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Applied Animal Behaviour Science 107 (2007) 66–77
APPLIED ANIMAL BEHAVIOUR SCIENCE
www.elsevier.com/locate/applanim

Effects of stress in hens on the behaviour of their offspring

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Accepted 13 September 2006
Available online 9 October 2006

Abstract

This experiment aimed at testing the hypothesis that exposing hens to stress increases their corticosterone secretion and leads to elevated corticosterone concentrations in their eggs. Furthermore, exposure to stress and the associated changes in egg hormone content was predicted to alter offspring behaviour. Parental hens were 40 White Lohmann Selected Leghorns, half of which were exposed to an unpredictable feed restriction treatment. After application of the treatment, birds in the treatment group had higher concentrations of faecal corticosterone metabolites than birds in the control group. The treatment had no effect on the weight of treated birds or on the concentration of corticosterone in the yolk or albumen of their eggs. Sixty progeny (30 per treatment) were tested for effects on growth and behaviour. No effects of the treatment on the hatchability of eggs or progeny body weight were registered, but treated birds had a higher duration of tonic immobility in response to manual restraint and spent less time eating, when competing with birds from the control group for access to feed in a novel environment. These results confirm that pre-hatch stress may influence behavioural development in chickens. The lack of an effect on the concentration of corticosterone in eggs suggests that other steroid hormones in addition to or rather than corticosterone may mediate the effects of pre-hatch stress in chickens.

Keywords: Chicken; Maternal effects; Prenatal stress; Corticosterone; Fear; Competition

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doi:10.1016/j.applanim.2006.09.016

1. Introduction

Recent studies of avian species demonstrate that the concentration of maternally derived steroid hormones in eggs may vary according to laying date as well as with environmental conditions such as competition, food availability, maternal parasite load, and exposure to novel or challenging surroundings (Downing and Bryden, 2002). Maternally derived corticosterone, androgens and oestrogens have been found in all avian eggs that have been analysed for these hormones (Groothuis and von Engelhardt, 2005; Groothuis et al., 2005) and thus represent a powerful mechanism by which the mother might influence the phenotype of her offspring. Most of the research on hormonally mediated maternal effects in wild avian species has focused on androgens (Schwabl, 1993, 1996; Sockman and Schwabl, 2000; Sockman et al., 2001; Daisley et al., 2005), which appear to speed up embryonic development, and boost muscle growth and the begging rate (Gil, 2003). Several studies have tested for the effect of embryonic corticosterone exposure in domesticated chickens. This treatment appears to reduce the growth rate, increase fearfulness, reduce aggressiveness, reduce competitive ability and reduce problem-solving or motor ability (Eriksen et al., 2003; Hayward and Wingfield, 2004; Janczak et al., 2006; Lay and Wilson, 2002). Although some research indicates that stress in laying hens may increase the concentration of corticosterone in incubating eggs (Downing and Bryden, 2002), other experiments indicate that this may be unlikely (Rettenbacher et al., 2005), illustrating that work documenting the relationship between environmental factors and the steroid content of eggs is notoriously difficult despite some promising findings (Gil, 2003). Although effects of embryonic hormone exposure on the behaviour of chickens has been documented no documentation is presently available on how exposure of laying hens to varying environmental conditions during the laying period might affect the behaviour of chicks hatching from their eggs. This type of study could have special relevance for the poultry industry, showing how non-genetic maternal effects in laying hen breeders might influence the development of behavioural and phenotypic characteristics that are related to progeny welfare and productivity (Janczak et al., 2006). Environmental factors clearly modify the secretion of glucocorticoids and androgens (Weinstock, 1996; Wingfield et al., 1990). In addition to the stressing effects of fear, induced by sporadic contact with humans (Jones, 1996), conventional laying hen breeders may also be exposed to social stress caused by high bird densities (Downing and Bryden, 2002) and a considerable amount of frustration caused by living in a restricted environment (Duncan and Wood-Gush, 1971, 1972). Laying hen breeders are thus regularly exposed to variation in several environmental factors that could potentially cause steroid-mediated maternal effects on the development of behaviour in their progeny. Documentation of such effects can be used as an incentive for further improving the living conditions of laying hen breeders.

Duncan and Wood-Gush (1971, 1972) showed that covering hen's feed with a Perspex cover induced escape attempts and stereotyped pacing, and de Jong et al. (2003) showed that chronic feed restriction in broiler breeders is associated with increased secretion of corticosterone. The effects of short term feed restriction, as for other forms of stress, may also be exacerbated by a lack of predictability (Weiss, 1970, 1971). In the present experiment we therefore chose to subject hens to an intermittent and unpredictable feed restriction treatment. The validity of this model of stress was then validated by testing for effects on the hen's physiological stress response as indicated by production of faecal glucocorticoid metabolites (Rettenbacher et al., 2004, 2005) as well as testing for effects of the treatment on the corticosterone content of eggs. Because we were interested in documenting effects of pre-hatch stress in adult laying hens the birds were only tested as adults. Effects on the behaviour of adult progeny were measured as tonic immobility,

which was used as an indicator of fear (Jones, 1986), and competition for access to feed, which was used as a measure of competitive ability (Janczak et al., 2006). These behaviours were chosen as they are affected by embryonic exposure to corticosterone, which is a model of pre-hatch stress in laying hens (Janczak et al., 2006), and because fearfulness and the ability to compete are generally altered by exposure to prenatal stress also in mammals (Braastad, 1998). Based on our earlier experiments (Janczak et al., 2006) the treatment was predicted to increase fear and reduce competitive ability in progeny.

2. Materials and methods

2.1. Housing of parent birds

Forty female White Lohmann Selected Leghorns were loose housed from hatching to 16 weeks of age in a solid-walled pen on the floor that measured 3.2 m × 3.0 m, at a density of 4.2 birds/m^2. Wood cuttings were used as bedding and conventional feed and water provided *ad libitum*. The temperature in the room was 34 °C when the chicks were placed into the pens and was then gradually reduced to 21 °C at 4 weeks of age. The light cycle was changed from 24 h light the first day of age to 8 h of light at 8 days of age. Birds were moved at 16 weeks of age to individual cages measuring 24 cm × 45 cm, where they were housed singly for the rest of the experiment. Birds in these cages had access to individual water nipples and feed troughs. At 18 weeks of age the light cycle was increased by 1 h/week until birds had 16 h of light. This was done in order to induce the laying cycle. Lights were turned on at 07:00 h.

2.2. Treatment of parent birds

At 26 weeks of age birds were allotted to a treatment or a control group, one treatment and one control bird living in adjacent cages where they had visual contact with neighbouring birds. Control birds were fed *ad lib* and thus had 16 h of access to feed, whereas treated birds had only 10 h of access to feed. This treatment was continued over 11 days. For treated birds the feed was covered with Plexiglass for 6 h/day. This was done to induce stress in the form of mild feed restriction and frustration, as birds could see and smell the feed but could not eat it during the time it was covered. The treatment was made unpredictable to ensure that birds would not so easily habituate to the treatment. The 6 h interval of the light cycle during which feed was not available to treated birds was distributed randomly over the 16 h light cycle, either during 07:00–13:00, 12:00–18:00 or 17:00–23:00 h. All treatment birds were exposed to this treatment at the same time period each day, and the period was chosen randomly prior to each day by drawing numbered bits of paper from a jar.

The day on which the treatment was started was termed day 0, and the last day on which the treatment was applied was called day 10. Faeces and egg samples, from 20 treated and 20 control hens, were collected on the day the treatment started (day 0), as well as days 4 and 10. Twenty eggs per treatment group (5 eggs/hen) were also collected for incubation on each of days 5–9. If a hen did not lay an egg on one of these days an additional egg from the same hen was collected for incubation on day 11. Rettenbacher et al. (2005) observed the highest concentrations of corticosterone in the eggs of hens 5 days after feeding them corticosterone. Eggs collected from the treatment group before day 5 were therefore not expected to have elevated concentrations of corticosterone, whereas eggs collected from this group on days 5–10 were expected to contain more corticosterone than eggs from control birds. Egg samples were collected from eggs on days 0, 5 and 10 in order to produce samples from all birds representative of basal (day 0) and treatment (days 5 and 10) conditions. Birds were weighed at 20 weeks of age, 6 weeks before the treatment was applied and again at 28 weeks of age, shortly following termination of the experimental treatment to test for effects of the treatment on body weight. All hens were inseminated every other day between 14:00 and 17:00, starting 5 days before the treatment started. Semen was collected from 10 different cocks immediately prior to insemination of hens. Insemination constituted removing hens from their home cages and holding them

around the body with the tail upwards. Pressure was then applied to the birds' body in order to expose the vagina into which 100 μl of semen was deposited from a syringe to a depth of 15 mm. After the end of the experiment the hens were distributed to several private persons who kept them under free-range conditions for egg production purposes. This experiment was part of a larger project aiming at understanding and reducing welfare problems related to the development of hysteria, feather pecking and cannibalism in hens. It was performed with the permission of the animal experiments committee of the Department of Animal and Aquacultural Sciences, Norwegian University of Life Sciences (approved by the Norwegian Government) under reference number 01/05.

2.3. Incubating eggs

The 200 eggs that were to be incubated were stored at 16–20 °C as suggested by North (1984) until they were placed into a preheated, shelf-type, forced-draft incubator designed for 700 eggs (America A/S-7700, Thisted, Denmark) at the end of the 11-day treatment. The incubator temperature was 37.8 °C, and the humidity was held at 55% from day E1 (day 1 of embryonic development, i.e. the day the eggs were placed in the incubator) to E17 and 65% from day E18 to hatching. On days E4 to E17, the eggs were automatically turned once a day. On day E18 they were put onto paper in hatching trays. The chicks were removed from the incubator 22 days after they were put in. Hatchability was registered as the number of eggs that produced viable chicks.

2.4. Housing of progeny birds

Treated ($N = 60$) and control ($N = 60$) birds were loose-housed throughout the experiment in the same pen on the floor as described above for parental birds. Because 65 control and 71 treated chicks were hatched, 5 control and 11 treated birds were removed from the experiment at hatching to standardize treatment sizes. The density of birds was thus 12.5 birds/m^2 until 4 weeks of age. For identification of individuals and groups, birds were fitted with numbered leg bands. Males were removed from the experiment when they could be identified based on comb size at 4 weeks of age. The number of females was thus reduced to 30 per treatment and the density was reduced to 6.3 birds/m^2. Lights were increased to from 8 to 12 h per day, starting when birds were 18 weeks old. The light cycle started at 05:00 h. Birds were weighed at hatch as well as at 1, 4 and 26 weeks of age. All birds were tested in a tonic immobility test at 22 weeks of age and in a competition test at 23 weeks of age as described below.

2.5. Egg and faecal sampling

Samples from yolk and albumen of eggs were taken on the day eggs were collected in order to measure corticosterone concentrations. After the egg was opened onto a plate a pipette was applied to the outermost layer of the albumen and then the yolk in order to collect a 5 ml samples of each, but neither the yolk nor the albumen were homogenized prior to sampling. After collection samples were stored at −20 °C for 1–2 months prior to analysis, which is a standard method of storing samples used for steroid hormone analysis. On the night before collection of faeces a cardboard plate was placed under the cage floor to catch faeces. Faecal samples were collected the next morning when lights came on at 7:00. The cardboard was first removed from all cages and placed on the floor. Samples were then placed into a Ziploc bag and stored for 2 months at −20 °C before analysis.

2.6. Assay of corticosterone in eggs and its metabolites in faeces

The radioimmunoassay (RIA) used for corticosterone measurement in egg samples is described in detail by Lofthus et al. (1986). Before assay the thawed chicken egg yolk and albumen samples were extracted with diethylether. A specific corticosterone antiserum was used in the RIA (Cat. No. 07-120016, ICN, Irvine, CA, USA). The intra-assay coefficient of variation was 8.0% and the inter-assay coefficient of variation was 10.0%. The lower limit of detection for the analysis used in the present study was 0.2 ng/ml albumen or yolk.

To analyse corticosterone metabolites in the faeces, 0.5 g of the homogenised sample was suspended with 5 ml of 60% (v/v) methanol by shaking for 30 min. Following extraction, aliquots (after dilution with assay buffer 1:10), of the supernatant were measured with a cortisone EIA to determine the amounts of corticosterone metabolites in the droppings. This EIA has been established and validated by Rettenbacher et al. (2004) for measuring metabolites with a 3,11-dione structure. The intra- and inter-assay coefficients of variation were 10.0 and 14.1%, respectively. Taking the dilution factor into account, the lower limit of detection was 10 ng/g faeces.

2.7. Tonic immobility testing

Each experimental bird was tested once in a tonic immobility test at 22 weeks of age. Testing was distributed over 3 days. Prior to testing the lights were dimmed to 4–5 lx as this was observed to attenuate flight reactions in birds and ease capture. The test bird was collected from the pen and carried out of the pen into the adjoining hallway. Lights were then raised to 20 lx and testing started. The light intensity during testing was held above the basal light intensity of 15 lx in order to ensure that the birds perceived and reacted to the human. The bird was placed on its back onto a V-formed cradle and held by the experimenter with one hand over the sternum and one gently covering the bird's head. The test bird was held for 15 s and then slowly released. A person sitting on a chair in front of the bird recorded the duration of tonic immobility from the time the bird was released. Up to three successive attempts to induce tonic immobility were made if the bird stood up within 10 s of release. The observer recorded the number of induction attempts. Birds were carried between 3 and 6 m from the room to the test apparatus and the experimenter's head was 1 m from the bird during behavioural observation. One control and one treatment bird were tested successively, 20 birds per day.

2.8. Competitive ability testing

Each experimental bird was tested once in a competition test at 23 weeks of age. Testing was started 5 days after tonic immobility testing was completed and was performed over a 3 day period between 10:00 and 14:00. A feeder designed for the test was placed into the bird's home pen starting 3 days before the first bird was tested in order for hens to habituate to its appearance. This feeder was identical to the round feeders in birds' home pen but was surrounded by wire. At one side was a slit in the wire that was 5 cm wide and 20 cm high, allowing only one bird to access feed within the feeder at a time. On each morning prior to testing all feeders were removed from the home pen at 8:00, 3 h after lights on, and the test feeder placed into the centre of a 1.5 m $\times$ 1.5 m $\times$ m test arena in the hallway just outside the bird's home pen. The opening in the feeder apparatus was placed at 90° to the front of the test arena. The arena had four solid walls and a front composed of a 150 cm high wire partition.

Before testing the lights in the room were dimmed as described for tonic immobility testing and one treated and one control bird was collected from the home pen. The lights were then turned up and the two birds were placed into the test arena and observed for 10 min from the time they were released. The observer sat 1 m from the front of the arena. Activities recorded included the duration of time each bird had its head inside the slit in the feeder apparatus, the duration of time each bird was on the half of the arena closest to the observer and the number of aggressive pecks (defined as directed at the head of the receiver and resulting in avoidance responses in the receiver) delivered by and received by each bird. The birds' position relative to the observer was defined on the basis of both its feet having crossed an imaginary line dividing the front and back half of the test arena. The duration of the time each bird had its head inside the feeder was used to calculate the total duration of time a pair of birds spent eating (total eating duration). These values were used to calculate the percentage of total eating duration that birds of each treatment spent eating, which was used as a measure of competitive ability. If neither bird in the same test ate they were assigned a percentage of total eating duration of 0. The duration of time spent near the observer was used to calculate the percent of the test duration spent near the observer.

2.9. Statistics

The behavioural data showed deviations from normality according to a Kolmogorov–Smirnov (K–S) test and treatment groups were therefore compared using the Mann–Whitney *U*-test (equivalent to the Wilcoxon rank sum test; Proc NPAR1WAY; SAS Institute Inc., 2000). Because the sample sizes were large, a normal approximation of the Mann–Whitney *U*-test was used and results are presented as *U*- and *z*-values. This test was one sided because we predicted prenatally stressed birds to be more fearful and less competitive based on earlier experiments (Janczak et al., 2006; Braastad, 1998; Weinstock, 1996).

The remainder of the data conformed to the assumptions of normality and were therefore analysed using analysis of variance (Proc GLM; SAS Institute Inc., 2000).

The data on the concentration of corticosterone in eggs and corticosterone metabolites in the faeces, as well as data on the weight of parental birds was analysed using a repeated measures ANOVA testing for the effects of treatment and day (or age) as well as the interaction between these factors. For models that were significant when testing for effects on corticosterone and its metabolites the treatments were subjected to a post hoc comparison of LSmeans testing for differences between control and stress treatment means for days 4 and 10. For data on the body weight of progeny, effects of age and treatment were tested. Effects on the hatchability of eggs were tested using the χ^2-test. A number of hens (day 0: control 2, treatment 2; day 4: control 2, treatment 1; day 10: control 5, treatment 3) did not lay eggs on the days on which they were collected for corticosterone measurement and were treated as missing in the statistical analysis.

3. Results

3.1. Hatchability of eggs

A total of 100 eggs from hens in the control-group and 100 eggs from hens in the treatment-group were incubated. The hatchability was 65% for control eggs and 71% for treated eggs and these values were not significantly different ($\chi^2 = 0.827$, d.f. = 1, $P = 0.36$). Equal numbers of male and female chicks were hatched.

3.2. Weight of treated birds and their progeny

Parental birds in the control group weighted 1641 ± 35 g (mean ± S.E.) and the weight of those subjected to the stress treatment was 1650 ± 31 g at 20 weeks of age. At 28 weeks of age the weight of control birds was 1851 ± 48 g and the weight of treated birds was 1782 ± 43 g. Bird weight increased over time ($F_{80,1} = 18.31$, $P = 0.0001$), and neither the treatment ($F_{80,1} = 0.56$, $P = 0.46$), or the interaction between treatment and age ($F_{(80,1)} = 0.98$; $P \leq 0.33$) affected body weight. The body weight of progeny at hatch, 1, 4 and 26 weeks of age are presented in Table 1. There was no effect of the treatment on body weight ($F_{238,1} = 0.00$, $P = 0.97$).

Table 1
Body weight of progeny at hatch, 1, 4 and 26 weeks of age

	Control	Treatment	*t*	*P*
Hatch	40.32 ± 0.62	39.42 ± 0.66	0.99	≤0.32
1 week	70.05 ± 2.10	67.10 ± 2.34	0.94	≤0.35
4 weeks	383.90 ± 8.47	383.80 ± 8.91	0.01	≤0.99
26 weeks	1788.37 ± 30.69	1806.10 ± 39.56	−0.35	≤0.72

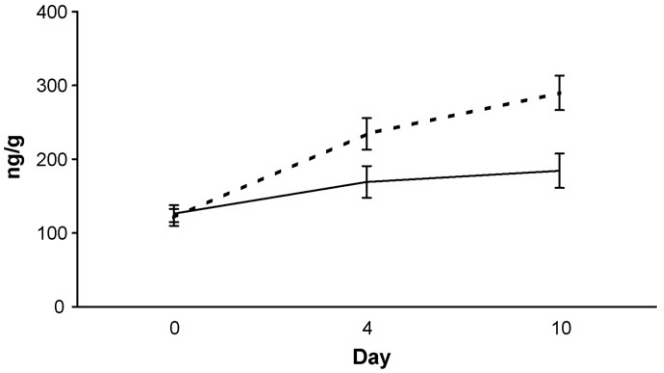

Fig. 1. The concentration of corticosterone metabolites in the faeces of hens measured on three different days, once before (day 0) and twice during (days 4 and 10) an 11-day period during which unpredictable feeding regime was applied to 20 treated hens (dotted line) starting on day 0 but not 20 control hens (solid line).

3.3. Faecal corticosterone metabolites and corticosterone in eggs

The effect of the treatment and time on the concentration of faecal corticosterone metabolites was significant ($F_{122,3} = 17.96$, $P = 0.0001$; Fig. 1). The treatment tended to affect the concentration of faecal corticosterone metabolites ($F_{122,1} = 3.36$, $P = 0.07$). The effect of time was significant ($F_{122,1} = 33.81$, $P = 0.0001$) and there was also a significant interaction between the treatment and time, the concentration of corticosterone metabolites rising only slightly over time for the control group but increasing more dramatically over time for the treatment group ($F_{122,1} = 8.07$, $P = 0.01$; Fig. 1). Post-hoc comparison of LS means for days 4 and 8 indicated that the level of metabolites was higher for the treatment group than for the control (control mean ± S.E.: 176.71 ± 1589, treatment: 262.02 ± 15.89, $t = -3.80$; $P = 0.0.001$). The variables treatment and time were not related to the concentration of corticosterone in the egg yolk ($F_{116,3} = 0.93$, $P = 0.43$; Fig. 2a) or albumen ($F_{116,3} = 0.68$, $P = 0.57$; Fig. 2b).

3.4. Effects of treatment on progeny behaviour

There was no difference between the control and treatment groups in the amount of inductions necessary to induce tonic immobility (Mann–Whitney U-test: $U = 902.50$; $z = -0.29$, $P = 0.39$, Table 2) but the duration of tonic immobility was higher for birds in the treatment group ($U = 753.00$; $z = -2.39$; $P = 0.01$; Table 2).

Eight of the 30 pairs of treated and control birds (16 subjects) that were tested in the competition test spent no time eating and were given a score of 0. The treatment reduced the hens' ability to compete for access to feed ($U = 1034.00$, $z = 1.87$; $P = 0.03$; Table 2). This effect did not seem to be related to aggressive behaviour as there was no effect of the treatment on the number of aggressive pecks birds delivered to the head of the opponent in this test ($U = 891.00$, $z = -0.41$, $P = 0.34$). There was no difference between treatment groups for the percent of time spent by hens closest to the observer in the test apparatus ($U = 977.00$, $z = 91.00$, $P = 0.18$).

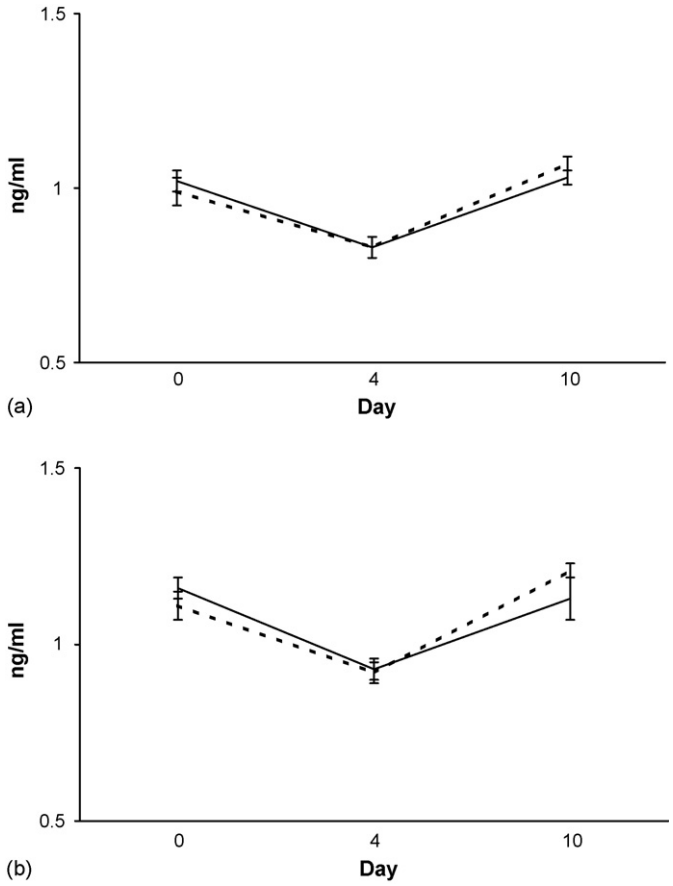

Fig. 2. The concentration of corticosterone in egg yolk (a) and albumin (b) samples collected on three different days, once before (day 0) and twice during (days 4 and 10) an 11-day period during which unpredictable feeding regime was applied to 20 treated hens (dotted lines) starting on day 0 but not 20 control hens (solid lines).

Table 2
Median, minimum and maximum values for behavioural records from the tonic immobility test (number of induction attempts and duration of tonic immobility) and competition test (percent of time spent eating, number of pecks and number of time spent in the half of the test arena closest to the observer)

Variable	Control			Treat			z	P
	Median	Minimum	Maximum	Median	Minimum	Maximum		
Inductions	1.0	1.0	3.0	1.0	1.0	2.0	−0.29	≤0.39
TI-duration (s)	69.0	0.0	249.0	120.0	13.0	407.0	−2.39	≤0.01
Eat (%)	33.5	0.0	100.0	0.0	0.0	100.0	1.87	≤0.03
Pecks	0.0	0.0	10.0	0.0	0.0	14.0	0.41	≤0.34
Near (%)	85.0	55.0	100.0	81.0	24.0	100.0	91.00	≤0.18

Thirty birds per treatment were tested.

4. Discussion

Hens having unpredictable access to feed were found to have increased concentrations of corticosterone metabolites in their faeces when compared to the levels found for *ad libitum* fed hens. This treatment of laying hens was also found to affect the behaviour of their adult progeny, increasing the duration of tonic immobility and reducing the percentage of time spent eating in the competitive ability test. Surprisingly, the treatment had no effect on the concentration of corticosterone in eggs.

Although the treatments and responses in the two experiments are somewhat different, the effect of unpredictable access to feed in hens on corticosterone metabolites in faeces in the present experiment is similar to the findings of de Jong et al. (2003), who showed that extreme feed restriction in broilers is associated with increased corticosterone secretion. Faecal samples were collected just after the lights were turned on in the morning in the present experiment. Because hens were subjected to no treatment or disturbance during the night it is therefore possible that the levels found in the treatment group reflect chronic elevations of corticosterone due to chronic stress. However, there are several other possibilities. Treatment on some days was applied between 17:00 and 23:00 h. This could cause corticosterone metabolite secretion during the night on some days even if birds were acutely but not chronically stressed. Another possibility is posed by the fact that feed restriction may elevate free corticosterone levels by decreasing circulating levels of corticosterone binding globulin without altering total corticosterone levels (Lynn et al., 2003). A third possibility is that the elevation in faecal corticosterone metabolites in our treatment group was caused by reduced gut motility instead of chronic elevations of corticosterone in the blood. These last two possibilities imply that the treatment may have caused an elevation of faecal corticosterone metabolites because of metabolic changes that were not necessarily associated with chronic stress. Further experiments would be necessary to rule out these alternative explanations.

The EIA used for analysis of faecal corticosterone metabolites in the present experiment has been successfully established and validated by Rettenbacher et al. (2004) for measuring the concentration of several metabolites with a 3,11-dioxo structure in chicken faeces. Compared with alternative methods it has the advantage that it detects large amounts of metabolites and shows a greater increase after administration of ACTH, thus allowing the detection of smaller changes in HPA activity (Rettenbacher et al., 2004). Corticosterone (4-Pregnene-11β-21-diol-3,20-dione) itself is not secreted by the chicken and kits designed for measuring corticosterone work because of antibody cross-reactions with reduced metabolites (Rettenbacher et al., 2004). This means that basal concentrations reported in the present study are not comparable to those reported by, for example Fraisse and Cockrem (2006; mean ± S.E. corticosterone: 12.45 ± 1.29 ng/g). The basal level of faecal corticosterone metabolites measured in the present experiment (control treatment mean ± S.E.: 126.29 ± 13.11 ng/g) is much higher than the basal metabolite concentrations for adult ISA brown laying hens reported by Rettenbacher et al. (2004; 44.64 ± 13.68 ng/g) and slightly higher than basal concentrations for in Lohmann traditional laying hen pullets reported by Jensen et al. (2006; 104.6 ± 10.5 ng/g). In addition to potential effects of strain and age, this difference in basal levels may partly be explained by the fact that all our experimental birds were inseminated every other day starting 5 days before the treatment in the present experiment. This handling could by itself be considered to be a rather strong acute stressor, as holding birds upside down has previously been shown to induce corticosterone secretion in hens (Eskeland and Blom, 1979). In addition to this putatively stressing and fear-inducing handling itself (Jones, 1996, 1997), the birds were also exposed to

daily visual contact with humans in connection with inspection, feeding, egg collection and sample collection. Aversive handling in conjunction with frequent visual contact with humans may explain the high basal corticosterone metabolite levels as well as the significant increase in the concentration of faecal corticosterone metabolites that was observed even for the control group. On the other hand, handling alone elevates serum corticosterone concentrations for only 40–45 min (Downing and Bryden, 2002), suggesting that long lasting elevations in corticosterone levels must be attributed to a combination of factors, and not to handling alone. Despite the high basal levels of faecal corticosterone metabolites found for the control group, it is clear that the feed restriction treatment did have an apparent effect above and beyond the insemination procedure itself. In light of this finding it is especially surprising that egg corticosterone concentrations were not elevated by the feed restriction treatment.

The prediction that the treatment should increase the corticosterone content of eggs was based on an earlier experiment indicating that maternally derived corticosterone is transferred in significant concentrations to the eggs of quail (Hayward and Wingfield, 2004). The egg levels of corticosterone we found were rather similar to previously reported blood levels of corticosterone (Cheng et al., 2001) and the lack of a treatment effect on egg corticosterone concentrations was unexpected. The lack of a treatment effect on egg corticosterone concentrations might be related to the fact that egg samples were not homogenized, which could be important given that the different layers of the egg may contain different concentrations of corticosterone (see Rettenbacher et al., 2005). The timing of the treatment relative to the timing of egg sampling could also be important because yolk is deposited over long time spans, whereas albumen is deposited during a few hours shortly following ovulation. The timing of a stressor applied to the hen can thus influence the concentration of corticosterone in the egg albumen. Another possibility is that high basal levels of stress, caused by insemination, could give a ceiling effect that could have masked the effect of the treatment on egg–corticosterone concentrations. However, the most obvious conclusion is that little corticosterone is transferred from hens' serum to their eggs as suggested by some previous studies (Hayward et al., 2005; Rettenbacher et al., 2005). It is thus impossible to explain the effects on progeny behaviour on the basis of variation in egg corticosterone concentrations in the present experiment.

Despite the lack of a significant treatment effect on the concentration of corticosterone in eggs in the present experiment, and the lack of a linear increase in corticosterone concentrations over time, it is clear that corticosterone concentrations at days 0 and 10 were different than the concentration of corticosterone measured in eggs on day 4. This shows that corticosterone concentrations in eggs may vary over relatively short time spans and be a factor that influences the behavioural development of the chicks. However, the cause of this variation is impossible to ascertain on the basis of the present experiment and should be the subject of future studies. Gil (2003) suggests in a recent review article on steroid mediated maternal effects in birds, that because there is strong evidence against a simple correspondance between steroid hormone levels in the female and her eggs studies on maternal effects should focus on the effect of direct modification of egg hormone content using injection procedures. Studies of this type are likely to shed considerable light on the effect specific maternally derived steroid hormones that are thought to vary with environmental conditions in birds.

Even though there was no effect of the treatment on egg corticosterone concentrations it did increase the duration of tonic immobility and reduced the percentage of time spent eating in a competitive situation in adult progeny. Tonic immobility was used as a measure of fearfulness (Jones, 1986). Because elevated fear is associated with increased activation of the HPA axis (Jones, 1996, 1997), this result indicates that maternal exposure to stress may increase the

sensitivity to fear-inducing stressors in progeny. The percentage of time that hungry birds spent eating in a novel environment was used as a measure of competitive ability under challenging conditions. It thus appears that pre-hatching stress has a negative effect on competitive ability in progeny. However, it should be mentioned that this behavioural response is probably also affected by underlying variation in fear induced by the novel surroundings. This finding thus corresponds well to the observed effect of the treatment on tonic immobility responses.

The observed effect of pre-hatch stress on tonic immobility and competitive ability in progeny is in line with previously documented effects of embryonic exposure to corticosterone in chickens (Janczak et al., 2006). However, because the present experiment shows no effect of the treatment on corticosterone content of eggs, future studies must be undertaken to illuminate the specific mechanisms underlying the observed effects. Taken together, the present findings support the view that pre-hatch stress may affect the development of behaviour in chickens. Future experiments should continue investigating whether different environmental factors and putative stressors influence the concentration of corticosterone in eggs, but should also test for environmental effects on other hormones such as gonadal steroids (Gil, 2003) and adrenal medullary hormones (Beuving and Blokhuis, 1997) as well as nutritional factors in chicken eggs.

Acknowledgements

This work was funded by the Norwegian Centre for Poultry Science and the Norwegian Research Council.

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