Journal of Animal and Veterinary Advances 6 (4): 500-504, 2007

Evaluation of Experimental Methods for Manipulating Chicken Egg Hormone Content Using Injections

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Abstract: To study the effects and mechanisms of pre-hatch stress in hens it is important to have reliable methodology that can be used to illuminate effects of embryonic hormonal exposure without seriously compromising the viability of embryos. This experiment aimed to develop methods that can be used for injecting corticosterone into eggs prior to incubation without greatly reducing hatchability. The injection method involved application of a small amount of silicone sealant to the apical end of eggs, insertion of a needle through this seal to a depth of 5 mm into the albumen and injection of a sesame oil vehicle with or without corticosterone. It is shown that corticosterone can be injected with a resulting mortality of 38%. The applicability of these methods are discussed and will be further used to test for effects of corticosterone injections prior to incubation on behaviour and morphology in laying hens.

Key words: Chicken, embryo, mortality, prenatal stress

INTRODUCTION

Prenatal or pre-hatch stress in chickens is found when stress applied to a hen affects the development of her progeny. There are several approaches to studying effects of pre-hatch stress in chickens. Hens can be exposed to stressors around the time of egg laying, followed by physiological and behavioural studies of their progeny. However, this approach has the disadvantage that there is a large variation in the physiological responses of different hens reacting to the same stressor (Eskeland and Blom, 1979). To circumvent this problem, pre-hatch stress can also be studied using artificial manipulation of hormones thought to mediate pre-hatch stress. A relevant model of pre-hatch stress is to inject relevant doses of stress hormones directly into eggs before incubation. This latter approach has the advantage that the mechanisms are manipulated directly, thus shedding light on the effects of specific hormones. The main stress hormone in hens is corticosterone, a lipophilic steroid secreted by the adrenal cortex during stress. Because of the lipophilic characteristic of corticosterone this hormone must be injected in lipid- (Sockman and Schwabl, 2000; Heiblum *et al*., 2001) or alcohol-based (Burke, 1996; Sui *et al*., 1997; Bossis and Porter, 2000; Eriksen *et al*., 2003) vehicles.

However, before injection of hormones into eggs can be used to model pre-hatch stress there are a number of methodological problems that must be addressed. One potential problem is that injections may result in high embryo mortality caused by different aspects of the injection procedure. Some researchers fail to report hatchability after injection of saline-ethanol (1-70%) vehicle into eggs, sometimes because effects are measured prior to hatching (Sui *et al*., 1997; Bossis and Porter, 2000). Others report between 30% (Burke, 1996) and 82% (Eriksen *et al*., 2003) mortality after similar treatment of eggs. In all cases it is difficult to judge what effect injection of the saline-ethanol vehicle might have had. Pre-hatch exposure to ethanol may produce a range of toxic effects on the embryo and lead to dysfunctions of the central nervous system resulting in a range of neurological and behavioural deficits (Abel, 1982; Clarren and Smith, 1978). Given the potential side effects of ethanol, it may be preferable to use oil as a vehicle for fat-soluble stress hormones. Sockman and Schwabl (2000) reported a 40% mortality after injection of sesame oil into the yolk of canary (*Serinus canaria*) eggs, whereas Heiblum *et al*. (2001) reported a mortality of 27% (sesame oil vehicle) or 20% (2 μg Corticosterone (CORT)) after injection into the albumen of poultry hen eggs. Taken together, these studies suggest that methods using a sesame oil vehicle may produce lower mortalities than when using ethanol.

Another cause of embryo mortality may be either egg trauma or egg contamination. Contamination may occur

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at the time of injection but may also be caused by the loosening of previously used but inappropriate sealants such as tape (Sui *et al*., 1997) wound dressing (Schwabl, 1996) or wax (Dean *et al*., 1999; Heiblum *et al*., 2001; Eriksen *et al*., 2003). These problems may be partly solved by injection through a pre-dried silicone seal applied prior to injection, as suggested by Johnston *et al*. (1997). Mechanical damage associated with handling, needle insertion and vehicle injection may also cause some embryo mortality. Another source of damage may be the mechanical or structural changes caused by the injected volume. Because a smaller volume of the vehicle may reduce adverse effects on hatchability, it is also important to investigate whether volumes lower than that normally used (100 μL) might further reduce mortality.

In order to study effects of embryonic exposure to fat soluble hormones in the hen it is important to establish experimental methodology and procedures for injecting hormones into eggs. The following experiments therefore examine the effect of different methodological components involved in injection of exogenous corticosterone into eggs on embryonic mortality. Components of the injection procedure that were evaluated included application of a silicone sealant, insertion of a needle, injection of a sesame oil vehicle and injection of corticosterone through the seal at two different dose levels and vehicle volumes. The results indicate parts of the procedure that are important for hatchability and thereby establish an injection protocol that may be used to test for effects of prenatal exposure to altered hormone levels in hens.

MATERIALS AND METHODS

Eggs: Lohmann-selected Leghorn eggs were obtained from a commercial producer and were from Lohmann Selected Leghorns. Hatchability of eggs from parent stock is estimated to be 83% according to the breeding company (Lohmann, 2004). Eggs were stored at 16-20°C until incubation. They were then treated according to the protocol described below and placed on trays in a preheated forced-draft shelf-type incubator built to accommodate 700 eggs (America A/S-7700 Thisted, Denmark). The day into which eggs were placed into the incubator was termed day E0. The temperature was held at 37.8°C throughout incubation. The humidity was held at 55% days E0-E17 of incubation and 65% days E18-E22. Eggs were automatically turned every hour on days E4-E17 by rotation around their long axis. On day E18 eggs were placed onto paper-lined hatching baskets and were not turned for the remainder of the incubation process. All eggs were candled on days 7 and 14 and dead eggs were removed to avoid contamination. Removed eggs were opened to assess their stage of development according to the Hamburger-Hamilton criteria (Hamburger and Hamilton, 1951). Chicks were removed from the incubator on day E22.

Treatments Serum corticosterone concentrations in unstressed hens normally vary between 0.8 and 1.5 ng mL^{-1} (De Jong *et al*., 2001). The concentration of corticosterone in the egg yolk and albumen from unstressed hens is about 1.17 and 1.55 ng mL^{-1}, respectively (Eriksen *et al*., 2003). Serum corticosterone concentrations may be as high as 30 ng mL^{-1} shortly after exposure to an acute stressor (Johnston *et al*., 1997). Because corticosterone is lipophilic, its concentration in developing eggs from stressed hens may be a mean function of serum concentrations. Thus, the effects on mortality of 10 and 20 ng CORT mL^{-1} egg were examined. A random sample of eggs were weighed indicating an average weight of 66 g, 10% shell and 90% egg content (Beauving *et al*., 1981). Based on this estimated egg content, injection of 0.6 and 1.2 μg of corticosterone should result in biologically relevant doses of approximately 10 and 20 ng corticosterone mL^{-1} egg content.

For experiment 1, 600 eggs were distributed among 6 treatments: 1) control, 2) silicone, 3) silicone+hole, 4) silicone+hole+100 μL sesame oil, 5) silicone+hole+100 μL oil+0.6 μg corticosterone, 6) silicone+hole+100 μL oil+1.2 μg corticosterone. For eggs to which the silicone sealant was applied, a 5×5 mm dab of Casco Glass Silicon was placed on the apical tip of the egg and left to dry for 48 h. Sealant was used to limit gas exchange through the egg shell. For eggs in treatment 3 a hole was made with an empty 23 gauge needle through the silicone seal to a depth of 5 mm into the albumen, but no solution was injected. Treatments 4-6 utilized the same technique as treatment 3 but eggs were injected with the designated suspension via 1 mL syringes. After egg manipulation, they were placed in an incubator. Eggs from the different treatments were evenly distributed over shelves to avoid potential effects of uneven airflow in the incubator. Experiment 2 was conducted with 84 eggs which were injected with 0.6 μg corticosterone in 50 μL oil while another 84 eggs were injected with 0.6 μg in 100 μL oil. This experiment was performed at a later time and to test whether a smaller volume of oil might reduce mortality.

Oil and corticosterone solutions were prepared using 2.5 mg corticosterone (Sigma)[1]which was dissolved in 2.5 mL sesame oil (Sigma), warmed to 100°C and then sonicated for 15 min. Then 0.3 or 0.6 mL stock solution was added to 50 mL sesame oil to make final concentrations of 0.6 and 1.2 μg corticosterone per 100 μL

oil. Sesame oil to which no corticosterone had been added and corticosterone solutions were sterilized in a 180°C oven for 30 min. Syringes were filled with this solution as soon as it had cooled. Attempts were made to sterilize solutions and avoid bacterial contamination because a pilot experiment indicated bacterial growth in all eggs that died during incubation after injection of non-sterile solution. Experiments were performed with the permission of the animal experiments committee of the Department of Animal- and Aquacultural Science, Agricultural University of Norway (approved by the Norwegian Government) under reference number 04/03 and 12/03. These experiments were part of a larger project aimed at improving the welfare of layers by increasing knowledge about prenatal factors influencing the major welfare problems of hysteria, feather-pecking and cannibalism. Animals that hatched were euthanised by exposure to CO_2. Eggs from which no chicks emerged were opened and surviving embryos were euthanised by cervical dislocation.

Observations and statistics: Observations are presented as the cumulative percentage of embryos dying within 7 (E7), 14 (E14) and 22 (E22) days after eggs were put into the incubator, E0 being the day on which incubation was started. Differences in mortality between treatment groups were analysed using pair-wise chi-squared tests (Uitenbroek, 1997) which was used to compare the number of dead embryos for all treatment pairs within age classes (E7, E14 and E22).

RESULTS

As indicated in Fig. 1, there were no differences in mortality at E7 between eggs that were untreated, treated with silicone or punctured. Eggs injected with 100 μL oil, on the other hand, had increased mortality relative to the above-mentioned treatment groups ($p = 0.01$) within E7 of incubation. No significant differences were observed for eggs injected with 0.6 μg corticosterone in 100 μL oil compared to oil injections alone. However, eggs injected with 1.2 μg corticosterone in 100 μL oil had increased mortality compared to treatments 1-4 ($p = 0.01$) within E7. Treatment differences were similar at E14. At E22 of incubation mortality remained the same for untreated, silicone treated and punctured eggs but all other treatments resulted in higher mortality and were also significantly different from one another ($p \delta 0.04$ for all treatment comparisons). There was thus a trend to increasing mortality with increasing severity of treatment beginning with vehicle injection.

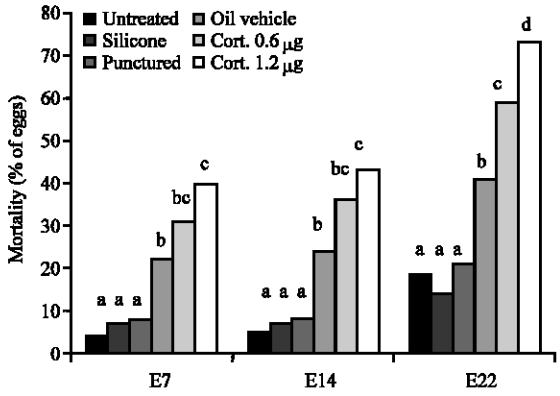

Fig. 1: Comparison of mortality over treatment groups presented as the cumulative percentage of eggs dying within E7, E14 and E22. 1) Untreated control, 2) silicone, 3) silicone and hole, 4) silicone, hole and oil, 5) silicone, hole, 100 μL oil and 0.6μg corticosterone 6) silicone, hole, 100 μL oil and 1.2 μg corticosterone. Values that were different within age class as indicated by pair-wise chi-squared tests are marked with different letters: a, b and c indicate significance at $p = 0.01$, whereas c and d are different at $p = 0.04$ ($N = 100$ per treatment)

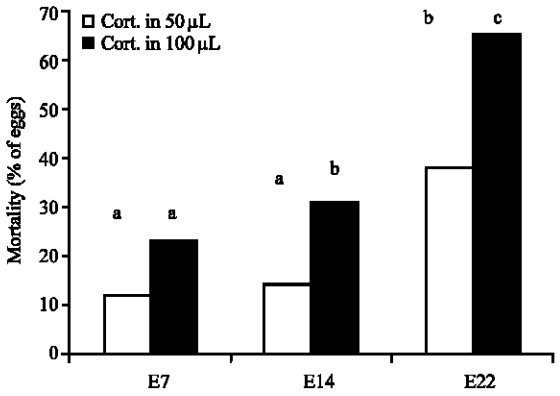

Fig. 2: Comparison of mortality over treatment groups presented as the cumulative percentage of mortality within E7, E14 and E22. Eggs were injected with 1) 0.6 μg corticosterone dissolved either in 50 ($N = 84$ eggs) or 2) 100 μL sesame oil ($N = 84$ eggs). Values that were different within age groups as indicated by pair-wise chi-squared tests are marked with different letters: a and b indicated a difference at $p \leq 0.05$, whereas b and c indicate a difference at $p \leq 0.04$

Figure 2 shows differences in mortality for eggs injected with either 50 or 100 μL of oil both containing

0.6 μg corticosterone. There were no differences in mortality between treatment groups within E7 ($p = 0.10$), but a smaller vehicle volume resulted in lower mortality both within E14 ($p = 0.05$) and E22 ($p = 0.04$).

DISCUSSION

Overall, parent stock hatchability is reported to be 83% (Lohmann, 2004). In this experiment, hatchability for untreated eggs was 82%, silicone treated eggs was 86% and punctured eggs was 79% and were not significantly different. The method of incubation used in the present experiment thus results in hatchability similar to that found under commercial conditions. Significant detrimental effects on hatchability were, however, found when sesame oil or corticosterone solutions were injected into eggs. Injection of the sesame oil vehicle increased mortality by 20% relative to untreated eggs, whereas injection of 0.6 or 1.2 μg corticosterone increased mortality by 40 and 50%, respectively. The second experiment indicated that a smaller oil volume further reduced mortality to 38%.

In a previous study performed in our laboratory (Eriksen *et al*., 2003) a diluted ethanol solution caused 82% mortality when the hole through which solution was injected was covered with wax after injection of a saline-ethanol solution. The use of sterile sesame oil as a vehicle in combination with the application of silicone sealant prior to injection of a small liquid volume thus resulted in a dramatic reduction of mortality. On the other hand, the methods used in the present experiment caused a higher mortality than that described by Heiblum *et al*. (2001) who showed that injection of sesame oil alone resulted in 27% mortality whereas the inclusion of 2 μg corticosterone resulted in a mortality of only 20%. However, these low estimates of mortality may be related to the fact that the results presented by these authors exclude eggs dying before E7, as injections were performed at this time. In the present experiment over 20% (vehicle), 10-30% (0.6μg corticosterone) and 40% (1.2 μg corticosterone) of embryos died during the first 7 days of incubation. This difference in the method of registering mortality between studies shows the importance of the present experiment. Had this early mortality also been excluded from our data set estimates of mortality would have been about 20% for oil, 26-30% for 0.6 μg CORT and 35% for 1.2 μg CORT and thus very close to the estimates of mortality produced by Heiblum *et al*. (2001). Johnston *et al*. (1997) injected a range of solutions into hens eggs and were apparently able to obtain hatchabilities very close to that found for uninjected controls. However, it is also difficult to compare the present findings with those of Johnston *et al*. (1998) because the authors did not include 'infertile' eggs in their calculations.

The main problem with the higher mortality of oil and corticosterone treated eggs is that chicks hatching from these eggs may have characteristics conferring resistance to detrimental vehicle or treatment effects. However, recent results from another of our studies suggests that CORT itself has no effect on hatchability if a 50 μL vehicle volume is used (unpublished data). This finding, in combination with the findings in the present experiment, suggests that detrimental effects of the oil vehicle may be controlled by injection of the vehicle in control groups.

CONCLUSION

The methods described here are judged by the authors to comprise a useful method for exposing chicken embryos to altered hormone levels and will be used in further experiments investigating effects on behaviour and morphology.

ACKNOWLEDGEMENT

An anonymous referee provided helpful comments to an earlier version of this study.

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