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Maternal corticosterone elevation during egg formation in chickens (*Gallus gallus domesticus*) influences offspring traits, partly via prenatal undernutrition

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ABSTRACT

The relationship between maternal stress during pregnancy in humans and the subsequent physical and mental health disorders in their children has inspired a wide array of studies on animal models. Almost all of these studies have used mammalian species, but more recently oviparous species in which the embryo develops outside the mother's body have received more attention. These new models facilitate disentangling of the underlying mechanism due to the accessibility of the prenatal environment, the egg. Studies in birds have found that maternal stress during egg formation induces phenotypic alterations in the offspring that hatch from these eggs. However, different offspring traits have been measured in different studies and potential underlying mechanisms are barely addressed. In this study we experimentally manipulated maternal corticosterone levels in laying hens. We found that mothers with experimentally elevated plasma corticosterone levels produced offspring that are smaller at hatching, less competitive, less fearful, have lower immunocompetence and higher plasma testosterone levels, as well as an alteration of visually guided behavioural lateralization. Earlier we have showed that eggs produced by these corticosterone treated mothers were lighter and contained lower concentrations of testosterone and progesterone in the yolk. While yolk hormones showed no correlation with any offspring traits, egg mass correlated positively with offspring's body mass from hatching until 10 days of age and hatching mass correlated positively with the offspring's ability to compete for food, indicating that prenatal under nutrition might mediate some effects of maternal stress.

1. Introduction

The environment can have profound and long lasting effects on the development of an animal's physiology and behaviour (Gilbert and Epel, 2009). During prenatal development animals are especially sensitive to such epigenetic regulation, which can happen when physiological changes in a female, due to changes in her environment, lead to alterations in the prenatal environment she provides for her offspring. Inspired by the potential detrimental effects of maternal stress during pregnancy on the offspring, maternal stress in mammals has been studied intensively over the past decades and several results show that the effects on offspring physiology and behaviour can be linked to the mothers' plasma glucocorticoid levels (for review see Weinstock (2005)).

Although most work on prenatally induced maternal stress has been conducted in mammals, it is evident from current literature (for review see Henriksen et al. (2011a)) that elevated plasma glucocorticoid levels in female birds during egg formation also can induce a variety of alterations in the offspring's phenotype. The use of birds as an alternative model can provide useful information due to their unique physiology and life history. In birds, embryonic development takes place outside the mother's body, in a sealed compartment, the egg, which is produced within a short time period. This facilitates correlating prenatal exposure to maternally derived hormones that are deposited into the egg (for review see Groothuis et al. (2005) and von Engelhardt and Groothuis (2011)) as well as nutrient supply to the embryo (e.g. yolk and albumen mass) with both the mother's stress levels during egg formation as well as with the offspring's phenotype.

So far, only a handful of studies (for review see Henriksen et al. (2011a)) have looked at the effects of elevated maternal plasma corticosterone (cort; the primary glucocorticoid in birds) during egg formation on offspring traits in birds. Maternal cort elevation has been reported to reduce offspring body mass (de la Cruz

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et al., 1987; Hayward and Wingfield, 2004) and (Love et al., 2005) but also to have no effect on body mass (Janczak et al., 2007) and (Satterlee et al., 2007). Maternal cort elevation has been reported to reduce the competitive ability of offspring (Janczak et al., 2007), increase fear behaviour (Janczak et al., 2007) or have no effect on fear behaviour but increase anxiety (Davis et al., 2008) and increase the HPA-axis response to a stressor (Hayward and Wingfield, 2004). Also, sex specific effects of maternal cort such as decreased immunocompetence in males (Love et al., 2005), reduced size and function of male reproductive organs (Satterlee et al., 2007) and reduced sex ratio at hatching (Love et al., 2005) have been reported. Since in most cases these findings are from single studies or are inconsistent between studies (for review see Henriksen et al. (2011a)), they do not allow one to draw general conclusions about the effects of maternal stress on avian offspring other than that such stress might have a wide array of effects on the chick. The inconsistencies between studies might be explained by differences in the duration and extent of maternal plasma cort elevation and in the time points during maternal stress at which eggs were collected for hatching.

Although several studies have looked at alterations in egg components in relation to the female's plasma cort levels (for review see Henriksen et al. (2011a)), no attempts were made to correlate these alterations in egg composition to phenotypic alterations in the offspring. Based on what is currently known, it is therefore not possible to resolve the mechanisms behind these maternal effects and whether all changes in behaviour or physiology can be linked to the prenatal environment (egg variables) or if alteration in one trait ultimately causes variation in another trait.

Overexposure of maternal glucocorticoids or reduced prenatal nutrition have been the main mechanisms used to explain effects of maternal stress on offspring in mammals, although frequent sex-dependent effects of maternal stress suggest a role also for sex-hormones (Weinstock, 2001). In an earlier study (Henriksen et al., 2011b) we found that female hens with artificially elevated plasma cort levels laid smaller eggs with lower concentration of yolk testosterone and progesterone. Effects of prenatal progesterone exposure have hardly received any attention, but lower concentrations of yolk testosterone are associated with reduced growth and competitiveness (Groothuis et al., 2005), which has also been found in offspring exposed to maternal stress. It therefore seems likely that prenatal testosterone could be involved in mediating the effects of maternal cort elevation.

We performed an experiment in which we collected eggs from both control laying hens and hens with experimentally elevated plasma cort concentrations. Part of these eggs were weighed and analysed for testosterone and progesterone concentrations (Henriksen et al., 2011b) and the others were incubated and hatched. In the offspring, we subsequently measured a wide array of behavioural (fear, anxiety, visual lateralization and competitive ability) and physiological traits (growth in terms of body mass and skeleton size, immunity, HPA-response to a challenge and testosterone production). Based on the available literature, we expected that elevated maternal plasma cort would increase fear (Janczak et al., 2007), anxiety (Davis et al., 2008), HPA-axis response to a challenge (Hayward and Wingfield, 2004) and reduce growth rate (de la Cruz et al., 1987; Hayward and Wingfield, 2004; Love et al., 2005), immune defence (Love et al., 2005), competitive ability (Janczak et al., 2007) and visual lateralization in the offspring. In addition, we correlated parameters of eggs laid immediately before and after the egg that produced an experimental chick with the physiological and behavioural traits of that chick, to assess whether maternal nutrition provisioning (egg mass, hatchling mass) or maternal hormone exposure (yolk hormone concentrations) or both would be part of the pathway translating maternal stress to the offspring.

2. Materials and methods

2.1. Treatment of mothers and egg collection

The design and details of the experiment have been extensively described in Henriksen et al. (2011b). In brief: In total, 40 egg laying chickens and 10 roosters were housed in 10 outdoor aviaries. Each aviary housed 2 white Leghorn hens, 2 ISA Brown hens and 1 Rhode Island Red rooster. One White Leghorn hen and one ISA Brown hen from each aviary were implanted subcutaneously at the right flank with a cort pellet (Innovative Research of America; Sarasota, FL, USA) designed to release 30 mg cort for a total duration of 60 days, the rest of the hens were implanted with a 30 mg placebo pellet. All the hens were implanted on the same day and blood sampled within 3 min after entering the aviary by puncture of the wing vein on day 0 (implantation day), 1, 3, 6, 9 and 12 after implantation. Plasma samples were analysed via enzyme immunoassays (EIA) for steroid hormones described in detail in Henriksen et al. (2011b). Implantation with cort-releasing pellets elevated the females' cort levels for almost 12 days, with a pronounced peak during the first 3 days.

Eggs from all females were collected from the day of implantation (day 0) until 19 days thereafter. Eggs laid on day 12, 13, 15, 16 and 18 after implantation were incubated and hatched, except eggs from 2 ISA Brown females whose cort pellet implantation failed to elevate plasma cort levels. We selected eggs from these days because egg formation takes about 8–11 days in chickens and we wanted to use eggs that were formed while mothers had elevated cort levels but avoid eggs that were formed during the first couple of days after the start of the treatment when plasma cort levels might have been supra-physiological. The rest of the eggs were weighed and analysed for yolk hormone concentrations and the analyses showed that maternal plasma cort elevation induced suppression of yolk testosterone and progesterone concentrations and reduced egg mass (for more details see Henriksen et al. (2011b)).

2.2. Offspring incubation and housing

Eggs were stored at 13 °C until they were all placed into a preheated incubator 18 days after pellet implantation. The incubator's temperature was 37 °C and the humidity was held at 58%. The eggs were turned automatically every 6 h by approximately 90°. After 19 days of incubation, the turning of the eggs was stopped and humidity was raised to 80%. Almost all the chicks hatched after 21 days in the incubator, only a few birds hatched the day before or after. The hatchlings were removed from the incubator as soon as their feathers were completely dry. The offspring were raised in the exact same aviaries as their parents but without their parents present. The aviaries measured 3×1.5 m, were equipped with perches, fresh water and food ad libitum. For the first 2 weeks of life, the chicks were provided heating lamps and confined within a 1×1 m solid-wall-open-ceiling arena built within the aviary. For identification, the chicks' feathers were coloured in different combinations with commercial water-resistant marker pens until the age of 10 days when they were given numbered wing tags. In total, 52 chicks from 15 cort treated mothers (Cort-offspring; 3.5 chicks per mother on average) and 68 chicks of 18 control mothers (Control-offspring; 3.7 chicks per mother on average) hatched successfully. Sample sizes for each test are given throughout the paper.

2.3. Offspring growth, structural size and bursa of fabricius mass (see Fig. 1)

Chicks were weighed to the nearest 0.01-g on day 0 (hatching day), 2, 5, 10, 17, 29 and at 13 weeks of age. At 13 weeks of age,

36 Cort-offspring and 50 Control-offspring of 15 and 18 mothers respectively were sacrificed. After weighing the birds, their back of the skull to tip-of-beak length, and tarsus length was measured with a slide calliper. In addition, the Bursa of Fabricius, an important tissue for producing antibodies in young birds, was dissected and weighed on a balance with a readability of 0.001 g.

2.4. Offspring behaviour (see Fig. 1)

2.4.1. Food competition test

At 9 days of age, 17 Cort-offspring of 12 mothers and 17 Control-offspring of 12 mothers were tested in a food competition test. One Control-offspring and one Cort-offspring of the same sex were placed together in a 40 × 20 arena. In the same arena, behind a wire mesh, was a stimulus bird of same age and sex as the test birds. The test began by giving the stimulus bird a mealworm and then quickly removing the wire mesh to allow the Control-offspring and Cort-offspring to chase after the stimulus bird to try and steal the mealworm from its beak. The test ended as soon as either the Cort-offspring or the Control-offspring ended up eating a mealworm and thereby winning the competition. In order to explore which behaviour would aid the chicks in winning the competition, the test was recorded on video. We later analysed from the video recordings (1) how many times the Cort- or Control-offspring would chase after the stimulus bird (2) how many times they would try to steal the mealworm by pecking at the stimulus birds beak and (3) how many times they would succeed in stealing the mealworm from the stimulus bird.

2.4.2. Novel object and visual lateralization

At the age of 11 days, 40 chicks (20 control offspring of 16 mothers and 20 Cort-offspring of 15 mothers) were confronted with a novel object. Four chicks from the same aviary (two Cort-offspring and two Control-offspring) were tested at the same time. They were placed in a 35 × 25 cm arena for 10 min to acclimatise. Thereafter, a novel object was placed in the middle of the arena. After 10 min, the first novel object was replaced by another novel object for an additional 10 min. Novel objects were made of plastic, one was green and square and measured 7 × 5 × 1.5 cm, the other was a white cylinder with a height of 10 cm and a diameter of 5 cm. We registered the amount of time each bird looked at the novel object facing it with either the left or right eye as visual fields hardly overlap in these birds. The percentage of time the birds spent more than 5 cm, within 5 cm but more than 2 cm and less than 2 cm from the novel object was also registered. Brain asymmetries are widespread within the animal kingdom and are supposed to have important adaptive value (Rogers et al., 2004). One example of this is visual lateralization that has been shown to provide cognitive advantages in chicken (Rogers et al., 2004). Since reduction in cerebral asymmetry has been reported in prenatally stressed mammals (Weinstock, 2001) we wanted to test if prenatal stress in birds also affects brain lateralization. Every 15 s it was noted which eye each bird had turned towards the novel object. The direction of lateralization was calculated using the laterality index (Li = (R − L)/(R + L)) in which R and L stand for the frequency of right and left eye used, respectively (Li > 0 means a preference for the right eye; Li < 0 means a preference for the left eye). The strength of lateralization was calculated as the absolute value of this preference score.

2.4.3. Anxiety test – open field test

At 14 days of age, 18 Cort-offspring of 12 mothers and 19 Control-offspring of 14 mothers were tested in a novel arena. The arena was a 2.25 m^2 round circle with an 80 cm high wall. The chicks were tested separately for 15 min and video recorded from above. Latency to start walking and vocalisation was noted. The duration of walking, standing, sitting and number of contact calls was also assessed. The arena was visually divided into 3 circles (inner, middle and outer) of the same width and the chicks' position in the arena was scanned every 10 s. The Open Field test is a standard test used to assess rodents' and chickens' level of anxiety by inducing a conflict between the fear that animals have of a novel and brightly lit open field versus their desire to explore new environments.

2.4.4. Fear test – tonic immobility

Tonic immobility is thought to be a defence strategy evolved to reduce a predator's interest in the prey, when the prey stops moving after it has been caught (Ratner, 1967). Fifty-two Cort-offspring and 68 Control-offspring of 15 and 18 mothers respectively were tested at 28 days of age and 28 of these birds (14 Cort-offspring of 11 mothers and 14 Control-offspring of 11 mothers) were also tested at 6 months of age. All birds within the two age groups were tested on the same day and both age groups were tested in the same way. The test bird was placed on its back and held by the experimenter with one hand over the sternum. The bird was held for 10 s and then the hand was slowly removed. The duration of tonic immobility was recorded up to 7 min. If the bird stood up within 10 s after the hand was removed from its sternum, new attempts to induce tonic immobility were made and the number of inductions necessary to induce tonic immobility was counted.

2.5. Offspring physiology (see Fig. 1)

2.5.1. HPA sensitivity

At 10 weeks of age, 18 Cort-offspring of 13 mothers and 20 Control-offspring of 16 mothers underwent a stress test to assess the reactivity of their HPA-axis by quantifying the cort response to a standard stressor (the bag protocol or capture stress protocol (Wingfield et al., 1992). Birds were blood sampled from the wing veins and baseline samples were obtained within 3 min after the person entered the aviary. After a blood sample was collected, each bird was placed in a cloth bag that allowed light to penetrate, in order to avoid a calming effect of darkness. The birds were blood sampled again 20 min and 45 min after being placed into the bag and returned to the aviaries after the last sampling. Blood was collected in EDTA-coated tubes, kept on ice and centrifuged within 2 h

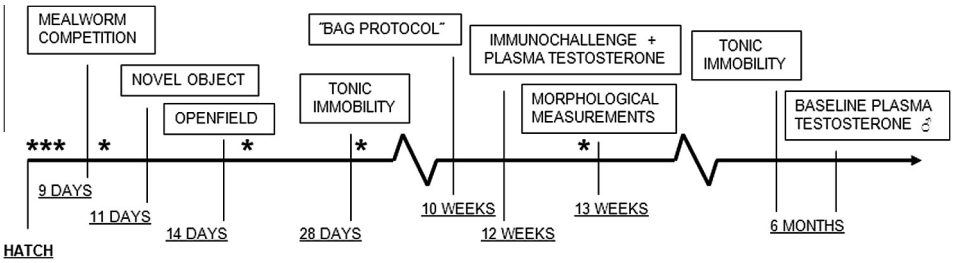

Fig. 1. Time line for tests and measurements on offspring. Time of body mass measurements is indicated with *.

of sampling and then stored at −20 °C until further analysis (see below).

2.5.2. Immunochallenge

At 12 weeks of age, the immune system of 52 Cort-offspring and 68 Control-offspring was challenged using sheep red blood cells (SRBC) as an antigen. This challenge stimulates the production of antibodies during subsequent days without causing illness (Hudson and Hay, 1989). Sheep red blood cells, stored in Alsever's solution at 4 °C were washed tree times in phosphate-buffered saline (PBS, ph = 7.5). Offspring were injected intraperitoneally with 1.5 ml of 2% SRBC diluted in PBS. A total of 1.5 ml blood was taken from the wing vein of all the offspring just before immunisation to test for any pre-existing (cross-reacting) antibodies against SRBC and again 6 days after immunisation. All birds of one aviary were sampled in random order within 15 min after the experimenter entered the aviary. Blood samples were centrifuged and serum was stored at −20 °C until tested. Haemagglutination tests were performed to determine initial and challenged anti-SRBC antibody concentrations (McCorkle and Glick, 1980; Ros et al., 1997). Antibody titers were expressed as the log2 of the reciprocal of the highest plasma dilution giving complete agglutination. The difference in titers between the final and initial blood sample was taken as the reaction to the challenge.

2.5.3. Plasma corticosterone and testosterone

Cort concentrations were assessed in the plasma samples collected from the offspring during the bag test and testosterone concentrations were assessed from the plasma samples collected during both blood samplings for the SRBC test. A meta-analysis by Boonekamp and colleges (Boonekamp et al., 2008) found that experimental immune activation decreases plasma testosterone levels, suggesting an overall strong suppressive effect of experimental immune activation on testosterone levels. We therefore tested whether a difference in the offspring's immunocompetence also might be reflected in their plasma testosterone levels after the immunisation. Baseline plasma testosterone concentrations were also assessed in seven adult Cort-males (of 7 mothers) and eight Control-males (of 8 mothers) at 6 months of age, when they had reached sexual maturity. For hormone analyses, 100 μl of plasma were extracted twice by adding 5 ml of diethyl ether twice and analysed in a cort and testosterone EIA, respectively (cort, Palme and Möstl, 1997; testosterone, (Palme and Möstl, 1994). Intra- and interassay variations were 12% and 27% for the cort EIA and 9% and 16% for the testosterone EIA. Detection limit was 0.06 ng cort per ml plasma for the cort EIA and 0.02 ng/ml for the testosterone EIA.

2.6. Exploring the underlying mechanism

We have previously reported (see Henriksen et al. (2011b)) that eggs collected from the Cort-implanted mothers during a 19 day period after implantation (minus eggs hatched in this study) weighed less than eggs from the placebo-implanted mothers and contained lower concentrations of testosterone and progesterone in the yolk.

As we could not analyse the eggs from which the chicks hatched (''chick-eggs'' laid on day 12, 13, 15, 16 and 18 after pellet implantation) we first performed a posthoc test (hierarchical modelling controlling for room, see statistical section) on eggs laid just before (day 11) or after (day 19) or in-between (day 14 and 17) the ''chick-eggs'' to see if maternal cort-treatment significantly reduced egg mass, yolk testosterone and progesterone on these days. Secondly, for each offspring we calculated a mean value for egg mass and yolk hormone concentration based on the values obtained from the egg laid prior to and after the egg from which the chick hatched. As a consequence chicks hatching from eggs laid on day 12 and 13 after implanting their mothers with cort- or placebo-releasing pellets were allocated mean values calculated from the values obtained from eggs laid day 11 and 14. Chicks that hatched from eggs laid day 15 and 16 had mean values calculated based on eggs laid day 14 and 17 and chicks that hatched from eggs laid day 18 had mean values calculated based on eggs laid day 17 and 19.

Whether corticosterone itself was transferred to the eggs was not assessed in our previous study (Henriksen et al., 2011b). Quantification of corticosterone in the yolk is not a straightforward procedure, since the high concentrations of gestagens present in the yolk of chickens interferes with the quantification of corticosterone via immunoassays (for more details on this methodological problem see Rettenbacher et al. (2009)). In this study we selected 12 eggs (6 from Cort-implanted mothers and 6 from placebo-implanted mothers) collected on day 14 after implantation. The entire yolk of each egg (minus 0.45 g used for analysis described in Henriksen et al. (2011b)) was carefully homogenised and prepared for analysis in separate HPLC runs as described by Rettenbacher et al. (2009).

2.7. Statistics

Since we had a nested design (offspring within mothers within aviaries), data was subjected to multilevel analyses (MlwiN 1.10.0007; Rasbash et al., 2000). Analyses for egg data were performed with a two-level model, with eggs nested within mothers (level 1) nested within aviary (level 2). Analyses of offspring data were performed with a three-level model, with measures of individuals (level 1) nested within mother (level 2) within the same aviary (level 3). Significance was tested using the Wald-statistics that follows a χ^2 distribution. For all dependent variables we examined the influence of mother's treatment (cort pellet-implanted mother or placebo pellet-implanted mother), mothers' strain (ISA Brown or white Leghorn), offspring sex (male or female), and the interaction between these variables. Variables were removed from the model if they showed no significance (unless involved in an interaction), except for offspring sex and treatment, which were always kept in the model as the main explanatory variables we were interested in. Residuals of the model were used to determine normality. Log transformation and square root transformation was applied in some cases in order to obtain normality. Binary data from the food competition test (eats or does not eat mealworm), hatching success (hatch or did not hatch) and offspring sex ratio (male or female) were transformed by the logit link function and analysed as a binomial distribution. For those chick traits where we found a significant effect of mothers' treatment, we performed further analyses. Using the egg mass and yolk hormone concentrations that we calculated for the egg that produced an experimental chick (''chick-eggs'') as predictors we examined the influence of the prenatal environment on the affected traits. The correlation among these three predictors was lower than 0.26. Data are shown as means and standard error of the mean (SEM), probability values are two tailed and hypotheses are rejected at $p < 0.05$. p-values for strain and sex are only given if they show significance.

3. Results

3.1. Hatchability and sex ratio

Hatching success was 74.32% for Cort-offspring and 77.85% for Control-offspring, which was not significantly different ($\chi^2 = 0.005$; df = 1; $p = 0.943$). Offspring of cort pellet-implanted

mothers consisted of 52.63% male offspring, for the placebo pellet-implanted mothers this was 56.52%, the difference being not significant ($\chi^2 = 0.117$; df = 1; $p = 0.731$).

3.2. Growth

Cort-offspring had significantly ($\chi^2 = 15.530$; df = 1; $p = 0.001$) lower body mass at hatching than Control-offspring and continued to be significantly ($p < 0.01$) lighter up until day 17 of age (Fig. 2). The difference in body mass between Cort- and Control-offspring at 29 days of age (Cort-offspring = 265.67 ± 5.45 g, Control-offspring = 275.41 ± 5.00 g) almost reached significance ($\chi^2 = 3.742$; df = 1; $p = 0.053$). There was no difference in body mass at 13 weeks of age between Cort-offspring (1318.26 ± 32.53 g) and Control-offspring (1321.58 ± 28.62 g; $\chi^2 = 0.455$; df = 1; $p = 0.499$). From 10 days of age, males were significantly heavier than females ($p < 0.05$) and offspring of ISA Brown hens were significantly heavier than offspring of white Leghorn mothers ($p < 0.05$), but two or three way interactions between treatment, mothers-strain and sex were not significant at any age. At 13 weeks of age, there was no effect of mothers' treatment on the offspring's' head plus beak length (Cort-offspring = 73.19 ± 0.48 mm, Control-offspring = 72.93 ± 0.39 mm, $\chi^2 = 1.834$; df = 1; $p = 0.175$) and tarsus length (Cort-offspring = 86.05 ± 1.31 mm, Control-offspring = 86.79 ± 0.846 mm $\chi^2 = 0.065$; df = 1; $p = 0.798$). Males had significantly ($\chi^2 = 51.091$; df = 1; $p = 0.001$) larger head plus beak length (males = 74.489 ± 0.313 mm; females = 70.94 ± 0.384 mm) and tarsus length ($\chi^2 = 30.071$; df = 1; $p = 0.001$; males = 89.88 ± 0.855 mm; females = 71.525 ± 0.710 mm) than females, but no interaction including treatment, sex and mothers strain was found.

3.3. Behaviour

3.3.1. Food competition

There was no significant difference between Cort-and Control-offspring in time spent chasing ($\chi^2 = 0.121$; df = 1; $p = 0.727$) or pecking ($\chi^2 = 0.015$; df = 1; $p = 0.902$) the stimulus bird in order to get the mealworm. There were no significant interactions including treatment on either chasing or pecking, but males chased the stimulus bird more ($\chi^2 = 14.163$; df = 1; $p = 0.001$) and pecked more often at the stimulus bird's beak ($\chi^2 = 8.752$; df = 1; $p = 0.003$) than females. Interestingly, Cort-offspring were significantly less successful in stealing the mealworm from the stimulus bird ($\chi^2 = 4.062$; df = 1; $p = 0.044$) and Cort-offspring were significantly less likely to end up eating the mealworm and thereby winning the competition than Control-offspring ($\chi^2 = 4.049$; df = 1; $p = 0.044$, Fig. 3). The offspring were not weighed on the day of the food competition, but their body mass the following day when the offspring were 10 days old did not predict the chicks' ability to win/eat the mealworm ($\chi^2 = 0.958$, df = 1; $p = 0.327$) or the chicks ability to steal the mealworm ($\chi^2 = 0.179$; df = 1; $p = 0.672$).

3.3.2. Novel object test and visual lateralization

There was no effect of treatment on the amount of time the birds spent in the vicinity of the novel object (>5 cm $\chi^2 = 0.177$; df = 1 $p = 0.67$; <5 cm and >2 cm $\chi^2 = 2.227$; df = 1; $p = 0.135$; <2 cm $\chi^2 = 2.293$; df = 1; $p = 0.129$). Overall the offspring tended to spend most of their time more than 5 cm away from the novel object (93.79 ± 0.97% Cort-offspring; 94.60 ± 1.23% Control-offspring). Cort-offspring spent in total 64 ± 3% of the time with either their left or right eye facing the novel objects while Control-offspring spent in total 60.13 ± 3.74% of their time facing the novel object, but the difference was not statistically significant ($\chi^2 = 0.155$; df = 1 $p = 0.694$). The difference in strength of visual lateralization (Cort-offspring 0.23 ± 0.05; Control-offspring 0.31 ± 0.06) between the two groups almost reached statistical significance ($\chi^2 = 2.978$; df = 1; $p = 0.060$). However, the direction of lateralization (see Fig. 4) was significantly different between the two groups ($\chi^2 = 9.569$; df = 1; $p = 0.001$). Post-hoc tests revealed that within the Control group offspring were significantly more likely to use their right eye to focus on the novel object ($\chi^2 = 12.073$; df = 1; $p = 0.001$) whereas cort-offspring were random with regard to eye preference within the group ($\chi^2 = 1.471$; df = 1; $p = 0.225$). Males were overall significantly more likely to focus their right eye at the novel object than females, independent of treatment (males: 0.284 ± 0.076; females: 0.049 ± 0.064; $\chi^2 = 4.281$; df = 1; $p = 0.038$).

3.3.3. Open field

Cort-offspring showed no difference from Control-offspring in latency to start vocalising ($\chi^2 = 0.014$; df = 1; $p = 0.906$) or number of contact calls ($\chi^2 = 0.240$; df = 1; $p = 0.624$). Females emitted more contact calls than males ($\chi^2 = 8.417$, df = 1; $p = 0.003$), but no significant interaction including treatment was found. Latency to start moving did not differ between the two groups of offspring ($\chi^2 = 0.583$; df = 1; $p = 0.445$), but Cort-offspring had a tendency

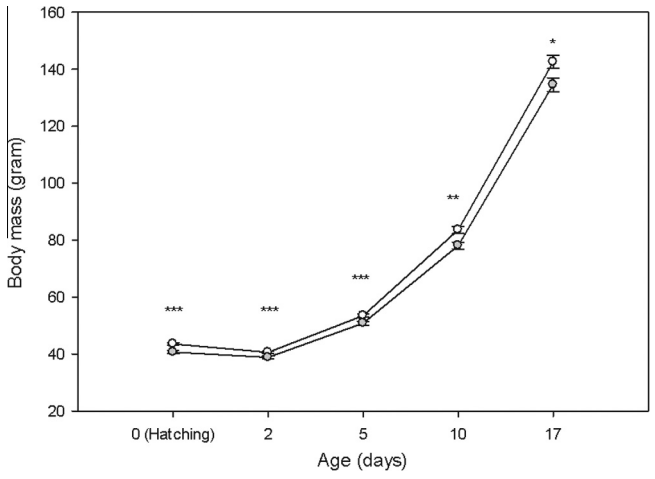

Fig. 2. Body mass (mean ± S.E.M.) of Control-offspring (○) and Cort-offspring (●) at hatching and age 2, 5, 10 and 17 days. X-axis not scaled to time for reasons of clarity. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

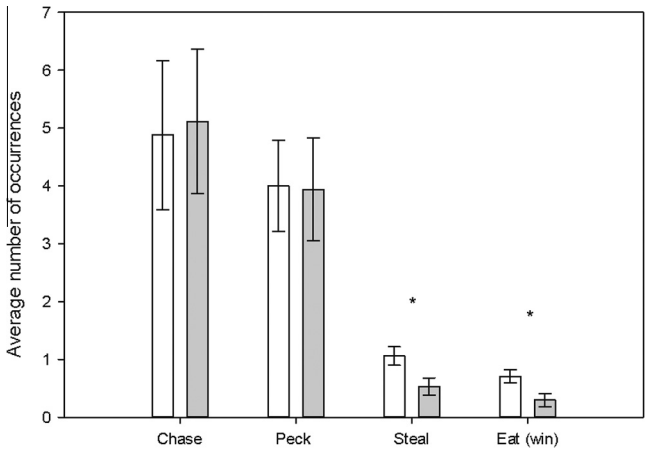

Fig. 3. Number of times (mean ± S.E.M.) during a mealworm competition test Control-offspring (open bars) and Cort-offspring (grey filled bars) would chase after a stimulus bird, peck at stimulus birds beak, steal mealworm from stimulus birds beak and win the competition. * indicates statistically significant difference $p < 0.05$.

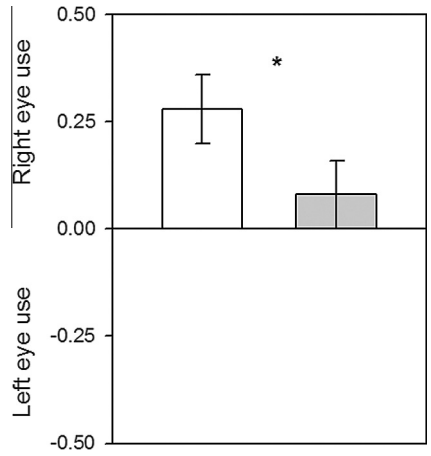

Fig. 4. Direction of visual lateralization (mean ± S.E.M). Lateralization index >0 right eye use, <0 left eye use of Control-offspring (open bar) and Cort-offspring (grey filled bar) at age 11 days. * indicates statistically significant difference $p < 0.05$.

($\chi^2 = 3.388$; df = 1; $p = 0.065$) to walk more during the test. Cort-offspring did not differ from the Control-offspring in the amount of sitting ($\chi^2 = 0.114$; df = 1; $p = 0.736$) or standing ($\chi^2 = 1.658$; df = 1; $p = 0.197$) and the two treatment groups did not differ in their use of the open field arena (inner circle, $\chi^2 = 0.994$; df = 1; $p = 0.318$; middle circle, $\chi^2 = 1.823$; df = 1; $p = 0.177$; outer circle, $\chi^2 = 0.002$; df = 1; $p = 0.964$).

3.3.4. Tonic immobility

Overall, taking both the test on day 28 and 6 months into account, Cort-offspring stayed in tonic immobility for a shorter duration of time than Control-offspring (48 ± 18 vs. 72 ± 10 s; $\chi^2 = 4.912$; df = 1; $p = 0.026$, see Fig. 5). At 6 months of age, offspring stayed in tonic immobility significantly longer than at 28 days of life ($\chi^2 = 34.281$; df = 1; $p = 0.001$), but there was no interaction between mother's treatment and the age of the offspring ($\chi^2 = 2.727$; df = 1; $p = 0.098$). Offspring sex and mothers' strain had no effect on the duration of tonic immobility. On average 1.72 ± 0.16 and 1.61 ± 0.14 inductions were needed to induce tonic immobility in Cort-offspring and Control-offspring respectively ($\chi^2 = 0.301$; df = 1; $p = 0.583$).

3.4. Physiology

3.4.1. Plasma corticosterone response

In the bag-test, four birds (one Cort-offspring and three Control-offspring) had baseline cort concentrations above 5 ng/ml (average values were 0.53 ± 0.10 ng/ml) and were therefore removed from the analysis as strong outliers. Baseline plasma cort levels did not differ between the Cort-offspring and Control-offspring ($\chi^2 = 0.754$; df = 1; $p = 0.652$). Concentrations increased significantly over time ($\chi^2 = 8.416$; df = 1; $p = 0.003$) but there was no significant difference in plasma cort levels between the two groups of offspring over time (treatment∗time $\chi^2 = 0.259$; df = 1; $p = 0.612$, Fig. 6).

3.4.2. Antibody response, mass of bursa of fabricius and plasma testosterone levels

Cort-offspring had a significantly ($\chi^2 = 4.487$; df = 1; $p = 0.034$) lower antibody response in the SRBC challenge than Control-offspring (Fig. 7). The mass of the Bursa of Fabricius was smaller in Cort-offspring than in Control-offspring (4.688 ± 0.30 and 5.127 ± 0.23 g, respectively), however the difference did not reach significance ($\chi^2 = 3.103$; df = 1; $p = 0.078$). Correcting mass of the Bursa of Fabricius for body mass by using the latter as a covariate revealed an almost significant effect of treatment ($\chi^2 = 3.017$; df = 1; $p = 0.054$) with Cort-offspring having lower bursa mass than Control-offspring. Cort-offspring had significantly ($\chi^2 = 7.189$; df = 1; $p = 0.007$) higher baseline plasma testosterone levels just prior to SRBC immunisation compared to the Control-offspring (Fig. 8) and experienced a decrease of 0.09 ± 0.08 ng/ml plasma testosterone during the 6 days after SRBC immunisation, whereas the Control-offspring experienced a 0.04 ± 0.03 ng/ml increase in plasma testosterone over the same period, but this difference in change over time was not significant (treatment∗time, $\chi^2 = 2.573$; df = 1; $p = 0.108$). At 6 months of age, sexually mature males from both treatments showed no difference in baseline plasma testosterone ($\chi^2 = 0.187$; df = 1; $p = 0.453$; Cort-offspring 4.7 ± 0.9 ng/ml, Control-offspring 5 ± 0.4 ng/ml). Since several studies on birds and other species have found a link between elevated testosterone and suppressed immunocompetence (Immunocompetence Handicap Hypothesis (Folstad and Karter, 1992) we tested if plasma testosterone levels on day 0 (just prior to the SRBC challenge) would predict the offspring's antibody response to the SRBC. We found that higher plasma testosterone concentrations

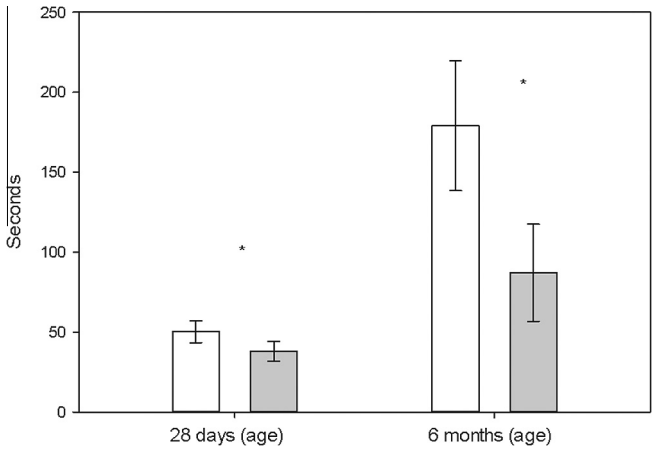

Fig. 5. Duration of tonic immobility (mean ± S.E.M.) for Control-offspring (open bar) and Cort-offspring (grey filled bar) at age 28 days and age 6 months. * indicates statistically significant difference $p < 0.05$.

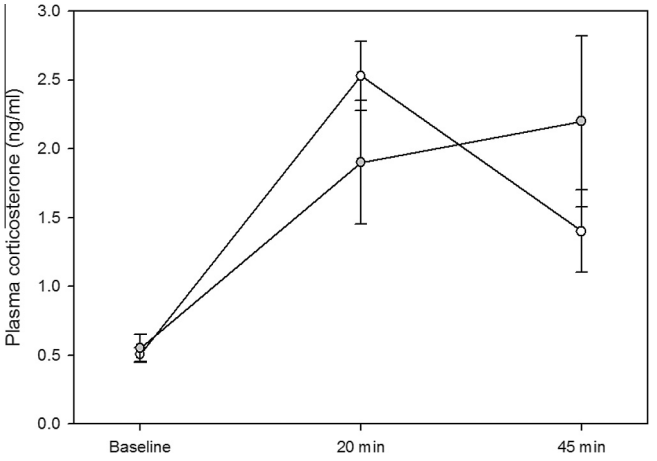

Fig. 6. Plasma corticosterone conc. (mean ± S.E.M.) of Control-offspring (○) and Cort-offspring (●) within 3 min. of catching (baseline) and after 20 min and 45 min of physical restraint stress at the age of 10 weeks.

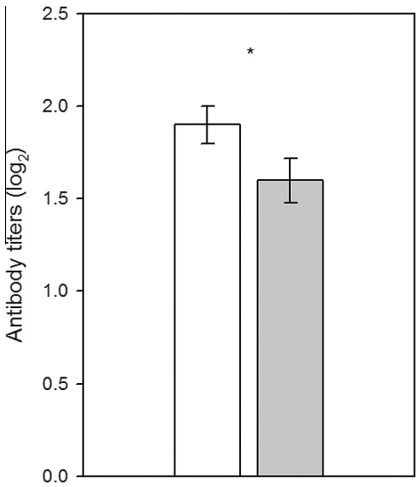

Fig. 7. Antibody titers (mean ± S.E.M.) after immunisation with sheep red blood cells in Control-offspring (open bar) and Cort-offspring (grey filled bar) at the age of 10 weeks. * indicates statistically significant difference $p < 0.05$.

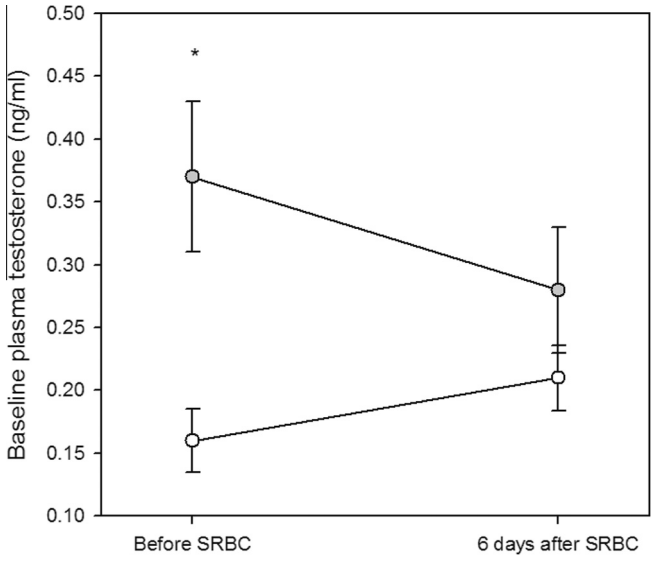

Fig. 8. Baseline plasma testosterone concentration (mean ± S.E.M.) in Control-offspring (○) and Cort-offspring (●) before and 6 days after immunisation with sheep red blood cells at the age of 10 weeks. * indicates statistically significant difference $p < 0.05$.

Table 1
Overview of significant effect (↑ increase, ↓ decrease, = none, between brackets: $p \leqslant 0.06$) of elevated maternal corticosterone on offspring traits.

Sex ratio	=
Chick body mass	↓
B-cell mediated production of antibodies	↓
Bursa fabricius mass	(↓)
Baseline plasma testosterone	↑
Skeleton size	=
Baseline plasma corticosterone	=
HPA axis response	=
Anxiety	=
Fearfulness	↓
Competitiveness	↓
Visual lateralization	(↓)

showed a tendency to predict lower antibody response ($\chi^2 = 3.733$; df = 1; $p = 0.053$).

3.5. Exploring mechanisms

Maternal cort-treatment significantly reduced egg mass (day 11, $\chi^2 = 4.657$; df = 1; $p = 0.030$; day 14, $\chi^2 = 8.974$; df = 1; $p = 0.003$; day 17, $\chi^2 = 9.653$; df = 1; $p = 0.002$; day 19, $\chi^2 = 4.025$; df = 1; $p = 0.038$) and yolk testosterone concentration (day 11, $\chi^2 = 4.653$; df = 1; $p = 0{,}030$; day 14, $\chi^2 = 5.497$; df = 1; $p = 0.019$; day 17, $\chi^2 = 7.597$; df = 1; $p = 0.011$; day 19, $\chi^2 = 3.010$; df = 1; $p = 0.083$) during the period when the eggs that produced the experimental chicks "chick-eggs" were laid. Yolk progesterone concentration was reduced to a lessor extent and only showed a significant decrease on day 17 (day 11, $\chi^2 = 1.090$; df = 1; $p = 0.296$; day 14, $\chi^2 = 2.008$; df = 1; $p = 0.156$; day 17, $\chi^2 = 3.109$; df = 1; $p = 0.043$; day 19, $\chi^2 = 2.103$; df = 1; $p = 0.141$). We found no significant differences in yolk cort concentrations in eggs collected from the placebo-mothers and cort-mothers (2.1 ± 0.57 ng/g and 1.6 ± 0.51 ng/g in cort and placebo eggs respectively, t-test, $t = -0.661$, $p = 0.524$). Egg mass showed a strong positive correlation with body mass during the first 10 days (hatching weight: $\chi^2 = 116.1$; df = 1; $p = 0.000$; age 2 days, $\chi^2 = 71.65$; df = 1; $p = 0.000$; age 5 days, $\chi^2 = 22.34$; df = 1; $p = 0.000$; age 10 days, $\chi^2 = 6.914$; df = 1; $p = 0.009$; age 17 days, $\chi^2 = 2.995$; df = 1; $p = 0.084$). There was no significant correlation of yolk testosterone or progesterone concentration ($p > 0.30$) with any of the affected offspring traits. Egg mass also showed a non-significant tendency to positively predict stealing a mealworm ($\chi^2 = 3.179$; df = 1; $p = 0.075$), and plasma testosterone concentrations ($\chi^2 = 2.904$; df = 1; $p = 0.088$). Correlating yolk mass (which was more affected by maternal cort elevation than albumen + shell, see Henriksen et al. (2011b)) produced the same results as the egg mass correlations. We also tested whether hatching mass, which correlated strongly with egg mass, would correlate with any of these traits. Hatching mass showed a significant correlation with the offspring's ability to steal a mealworm ($\chi^2 = 4.588$; df = 1; $p = 0.032$), but not the offspring's baseline plasma testosterone ($\chi^2 = 0.247$; df = 1; $p = 0.619$).

4. Discussion

The study of stress-mediated maternal effects has been an important field of research for a long time, but only recently have oviparous species such as birds been used as a model. Using birds facilitates the investigation of underlying pathways due to the accessibility of the prenatal environment. However, the few avian studies available often measure only a limited array of offspring traits and the results are not always consistent, perhaps due to differences in design (for a review see Henriksen et al. (2011a)). In this study we carefully collected eggs that had been formed when the mothers' plasma cort levels were elevated within the physiological range and measured a wide array of offspring traits. In order to explore underlying pathways we also related variation in the composition of eggs to variation in offspring traits.

We were able to demonstrate that offspring that hatched from eggs produced while the mother had elevated plasma cort levels show a range of alterations in phenotype (see Table 1). Although not all traits were affected, those that were showed effects in the expected direction and were consistent with one another, (see introduction and Table 1), except for fear behaviour. Our study shows that elevated maternal plasma cort concentrations during egg formation impair the prenatal growth of the chick, resulting in lower body mass at hatching. We found a significant correlation between egg mass and hatchling mass, indicating that the Cort-offspring's smaller body mass at hatching was caused by reduced prenatal nutrition. Cort-offspring continued to be smaller than Control-offspring during the first 3–4 weeks of life. Although lower

body mass is a recurring finding in maternal stress studies in birds (see de la Cruz et al. (1987), Hayward and Wingfield (2004) and Love et al. (2005)), lower hatching body mass has only been reported in one other study so far (Love et al., 2005). Cort-offspring caught up in growth to that of the Control-offspring in about 4 weeks. Catch-up growth has been reported across a wide range of taxa (for review see Metcalfe and Monaghan (2001)) and usually follows a nutritional deficit (Gurney et al., 2003). A quick catch-up growth might be beneficial for the offspring, since being smaller could have potential fitness cost, like reaching sexual maturity later or being less able to compete for resources. However, the fast catch-up growth in the Cort-offspring might have come at a cost: Studies in humans, rodents and birds have found long-term cost associated with compensatory growth (see Fisher et al. (2006), Metcalfe and Monaghan (2001) and Ozanne and Hales (2005)) some of which include increased risk for developmental health problems and reduced cognitive abilities. It is therefore likely that some of the effects on the offspring from mothers with elevated cort, expressed after compensatory growth was completed, is due to compensatory growth instead of lower body mass at hatching. We did a simple test of correlating offspring traits with growth rate (body mass day 29 divided by body mass at hatching) and found no correlations indicating that catch-up growth was responsible for these effects, but this requires further experimentation.

At 12 weeks of age, Cort-offspring had a lower antibody response to the antigen challenge, indicating a lower immunocompetence in these offspring. The only other study that looked at immunocompetence in offspring of female birds with elevated plasma cort found that European Starling male but not female offspring had a lower T-cell mediated inflammatory response (Love et al., 2005). Interestingly, the same study found that males of mothers with elevated cort were smaller at hatching, but caught-up in growth long before sexual maturity, while females' body mass was unaffected by maternal plasma cort levels. Together, the study by Love et al. (2005) Love and colleagues and our study suggest a link between reduced prenatal growth (followed by catch up growth) and lower immunocompetence in prenatally stressed birds. We did however not find a link between prenatal undernutrition (egg mass – which correlated strongly with hatchling mass) and immunocompetence, but in accordance with the Immunocompetence Handicap Hypothesis we found an almost significant negative correlation between the offspring plasma testosterone levels before the immunisation and their antibody response. The fact that Cort-offspring, which showed a fast catch-up growth also had higher plasma testosterone concentrations, is consistent with the idea that testosterone, as an anabolic hormone, is important for growth and muscle development. This is the first study to explore the effect of maternal stress on offspring's plasma testosterone levels in birds, but prenatal stress has been reported to increase plasma testosterone concentrations in female offspring of guinea pigs (Kaiser and Sachser, 1998).

Cort-offspring were significantly less likely to win in a food competition test at nine days of age. Janczak and colleges (Janczak et al., 2007) found similar results when testing offspring of stressed chickens at maturity, which suggests that elevated plasma cort in female birds, induce a permanent negative effect on their offspring's competitive ability. Reduced competitiveness in offspring of stressed mothers could mean a potential reduction in the offspring's fitness, since in an environment where food is limited, a reduced ability to compete for food in a social species like chickens would come at a cost. The underlying behavioural or physiological difference that caused the offspring to be less successful when competing for food is unclear. The Cort-offspring were just as likely to chase after the stimulus bird and try to peck the mealworm out of its beak as Control-offspring, but Cort-offspring were less likely to actually succeed in stealing the mealworm from the stimulus bird. The lower body mass of Cort-offspring at the time of the test did not explain the lower competitiveness of Cort-offspring but the offspring's ability to steal a mealworm from the stimulus bird did correlate with hatchling mass, indicating an effect of prenatal growth on early life competitiveness. The embryonic stage is crucial for skeletal muscle development because there is no increase in muscle fibre numbers after prenatal development (Du et al., 2010) and reduced prenatal growth has been linked to altered body composition at 6 weeks of age in chickens (Van der Waaij et al., 2010). It is therefore possible that reduced muscle mass and thereby physical strength, due to prenatal undernutrition caused the cort-offspring to be less successful when competing for food.

The reduced competitiveness of the Cort-offspring might also have been due to impairment of fine motor control or some sort of cognitive function induced by prenatal undernutrition. Metcalfe and Monagan (Metcalfe and Monaghan, 2001) have argued that precocial birds might be more affected by prenatal nutrition deficits than altricial species, since the individuals cannot compensate during this important developmental period for any nutritional shortcomings. In mammals, maternal stress has been shown to affect the limbic system (a set of brain structures responsible for e.g. behaviour and long-term memory) and the prefrontal cortex, a brain region involved in planning complex cognitive behaviour (Weinstock, 2008). We have previously found that the reduced egg mass of these cort implanted females is mainly due to reduced yolk mass and it is therefore possible that egg substances, such as vitamins, minerals, carotenoids or dietary proteins are part of mediating these effects and not just reduced nutrition. The effect of maternal cort elevation on visual lateralization in the Cort-offspring suggests that maternal cort elevation in birds can indeed induce functional changes in certain brain regions. In birds the two eyes project massively to the contralateral hemisphere with little inter-hemispheric coupling (Güntürkün, 1997). This means that visual input to the chick brain can be restricted to one hemisphere and birds can use their eyes independently thereby allowing them to keep an eye out for predators while at the same time searching for food on the ground (Rogers et al., 2004). The novel object test showed that the Control-offspring had a tendency to be more strongly visually lateralized than Cort-offspring and that Control-offspring preferred to look at the novel object with their right eye, whereas Cort-offspring were more random. Interestingly, Rogers and Deng (Rogers and Deng, 2005) found that chicks hatching from eggs injected with cort 3 days prior to hatching were more random with respect to direction of lateralization. We found no difference in yolk cort concentration, and also no indication of altered HPA-axis activity in the Cort-offspring since they did not differ in their postnatal baseline cort levels or cort response after being physically restrained in a cloth bag for 45 min. at 11 weeks of age. It can however not be excluded that a different or more intense stressor would have caused a difference between Cort-offspring and Control-offspring HPA-axis sensitivity.

The lack of differences in the Cort-offspring's cort production is contrary to the findings of Hayward and Wingfield (Hayward and Wingfield, 2004) who found that adult offspring of stressed quails had an enhanced HPA-axis response when stressed in a similar test. Our finding that Cort-offspring were less fearful when placed into tonic immobility and were slightly more active in an open field test ($p = 0.065$) is also contrary to earlier findings where maternal stress has been reported to increase offspring's level of fear (Janczak et al., 2007) or to have no effect on fear but increase anxiety (Davis et al., 2008). Finally, in contrast to findings in other bird species, (Pike and Petrie, 2006, (Bonier et al., 2007) and (Love et al., 2005) the sex ratio of offspring from mothers with elevated plasma cort levels did not differ from controls.

In general, our findings were independent of the offspring's sex and the mothers' strain since no interaction including mother's

treatment, mothers' strain and the sex of the offspring showed any effects. However, we found that males were generally more right eye lateralized and more competitive, while females emitted more contact calls during the open field test.

We did not find any correlation between the traits affected in the Cort-offspring and the concentration of prenatal testosterone or progesterone exposure. The small effect maternal cort elevation had on yolk progesterone concentration indicates that this hormone was not a main mediator of these maternal effect, but it is surprising that testosterone which was affected by maternal cort elevation to a much larger degree and which has been shown to be a potent modifier of prenatal development in birds showed no effect either. We suggest three possible reasons for this: (1) reduced testosterone hormone exposure did not induce the alterations seen in the Cort-offspring; (2) we did not have the exact values for the actual egg from which the offspring hatched but instead calculated mean values from eggs laid before and after that egg. This might also explain why we found a correlation between the offspring's ability to steal a mealworm and hatching mass but not between the offspring's ability to steal a mealworm and egg mass; (3) testosterone work in synergy with other egg components (e.g. vitamins, minerals or carotenoids) that together induce the effects seen in the Cort-offspring.

5. Conclusions and future perspective

The current findings do not suggest that stress-mediated effects in birds are due to alteration in prenatal testosterone, progesterone or corticosterone concentrations, but do indicate that prenatal undernutrition might mediate some of these effects. The Cort-offspring's reduced ability to compete for food and lower antibody response indicate, like in earlier studies, that maternal stress has costs for the offspring. However, it has also been suggested that the prenatal environment provides the developing embryo with an important source of information for predicting the environment it will be exposed to postnatally. If the offspring adapts its physiological development in a way that is advantageous in the predicted future environment, then the reduced quality of the Cort-offspring might have been due to a mismatch between the prenatal and postnatal environment. Matching and mismatching the offspring's postnatal environment to the environment the mother experiences during egg formation in future experiments will give more clarity about the potential adaptive effects of maternal stress.

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