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Developmental programming: Cumulative effects of increased pre-hatching corticosterone levels and post-hatching unpredictable food availability on physiology and behaviour in adulthood ☆

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ABSTRACT

Prolonged exposure to stress during development can have long-term detrimental effects on health and wellbeing. However, the environmental matching hypothesis proposes that developmental stress programs physiology and behaviour in an adaptive way that can enhance fitness if early environments match those experienced later in life. Most research has focused on the harmful effects that stress during a single period in early life may exert in adulthood. In this study, we tested the potential additive and beneficial effects that stress experienced during both pre- and post-hatching development may have on adult physiology and behaviour. Japanese quail experienced different stress-related treatments across two developmental life stages: pre-hatching corticosterone (CORT) injection, post-hatching unpredictable food availability, both pre- and post-hatching treatments, or control. In adulthood, we determined quails' acute stress response, neophobia and novel environment exploration. The pre-hatching CORT treatment resulted in attenuated physiological responses to an acute stressor, increased activity levels and exploration in a novel environment. Post-hatching unpredictable food availability decreased adults' latency to feed. Furthermore, there were cumulative effects of these treatments across the two developmental stages: quail subjected to both pre- and post-hatching treatments were the most explorative and risk-taking of all treatment groups. Such responses to novel environments could enhance survival in unpredictable environments in later life. Our data also suggest that these behavioural responses may have been mediated by long-term physiological programming of the adrenocortical stress response, creating phenotypes that could exhibit fitness-enhancing behaviours in a changing environment.

Introduction

Prolonged exposure to adverse conditions during development can have serious long-term effects on an individuals' physiology and behaviour, leading to significantly higher risks of many health pathologies and behavioural disorders later in life (Cottrell and Seckl, 2009; Lupien et al., 2009; Sachser et al., 2011; Welberg and Seckl, 2001). For this reason the role of developmental environments in shaping adult phenotypes has received a lot of attention in the last decade (Lindstrom, 1999; Monaghan, 2008). In contrast, one recent view proposes an adaptive framework of developmental programming, where shaping of physiology and behaviour by early-life conditions can enhance fitness if early environmental conditions match those experienced across life stages. According to this 'environmental matching hypothesis', negative effects of developmental adversity may occur due to a mismatch between environmental conditions at different life stages (Bateson et al., 2004; Gluckman et al., 2005; Monaghan, 2008). Whilst this is an intriguing hypothesis that has prompted theoretical studies (Monaghan, 2008), there is currently a lack of studies that have tested it empirically (Gluckman et al., 2005; Monaghan et al., 2012). No study to date has explored the interaction between different developmental stages in shaping these potentially adaptive responses in adulthood. In addition, we have little information about the effects of developmental conditions on behaviours, such as risk-taking behaviour, that may be related to coping with adverse conditions.

One fundamental physiological system that links an individual to changes in the environment is the hypothalamic–pituitary–adrenal (HPA) axis or stress axis (Cottrell and Seckl, 2009; Monaghan, 2008). This axis is activated during adverse conditions in both development and adulthood and, in vertebrates, results in the release of glucocorticoids (Weinstock, 2008; Welberg and Seckl, 2001). This stress response facilitates a switch of physiological processes and behaviours from non-essential activities to those that promote short-term survival, such as increased locomotion and mobilisation of energy stores (Wingfield and

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Romero, 2001). This axis is therefore a prime candidate for a mechanism by which adversity over different developmental stages could program an individual's behaviour in an adaptive manner. Indeed, many studies have already shown a link between stress during development and later behavioural effects, such as sexual signalling, longevity, breeding behaviour, learning and memory (Henriksen et al., 2011; Lupien et al., 2009; Monaghan, 2008; Monaghan et al., 2012; Sachser et al., 2011; Spencer and Verhulst, 2007; Weinstock, 2008). Stress during the last days of pregnancy can also result in alterations to behaviours linked to risk-taking, such as higher fearfulness (Henriksen et al., 2011) and reduction in exploration levels (Champagne and Meaney, 2006) in rats (*Rattus norvegicus*).

Many of these effects appear to be mediated by permanent changes in HPA axis functioning, mainly through perturbation of the negative feedback systems that regulate glucocorticoid secretion, such as the intracellular glucocorticoid receptors (GR) and mineralocorticoid receptors (MR) (Banerjee et al., 2012; Cottrell and Seckl, 2009; Lupien et al., 2009; Welberg and Seckl, 2001). In mammals, pre-natal stress through immobilisation during six hours from day 8.5 of pregnancy to birth in mice (*Mus musculus*) resulted in a prolonged response to an acute stress (Chung et al., 2005). However, post-natal glucocorticoid treatment in rats led to a decreased stress response later in life (Vázquez et al., 2012). In birds, injection of glucocorticoids into starling (*Sturnus vulgaris*) eggs resulted in a reduced stress response (Love and Williams, 2008). Conversely, post-hatching deprivation of maternal care in zebra finches (*Taeniopygia guttata*) resulted in a prolonged response to an acute stressor (Banerjee et al., 2012). In both mammals and birds, the prolonged response to acute stress is linked to a decreased expression of glucocorticoid receptors in areas of the brain linked with HPA negative feedback (Banerjee et al., 2012; Chung et al., 2005). When living in an environment with frequent exposure to stressful stimuli, an attenuated stress response could allow an individual to better cope with these conditions; repeated elevated levels of glucocorticoids have detrimental effects, and a quicker return to baseline levels may facilitate more adaptive behaviours (Love and Williams, 2008; Weinstock, 2008). Moreover, in stressful situations increased exploration and risk-taking associated with an attenuated stress response could also be adaptive (Blas et al., 2007; Cavigelli and McClintock, 2003; Dingemanse et al., 2004; Love and Williams, 2008; Martins et al., 2007; Smith and Blumstein, 2008). The overwhelming majority of experimental studies conducted to date have focused on possible detrimental effects that developmental stress during a single developmental stage, either pre- or post-natal, may exert on adult traits. However, to test the environmental matching hypothesis, empirical data are required that address how a combination of pre- and post-natal stressors affects phenotypic traits.

In this study, we exposed test subjects to stress-related treatments during pre- and/or post-hatching development and examined the effects on behavioural traits related to risk-taking behaviour and HPA axis functioning in adulthood. We used a precocial avian species (Japanese quail, *Coturnix japonica*) as it allowed us to easily manipulate pre-hatching glucocorticoid levels by injecting eggs and avoid the confounding effects of post-hatching/-natal maternal care as present in altricial birds and mammals, respectively (Henriksen et al., 2011; Spencer et al., 2009). To test the effects of environmental matching between developmental and adult conditions, and the importance of the developmental timing and nature of the stressor(s) in programming adult physiology and behaviour, we created four treatment groups: we treated one group in the pre-hatching phase only by injecting corticosterone (CORT) into the egg yolks, thereby simulating the transfer of elevated CORT from the mother into her eggs, which is known to occur in several bird species, including Japanese quail (Henriksen et al., 2011). We treated another group in the post-hatching phase only by exposing chicks to an unpredictable food availability paradigm (Buchanan et al., 2003; Cuthill et al., 2000), thereby simulating a stressful foraging environment. A third group was exposed to both treatments and the final group was maintained as a control. We hypothesized that both pre- and post-hatching treatments would affect physiology and behaviour as seen in previous studies. If the environmental matching hypothesis is correct, we predicted that birds in the pre- or post-hatching treatment groups would exhibit behaviours that could potentially enhance fitness under stressful adult conditions, such as reduced neophobia and more exploratory or risk-taking behaviours in novel environments (Blas et al., 2007; Cavigelli and McClintock, 2003; Dingemanse et al., 2004; Smith and Blumstein, 2008). Additionally, if stress experienced during early life matched across both developmental stages and with conditions later in life, we also predicted that birds in the pre- and post-hatching treatment group would show stronger behavioural responses when exposed to stressful conditions as adults than birds exposed to only one of the treatments. Finally, we expected that these behavioural changes would be associated with changes to the acute CORT response to stress (Cavigelli and McClintock, 2003; Love and Williams, 2008; Spencer and Verhulst, 2007).

Methods

Pre- and post-hatching treatments and stress response measurements

Unrelated Japanese quail eggs ($n = 76$) were obtained from Moonridge Farm, Exeter, UK and placed in an incubator (Ova-Easy 190A, Brinsea Products Ltd, UK) at 37.5 °C and 55% humidity. After 5 days of incubation, half of these eggs (CORT: $n = 38$) were injected with 10 μl CORT (Sigma Aldrich, Poole, UK; concentration CORT: 850 ng/ml) dissolved in sterile peanut oil at the egg apex under sterile conditions. This gave a dose of 8.5 ng of CORT, which increases endogenous CORT concentrations in the yolk within 1.8 SD above control yolks, which is similar to previous studies that have increased CORT levels within physiologically relevant ranges (e.g. Hayward et al., 2006; Love and Williams, 2008). CORT levels were quantified in a sample of yolks from eggs from the same mothers used in this study ($n = 8$) and basal levels were validated using both radioimmunoassay and liquid chromatography-mass spectroscopy (LC–MS–MS). Experimental injection of 8.5 ng of CORT increased whole yolk CORT levels to 17.1 ± 8.3 (SD) ng/ml (RIA analysis, for details see section below). Control eggs were injected with peanut oil alone (Ctrl: $n = 38$). Punctures were sealed with a transparent wound dressing (Germolene New Skin, UK) and each egg was given a unique mark. On day 14 of incubation, eggs were moved to two treatment-specific hatchers (Hatchmaker, Brinsea Ltd, UK), where they were maintained at 37 °C and 75% humidity until hatching on day 18. Fifty-nine eggs hatched, with hatching success for control eggs 74% and for CORT injected eggs 82%. Upon hatching, each chick was given a unique nail polish mark and was returned to the hatcher for 24 h to allow feathers to dry. Chicks of each pre-hatching treatment were subsequently randomly allocated to four pens (114 × 114 × 58 cm) with *ad libitum* food (minced Turkey crumb, BOCM, UK), water and a heat lamp. Pens were maintained at 30 °C for the first 4 days post-hatching, followed by a reduction of 2 °C per day until chicks were 10 days of age, when all additional heat sources were removed and birds were moved to treatment-specific enclosures ($n = 8$; 2 pens per treatment, 100 cm × 86 cm) that were maintained at 20–22 °C throughout the rest of the experiment. The photoperiod was 14 L:10D at all times. When chicks were 4 days old, one pen of each pre-hatching treatment (CORT or Ctrl) was assigned to one of two post-hatching food treatments: either food removal for 25% of daylight hours (3.5 h) on a random daily schedule for 15 days (Food −: $n = 28$) or *ad libitum* food at all times (Ctrl: $n = 31$). Random removal of food has been shown to increase peak CORT levels in starlings (Buchanan et al., 2003), without causing food restriction (Buchanan et al., 2003; Cuthill et al., 2000). Moreover, this treatment is ecologically relevant since precocial birds are not fed by their parents and have to find food by themselves. Thus, while the CORT treatment simulated maternal 'programming' of the offspring regarding a future

stressful environment, the Food− treatment simulated this stressful environment, allowing us to match environmental conditions across developmental stages in an ecologically relevant way. At 20 days post-hatching, all birds were provided with access to *ad libitum* food (Standard Layer Pellet, BOCM, UK and daily supplement of dried mealworms). We thus created four treatment groups: pre-hatching control/post-hatching control (Ctrl/Ctrl: n = 15, 9 males, 6 females); pre-hatching control/post-hatching unpredictable food availability (Ctrl/Food−: n = 13, 7 males, 6 females), pre-hatching CORT-injection/post-hatching control (CORT/Ctrl: n = 16, 4 males, 12 females) and pre-hatching CORT-injection/post-hatching unpredictable food availability (CORT/Food−: n = 15, 9 males, 6 females). The experiment was repeated and conducted in two batches (batch 1 = 31 chicks; batch 2 = 28 chicks). Japanese quail become sexually mature between 4 and 6 weeks post-hatching (Hazard et al., 2005). At this time, we tested the potential long-term effects of both early treatments on the acute stress response, neophobia and exploration of a novel environment.

Between 42 and 44 days of age (43 ± 0.6 days), stress responses were assessed using a standardized capture-handling-restraint stress protocol (Wingfield, 1994). Specifically, between 0900 and 1200 h each bird was captured from its home cage and blood (70 μl) was collected within 2 min of capture to determine basal CORT levels. Birds were then placed in an opaque box and two more blood samples were collected 10 and 30 min after initial capture. Samples were taken by venipuncture of a brachial vein. Blood was collected in a heparinised capillary and then transferred into a microtube and kept on ice until centrifugation (less than 3 h). Samples were centrifuged for 5 min at 3500 rpm and plasma stored at −20 °C for later analysis. All experimental procedures were carried out under Home Office Animals (Scientific) Procedures Act project licence 60/4068 and personal licence 70/1364.

Hormone assays

CORT concentrations were measured after extraction of 20 μl aliquots of plasma in 1 ml of dichloromethane by the radioimmunoassay method (Spencer et al., 2009), using anti-CORT antiserum code Esoterix Endocrinology, USA B3-163 and [1,2,6,7-3H]-CORT label (Perkin Elmer, UK). The cross-reactivity of the antibody is 0.01–1%, depending on the compound. CORT levels of all birds were above the detection limit (0.08 ng/ml). For all samples, extraction efficiency was estimated and ranged between 68 and 100%. All samples were run in duplicate in two assays and intra-assay and inter-assay coefficients of variation were 0.08 and 0.09, respectively. CORT concentration at 50% binding was 1.92 ng/ml. All samples from a single individual were quantified in the same assay and treatment groups were equally represented within each assay.

Behavioural measurements

One day before the start of behavioural tests birds were placed in individual cages (76 × 48 × 3 cm).

Neophobia

Neophobia was measured when quail were 50–58 days of age (53.6 ± 2.9 days). Tests assessed individual responses to two novel objects placed during two test sessions in the familiar feeding dish (an oval white opaque dish 11 cm long × 3 cm high). The first novel object consisted of two plastic balls (1 pink, 1 blue, 6 cm diameter), taped to a piece of paper. The second object was a coloured block tower made with 8 plastic bricks (yellow, blue, green and red bricks, 10 cm high). Both objects were the same for all birds. Tests took place in individual cages and all birds were food-deprived 90 min before the beginning of each test session to standardize hunger levels. Birds were allowed to recover for 2 h with *ad libitum* food access after the end of the first test (started at 1030 h) and the beginning of food deprivation for the second test (starting at 1430 h). Each novel object was tested in a control—novel object—control design. In control trials, the feeding dish contained 3 dried mealworms. In novel object trials, the feeding dish contained 3 dried mealworms and the novel object. For each trial, quail had access to the feeder for 10 min and their behaviour was recorded with a camcorder. The inter-trial interval was 3 min. From the videos, we recorded the latency (in seconds) to eat mealworms from the feeding dish during each trial.

Novel environment exploration

Novel environment exploration was measured after individuals had been tested for object neophobia, when they were 51–60 days of age (55.9 ± 3 days). Quail were first deprived of food for 80 min in their individual cages. They were then moved to the introductory compartment of the novel environment and allowed to habituate for another 10 min without food (see insert Fig. 3). The novel environment was a cage (120 × 75 × 75 cm) containing four novel objects (2 orange plastic traffic cones (17.5 cm high × 10 cm wide on the basis), a multi-coloured enclosed feeder (26 cm × 19 cm × 15 cm), a cloth ring with coloured fabrics (28 cm high × 30 cm wide) and two white opaque plastic panels, one of which hid one of the traffic cones while the other panel hid the cloth ring with coloured fabrics. The second traffic cone was positioned close to the exit of the introductory compartment. Three small white opaque plastic dishes (7 cm diameter × 4 cm high) containing three dried mealworms each were placed near both hidden objects and in the enclosed feeder (see insert Fig. 3). After the 10 min habituation period, a sliding door opened to allow the test subject to enter the novel environment, and the experimenter left the room. The behaviour of the test subject was recorded for 15 min with two camcorders: one in front of the cage and one above the cage. For video analyses, we imagined the cage to be divided into three zones (see insert Fig. 3). From the videos, we recorded the latency to exit the introductory cage, the latency to enter each of the three virtual zones, latency to feed from each of the three feeders, the time spent in each zone and in the introductory compartment, the number of feeders visited, the number of mealworms eaten and the time spent moving.

Statistical analyses

We assessed whether CORT concentrations during the capture-handling-restraint stress response changed with sampling time and pre- and post-hatching stress-related treatments using generalized linear mixed models (GLMM) fitted with a gamma error distribution. Pre-hatching treatment, post-hatching treatment, sex, batch and sample time (0, 10 or 30 min) and all interactions between these variables were specified as fixed factors. Sample time was also included as a repeated factor to take into account the non-independence between samples. Individual identity was used as a random factor to account for inter-individual differences. To further describe the stress response for each bird, we calculated the peak CORT (highest CORT concentration at either 10 or 30 min minus basal CORT) and the change in CORT between 10 and 30 min after capture. The effects of CORT and Food− treatments on both of these dependent variables were analysed with General Linear Models (GLM). In each model, pre-hatching treatment, post-hatching treatment, sex and batch and all interactions between these variables were included as fixed factors. To take into account the potential effects of bleeding day, time of the day of bleeding, time between capture and basal bleeding and basal CORT on the dependent variables, these factors were added as covariates in the models.

We tested whether CORT and Food− treatments affected the latency to feed in the neophobia tests with a GLMM after checking residuals' normality. Pre-hatching treatment, post-hatching treatment, sex, batch and trial (controls and novel object for each object) and all interactions between these variables were included as fixed factors. Trial was also specified as a repeated factor and birds' identity as a random effect.

We assessed how CORT and Food − treatments affected quail behaviour in the novel environment test with GLMs after checking residuals' normality. For each model, pre-hatching treatment, post-hatching treatment, sex and batch and all interactions between these variables were specified as fixed factors. For the number of feeders visited and the number of mealworms eaten, models were fitted using the Poisson error distribution. The impact of both CORT and Food − treatments on the proportion of birds entering feeder 2, which should be the most stressful to enter since it was enclosed by a plastic brick construction, was determined by using a G-test. The G-test was calculated with the UNIVARIATE procedure in SAS 9.2 (SAS Institute Corporation).

GLMMs were fitted using the GLIMMIX procedure, GLMs, after checking for residuals' normality, using the GLM procedure for normal models and the GENMOD procedure for the model based on Poisson error distribution in SAS 9.2. In the GLMMs, the MSPL (Maximum Subject-specific Pseudo-Likelihood) was used as the estimation method. For all GLMMs and GLMs, Tukey–Kramer multiple comparison adjustment was applied to obtain corrected p-values. Probability levels < 0.05 were considered as significant. Data presented are means ± SEM.

Results

There were no sex differences in any of our test measures ($F_{1,58} < 2.12$, $p > 0.15$), and no significant interactions between sex and treatments ($F_{1,58} < 3.61$, $p > 0.061$).

Stress response

CORT levels differed significantly between sample times ($F_{2,117.1} = 44.64$, $p < 0.0001$): basal CORT (8.16 ± 1.03 ng/ml) was lower than CORT levels at 10 (19.52 ± 2.15 ng/ml) and 30 (16.26 ± 1.85 ng/ml) minutes after capture ($t < -7.53$, $df = 117$, $p < 0.0001$). This stress response over time was influenced by pre-hatching treatment (pre-hatching treatment × time: $F_{2,117.1} = 4.55$, $p = 0.013$; Fig. 1) but not post-hatching treatment (post-hatching treatment × time: $F_{2,117.1} = 0.14$, $p = 0.87$). Pre-hatching treatment influenced the change in CORT, with a larger decrease in CORT levels between 10 and 30 min after capture in CORT-treated quail (−6.2 ± 3.1 ng/ml) than in pre-hatching control ones (0.8 ± 3.3 ng/ml) in which CORT levels remained high ($F_{1,58} = 4.54$, $p = 0.039$; Fig. 1).

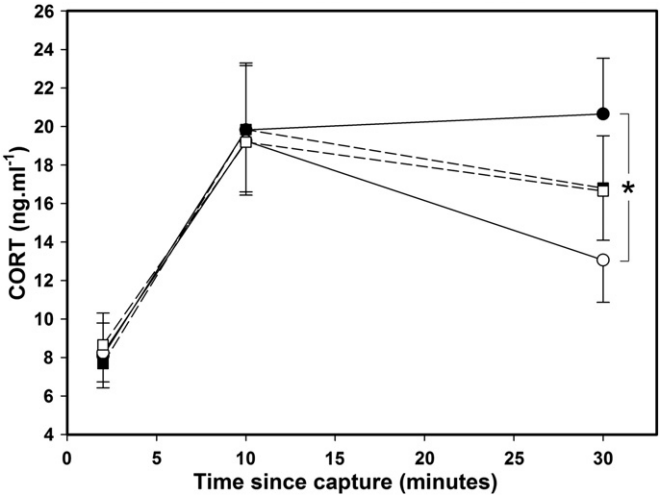

Fig. 1. CORT level modification in response to a standardized capture-handling-restraint stress protocol in pre-hatching control quail (black dots and solid line, n = 28), in pre-hatching CORT-treated quail (white dots and solid line, n = 31), in post-hatching control quail (black squares and dashed line, n = 30) and in post-hatching food-manipulated quail (white squares and dashed line, n = 29). Dots are means ± SEM. * indicate significant differences.

Neophobia

Latencies to eat mealworms from the normal food dish in the individual home cage were higher during the novel object trials compared to the pre- and post-object control trials ($F_{5,290} = 187.29$, $p < 0.0001$; Fig. 2). The latencies during control trials were not different before versus after the presentation of each new object or across objects ($t_{290} < 2.61$, $p > 0.10$; Fig. 2), suggesting quail were highly motivated to eat throughout all trials. Latencies during novel object trials were not different between the two objects ($t_{290} = 1.31$, $p = 0.78$; Fig. 2). Pre- and post-hatching treatments had no effects on the latency to eat mealworms in any of the trials ($F_{1,58} < 0.4$, $p > 0.55$).

Novel environment exploration

The latency to exit from the introductory compartment into the novel environment was affected by pre-hatching treatment ($F_{1,58} = 4.92$, $p = 0.031$) as CORT-treated birds were faster to exit than pre-hatching controls (Fig. 3). Consequently, CORT-treated quail spent less time in the introductory compartment (380 ± 55 s) and more time in Zone 1 of the novel environment (327 ± 46 s) than controls (543 ± 72 s, 207 ± 53 s, respectively) (introductory compartment: $F_{1,58} = 5.01$, $p = 0.029$; zone1: $F_{1,58} = 4.00$, $p = 0.05$). CORT-treated quail spent also more time moving in the novel environment (253 ± 42 s) than did pre-hatching controls (124 ± 36 s) ($F_{1,58} = 8.08$, $p = 0.006$).

One of the three feeders in the novel environment, feeder 2, was designed so that birds had to enter an enclosed space to gain food. We hypothesised that this would be the feeder perceived as the riskiest to enter. Post-hatching CORT exposure significantly reduced the latency to feed from feeder 2 compared to controls (813 ± 34 s, 893 ± 6 s, respectively) ($F_{1,58} = 6.47$, $p = 0.014$).

Exposure to both pre- and post-hatching treatments (CORT/Food −) affected the latency to feed in the novel environment (interaction pre-hatching × post-hatching treatments: $F_{1,58} = 4.84$, $p = 0.032$), the time spent in zone 3 (interaction pre-hatching × post-hatching treatments: $F_{1,58} = 4.95$, $p = 0.031$) and the number of mealworms eaten (interaction pre-hatching × post-hatching treatments: $\chi^2_{1,58} = 4.45$, $p = 0.035$). Although multiple comparisons did not show significant differences between treatment groups for these behaviours, birds experiencing both pre- and post-hatching treatments (CORT/Food −) tended to exhibit the shortest latency to feed from any of the three feeders (Fig. 4a). These birds also spent more time in zone 3 of the novel environment than birds in the other three groups (Fig. 4b) and

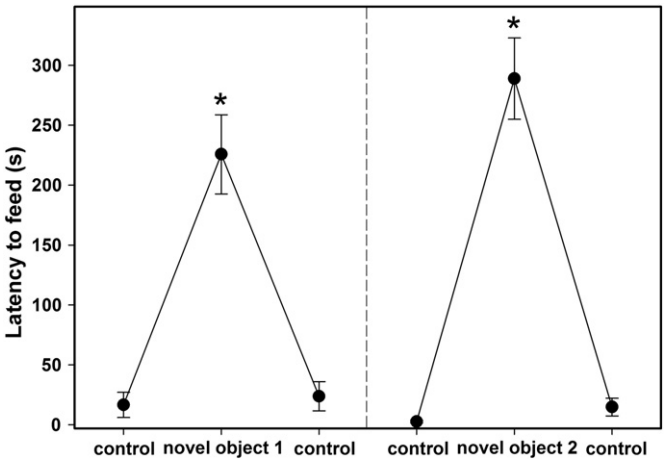

Fig. 2. Mean latency in seconds ± SEM to eat mealworms from the feeding dish in the object neophobia test for both novel objects. Each object was tested in a control–novel object–control design. Novel object 1 consisted of two coloured balls and novel object 2 was a coloured plastic brick block. * indicate significant differences.

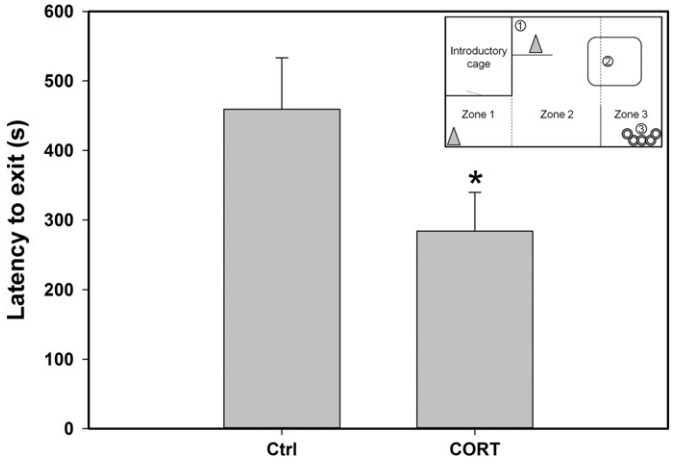

Fig. 3. Mean latency in seconds ± SEM to exit from the introductory compartment for pre-hatching control quail (Ctrl) and pre-hatching CORT-treated quail (CORT). * indicate significant differences. Insert: Plan of the cage used for the novel environment exploration test. Circles with numbers represent feeders. The solid line in zone 2 and the one between zones 2 and 3 represent opaque panels. Triangles represent cones, the five linked circles represent the cloth ring with coloured fabric and the open rectangle represents an enclosed multi-coloured feeder. Dashed lines represent the virtual separations between the different zones.

were more likely to enter feeder 2 (40%) than quail from any other group (Ctrl/Ctrl = 0%, Ctrl/Food− = 7%, CORT/Ctrl = 7%) ($G = 12.09$, $df = 3$, $p = 0.0071$; Fig. 4c). Finally, quail exposed to both developmental treatments obtained more mealworms from the feeders in the novel environment (Fig. 4d).

Discussion

In this study, we show that pre-hatching CORT injection and post-hatching unpredictable food availability led to long-term changes in the physiological stress response and behaviour. Both pre- and post-hatching treatments had effects on behaviour, enhancing exploration of a novel environment. The pre-hatching CORT-treatment significantly altered physiological responses to stress, potentially mediating some of the behavioural effects observed. Most importantly, the effects of combined pre- and post-hatching treatments seemed to be cumulative, leading quail exposed to both to be the most explorative and most likely to find food in the novel environment of all treatment groups. This increase in risk-taking behaviour in a novel, and presumably stressful, environment resulted in a higher food acquisition. We suggest that this is evidence in favour of the environmental matching hypothesis.

In the novel environment test, pre-hatching CORT-treated adult quail exited the introductory cage earlier, spent more time in the novel environment, and spent more time active than controls. Pre-hatching CORT-treatment thus induced increased levels of activity and exploration in adulthood, which has also been found in mammals (Emack and Matthews, 2011; Meek et al., 2000). For instance, exposure of pregnant guinea pig females to chronic stress every second day from gestational days 22 to 66 resulted in an increase in ambulatory activity in male offspring in an open-field test on post-natal day 80 (Emack and Matthews, 2011). In our quail, pre-hatching CORT-treatment also resulted in a permanently attenuated acute stress response. This attenuated stress response may underlie the observed increase in exploration in these birds. Indeed, it has been shown in various mammal and bird species that individuals showing more explorative behaviour and taking more risks in a novel environment have an attenuated stress response to an acute stress as compared to individuals that do not explore (Cavigelli and McClintock, 2003; Macrì and Würbel, 2007; Martins et al., 2007; Stöwe et al., 2010). This suggests increased negative feedback efficiency within the HPA axis. In rats, early-life stress resulting in an attenuated stress response is inextricably linked to a higher expression of glucocorticoid receptors in brain regions involved in negative feedback, such as the paraventricular nucleus of the hypothalamus and the hippocampus (Catalani et al., 2000; Cottrell and Seckl, 2009;

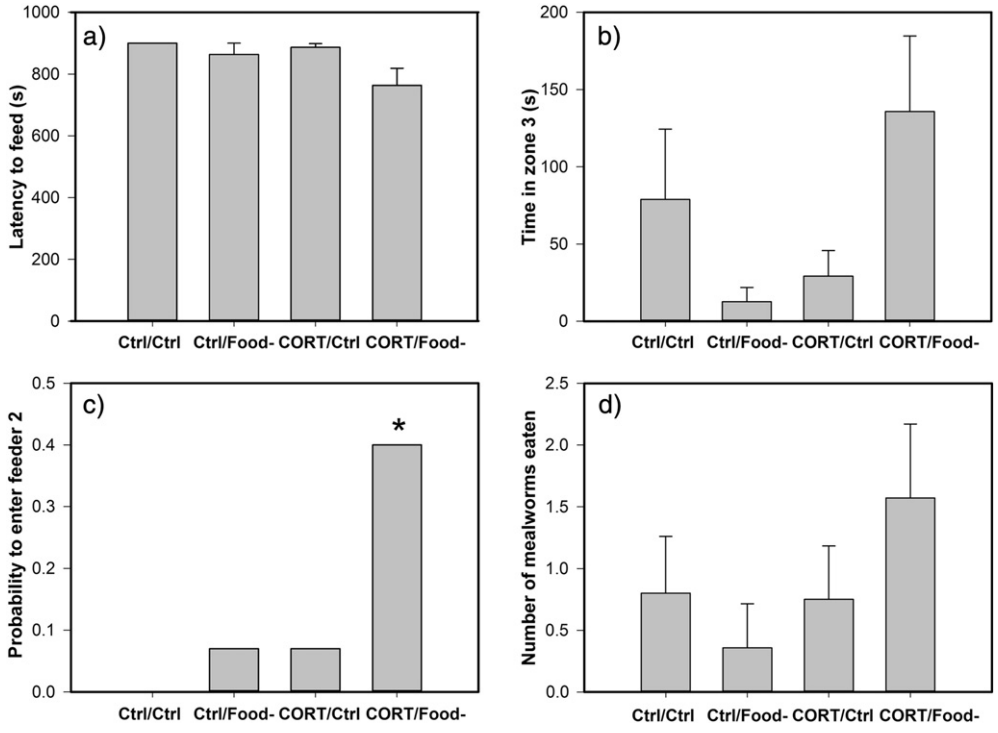

Fig. 4. Effects on behaviour during the novel environment test in the four treatment groups: pre-hatching and post-hatching control (Ctrl/Ctrl), pre-hatching control and post-hatching unpredictable food availability (Ctrl/Food−), pre-hatching CORT-treated and post-hatching control (CORT/Ctrl) and both treatments (CORT/Food−). (a) Mean latency in seconds ± SEM to feed in any feeder. (b) Mean time in seconds ± SEM spent in zone 3. (c) Probability to enter the enclosed feeder (feeder 2). (d) Mean number of mealworms eaten ± SEM. * indicate significant differences.

Kapoor et al., 2008). It has also been shown in birds that glucocorticoid receptor expression in the brain can be affected by stress (Banerjee et al., 2012; Hodgson et al., 2007). Thus, we suggest that increased pre-hatching exposure to CORT modified the HPA axis activity in our quail chicks through a higher expression of glucocorticoid receptors in the brain, a hypothesis that we are currently testing.

Unpredictable food removal during the post-hatching phase had no effects on stress physiology in adulthood. However, it did exert specific effects on feeding behaviour: birds in the post-hatching treatment group exhibited decreased latencies to feed from an unfamiliar and stressful enclosure (feeder 2). Hence, it appears that birds exposed to unpredictable food availability post-hatching took more risks to find food in adulthood than post-hatching controls. Similar effects of post-hatching stress on adult risk-taking behaviour have been reported in different species (e.g. rats: Macrì and Würbel, 2007; Roman et al., 2006; great tits *Parus major*: Carere et al., 2005; domestic chicken *Gallus gallus domesticus*: Freire et al., 2006; zebra finch: Krause et al., 2009) and may be linked to increased nutritional needs (Krause et al., 2009). Such behavioural responses in novel environments, where food availability is unknown, may allow individuals to better cope with periods of food shortage even in more familiar environments and as such could be considered adaptive (Carere et al., 2005).

In mammals, it has been shown that exposure to pre- and post-natal stress can have cumulative effects, resulting in greater negative consequences than exposure to stress during a single developmental stage, or having opposite effects (Chung et al., 2005; Koo et al., 2003; Meaney et al., 2007; Vallée et al., 1999). In our case, the effects of pre- and post-hatching treatments on physiology and behaviour appeared to lead to cumulative effects in our birds that experienced both treatments: these birds were the fastest to feed in the novel environment, spent more time away from the introductory cage and in the zone of the enclosed feeder, were more likely to enter this feeder, and tended to eat more mealworms than control quail and those treated during a single developmental period. We suggest that exposure to both early stress-related treatments resulted in an overall increase in exploration and higher risk-taking when exposed to stressful situations later in life, which has been demonstrated to have significant fitness advantages in several species (Dingemanse et al., 2004; Smith and Blumstein, 2008). It remains to be determined whether Japanese quail that show more explorative and risk-taking behaviours will experience similar fitness advantages when subjected to natural selection pressures in the wild.

Contrary to our predictions we found no treatment effects on neophobia. In contrast, studies on other species have shown that pre-natal and post-natal stress can decrease neophobia. In chickens for example, CORT injection in eggs reduced the probability that hatchlings crossed a barrier to access food (Janczak et al., 2006), and CORT-treated zebra finch male nestlings showed less neophobia as adults (Spencer and Verhulst, 2007). In rats, post-natal maternal separation resulted in increased object exploration compared to controls (Hensleigh et al., 2011; Roy and Chapillon, 2004). However, rearing environment had no effect on neophobia in starlings (Feenders et al., 2011). It has been proposed that differences in neophobia due to developmental history might only be evident when individuals experience intense fear (Feenders et al., 2011). As CORT-treatment and unpredictable food availability affected quail behaviour in the novel environment test (see above) but not in the object neophobia test, it seems likely that exposure to novel objects in the individual cage did not induce as much fear as exposure to a completely different environment filled with novel objects. Hence, object neophobia and responses to a novel environment were not correlated in our birds or in other avian studies (Boogert et al., 2006; Feenders et al., 2011).

Although other studies have already shown that early-life stress can have long-term effects on behaviour and physiology, to our knowledge this is the first study to show long-term persistent cumulative effects of stress-related treatments experienced during both pre- and post-hatching stages on adult behaviour. We show that early life conditions can program physiology and behaviour into adulthood in a way that could potentially enhance fitness. Therefore, our results are in accordance with the environmental matching hypothesis. Exposure to stress-related events across different developmental stages appears to program the HPA axis and behaviour in such a way that individuals encountering novel environments obtain more resources, which in turn may result in an increase in fitness.

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