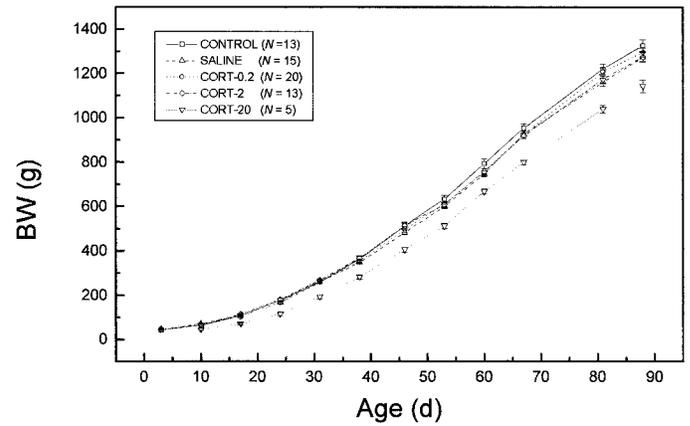


FIGURE 5. Mean body weight of male chicks that were injected with saline (SALINE) or 0.2, 2 and 20 µg cortisol (CORT) per egg on Day 7 of incubation in Experiment 1. CONTROL = uninjected-uninterrupted controls; *N* = the number of chicks.

out the first 3 mo posthatch, $F_{4,61} = 5.94$, $P < 0.0005$ (GLM on predicted weight at the age of 46 d). In Experiment 2, treatment with 0.2 µg GC injected in saline + 0.1% ethanol did not affect hatch weight, which was approximately 40.0 g in all the groups. In Experiment 3, hatch weights of CORT-20 ($n = 3$) and CORTIC-20 ($n = 2$) were 32.0 ± 1.0 and 31.0 ± 0.0 g, respectively, and were significantly lower than 40.1 ± 1.1 g, 42.3 ± 0.5 g, and 41.1 ± 0.5 g for control ($n = 21$), X-INCUB ($n = 23$), and saline2 ($n = 12$), respectively ($F_{4,56} = 7.72$, $P < 0.0001$). Growth of CORT-20 was inhibited throughout that experiment, $F_{3,34} = 4.92$, $P < 0.01$, whereas BW of X-INCUB and saline2 were no different than control (GLM on predicted weight at the age of 45 d, Figure 6). BW of the single surviving CORTIC-20 chick at the four time points was close to those of CORT-20 (Figure 6). Hatch weight of CORT-10 ($n = 36$) in Experiment 5 was also significantly lower than that of the other groups [37.9 ± 0.5 g versus 42.4 ± 0.5 , 41.5 ± 0.5 , and 40.9

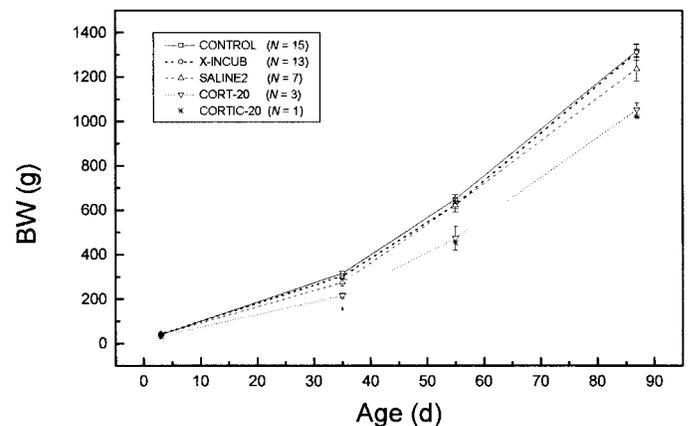


FIGURE 6. Mean body weight of male chicks that were injected with 20 µg cortisol (CORT) or corticosterone (CORTIC) per egg or with vehicle of saline + 4.3% ethanol (SALINE2) on Day 7 of incubation (Experiment 3). CONTROL = uninjected-uninterrupted controls; X-INCUB = uninjected with incubation interrupted for 30 min; *N* = the number of chicks.

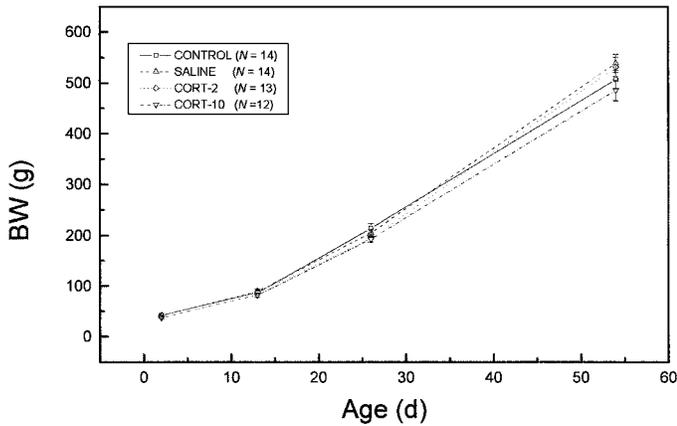


FIGURE 7. Mean body weight of male chicks that were injected with saline (SALINE) or 2, 10 and 20 μg cortisol (CORT) per egg on Day 7 of incubation in Experiment 5. CONTROL = uninjected-uninterrupted controls; N = the number of chicks.

± 0.7 g in control ($n = 50$), saline ($n = 46$), and CORT-2 ($n = 34$), respectively, $F_{3,162} = 11.92$, $P < 0.0001$]. However the growth curve of CORT-10 chicks (Figure 7) did not differ from that of other groups (GLM on predicted weight at the age of 26 d).

DISCUSSION

Five experiments were conducted. In Experiment 1, the effects of a wide range of doses of cortisol on embryonic mortality and posthatch growth were examined. In Experiments 2 and 3, the effects of low and high doses of corticosterone and cortisol were compared, when both were injected using the same vehicle; whereas, in Experiment 4, corticosterone was injected in oil and cortisol in saline. In Experiment 5, only posthatch growth, as affected by embryonic injections of cortisol, was monitored. Our results indicate that cortisol increases embryonic mortality rates in a dose-response manner with an LD_{50} at 10 μg per egg, that cortisol and corticosterone are equally as lethal when injected in the same vehicle, and that injection of corticosterone in oil is less detrimental than injection of cortisol in saline. BW at hatch was reduced by embryonic treatment with 10 and 20 μg GC, whereas BW during the first 3 mo posthatch was lower only in the embryos injected with 20 μg GC. Lower doses of GC did not affect hatch weight or growth throughout the assay period.

The doses of GC that were used in this study were estimated to range from around endogenous level up to 1,000 times that amount. Several authors could not detect any GC levels in the plasma of chicken embryos at Day 7 of incubation (Kalliecharan and Hall, 1974; Marie, 1981; Tanabe et al., 1986). However, Ericson and Domm (1969) have detected activity of 3β -hydroxysteroid dehydrogenase in primordial adrenocortical cells of 4-d-old embryos, and Siegel and Gould (1976) measured approximately 10 ng corticosterone/mL in the plasma of embryos of that age. Lower levels of corticosterone at 1.28 ng/mL and 0.5 ng/mL were found in the plasmas of 10- and 12-d-old

chick embryos, respectively (Tanabe et al., 1986; Marie, 1981). Assuming an even distribution of the injected GC in a 50-mL egg, the concentration of GC resulting from the injection of the lowest dose, 0.02 μg per egg, would be 0.4 ng/mL. This value is similar to the lowest reported endogenous level for chick embryo (Marie, 1981). Based on the same calculation, the highest dose, 20 μg per egg, would yield a GC concentration of 1,000 times the endogenous value.

Cortisol administered on Day 7 of incubation increased embryonic mortality rates in a dose-response manner. The mortality rate caused by injection of 10 μg cortisol/egg was 50%—similar to that obtained after administration of 12 μg corticosterone to fertilized eggs on Day 3 of incubation (Mashaly, 1991). The greatest lethality following GC administration occurred at Days 16 to 18 and also at Days 10 to 15 (Experiment 4). GC can bind to high affinity-low capacity corticosteroid-binding globulin (CBG) and to low affinity-high capacity albumin (Orth et al., 1992). Proteins in the egg albumen could have bound the GC and slowed down their immediate diffusion into the embryo. It is possible that with the progress of incubation, GC availability to the embryo increases, as between Days 10 to 12 of incubation, albumen begins to invade the amniotic fluid and the embryo ingests it (Romanoff, 1960). In the embryo, high affinity CBG, which is at zero prior to Day 10, rises sharply between Days 10 and 12 (Siegel and Gould, 1976), thus facilitating the transport of the steroid to target cells. It is also possible that teratogenic activity of GC increases during development, because of enhanced transcriptional activity. In the chick embryo, receptors for GC are present in the limb buds and face as early as the third day of incubation (Pavlik et al., 1986). However, the transcriptional activity of GC receptors may be developmentally controlled. For example, in the chick retina, GC can induce glutamine synthetase on Day 10 but not on Day 6 of incubation, although the level of GC receptor is similar at both ages (Ben-Dror et al., 1993). According to Woods et al. (1971), the hypothalamic-pituitary-adrenal axis in the chicken embryo is established at the beginning of the last week of incubation. Therefore, one may expect that sensitivity to GC will be manifested fully during that period, leading to high mortality at toxic doses of GC.

As mentioned earlier, GC are essential for embryogenesis. However, high exogenous levels of GC are teratogenic and toxic for the avian embryos in that they affect a wide range of systems and functions (Pavlik et al., 1986; Mashaly, 1991; Kaltner et al., 1993). In Japanese quail embryos, dexamethasone caused histological changes in the kidneys, depressed protein synthesis, and uric acid excretion (Kaltner et al., 1993). Cortisol may change elastin production and, therefore, can damage aorta and other blood vessels in embryos of chicken (Keeley and Johnson, 1987). Cortisol may also inhibit feather development in chicken embryos (Turque et al., 1997). These findings could explain the hemorrhages and abnormal feathering observed in embryos that were treated with high doses of GC in the present study.

Although corticosterone is the major GC in chickens, administration of cortisol or corticosterone, using the same vehicle, resulted in similar mortality rates. The similar activity of the two GC is in accordance with the fact that endogenous cortisol is present in chick embryo plasma, until Day 19 of incubation, at levels lower or similar to those of corticosterone (Kalliecharan and Hall, 1974; Tanabe et al., 1986). However, the vehicle in which GC is administered influences its lethality. Whereas mortality rates as a result of injection of oil or saline vehicles were similar, corticosterone in oil was less lethal than cortisol in saline, perhaps because the rate of release from the oil vehicle was much slower compared with the fast diffusion of the aqueous solution of GC.

We have found that high doses of GC were not only lethal to embryos, but also permanently reduced BW in the surviving chicks. Lower weights of chick embryos were also observed at doses of 12 μg corticosterone and higher (Mashaly, 1991). In general, GC cause growth retardation during development, probably through direct inhibition of bone, muscle, and connective tissues (Orth et al., 1992). It has been reported that inhibition of growth by pharmacological doses of GC involves suppression of release and activity of the pituitary growth hormone and of insulin-like growth factor and suppression of synthesis of local growth factors (Tonshoff and Mehls, 1997). The effect of prenatal GC on growth throughout life is a result of permanent changes in the hypothalamic-pituitary-adrenal axis (Seckl et al., 2000). However, growth was significantly retarded throughout the experimental period only in cockerels treated with 20 μg of cortisol, a dose that also resulted in a rate of 82 to 93% mortality during incubation, which indicates that BW is very highly conserved and cannot be easily manipulated by embryonic GC administration. According to the free hormone hypothesis (Orth et al., 1992), the biological activity of the steroid is proportional to the concentration of its free form. Binding to CBG and albumin and metabolism by the liver reduce the amount of free steroid (see Siegel and Gould, 1976). It could be that only at the highest dose was enough GC present in a free form at the critical period to affect the differentiation of the hypothalamic-pituitary-adrenal axis.

Our results indicate that the injection procedure itself was detrimental, as mortality in vehicle-injected embryos was significantly higher than that in uninjected controls. Microorganism contamination was not the major cause of mortality due to vehicle injection, as might be suspected. We suggest that a combination of aversive factors could have been deleterious to the embryo. Although the increase in NaCl in the egg due to saline injection was only 0.5%, ionic changes in the albumen could affect the density of the subembryonic fluid, which would affect the density of the yolk and impair transfer of nutrients to the embryo (Babiker and Baggott, 1992). Likewise, changes in the density of the albumen caused by the oil could also disrupt the other compartments of the egg. During the injection procedure, embryos were exposed to a lower ambient temperature for about 20 to 30 min. According to Suarez et al. (1996), this exposure was not sufficiently long to

affect mortality. Whereas embryonic mortality rate in their study increased significantly under continuous cold stress, starting on Day 8 of incubation, it did not under intermittent cooling (6 h every 48 h). Nevertheless, changes in heart rate and amnion contractions were observed in 6-d-old chick embryos during 30 min of exposure to a colder environment (Oppenheim and Levin, 1975). Exposure to a lower temperature could explain the increased mortality observed in X-INCUB embryos. Inversion of eggs, so that their small end was up during injection, could be another aversive factor. Hatchability of fertilized eggs incubated with their small end up is lower than for eggs set with their large end up (Bauer et al., 1990). Although in our study eggs were inverted only for 20 to 30 min, the turbulence during the inversion could have constituted an additional stress. Drilling and disruption of the membranes could constitute another stressful event. Fineman and Schoenwolf (1987) found that a brief (less than 10 s) perturbation during a windowing procedure in the eggshell led to death of chicken embryos. Windowing is highly teratogenic with predominantly neural tube defects in chicken embryos as a result (Mann and Persaud, 1979).

Epple et al. (1992) found accumulation of catecholamines in the allantoic fluid of 10- and 14-d-old chicken embryos in reaction to various stresses such as opening of the egg shell, asphyxia, handling, disturbance of allantoic fluid, and cooling (10-min exposure to 22 C). Adrenal catecholaminergic activity was found in 8-d-old chick embryos (Romanoff, 1960). Catecholamines can affect neural growth (Lankford et al., 1988) and can function as morphogens (Lauder, 1988). Hence, a sharp increase in embryonic catecholamines could alter development and might cause death. Another possible mechanism for the detrimental effects of stress is via cytokines that are released during tissue stress (Turnbull and Rivier, 1999). Prenatal administration of interleukin-1 β in rats caused permanent somatic and hormonal changes in fetuses (Gotz et al., 1993).

In Experiment 1, mortality around injection time was significantly lower in CORT-0.02 and CORT-2 compared with saline treatment. This phenomenon was not observed in subsequent experiments. The injection procedure in the first experiment lasted longer due to lack of experience, which could explain this discrepancy. Possibly, the increase in GC level helped the embryos to better withstand the stress involved in the injection procedure. Secretion of GC in response to stress has been suggested to reduce the ill effects of that stress (Munck et al., 1984), including suppression of cytokines (Turnbull and Rivier, 1999). Cortisol inhibition of catecholamine production (Kvetnansky et al., 1995) may be part of the mechanism. This point merits further investigation, as the chick embryo could serve as a model for investigation of the interaction between the adrenocortical system, cytokines, and the sympathetic system during the stress response.

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