# RESEARCH ARTICLE

# Pre- and post-natal stress in context: effects on the stress physiology in a precocial bird

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## SUMMARY

**Developmental stress can significantly influence physiology and survival in many species. Mammalian studies suggest that pre- and post-natal stress can have different effects (i.e. hyper- or hypo-responsiveness) on the hypothalamic–pituitary–adrenal (HPA) axis, the main mediator of the stress response. In mammals, the physiological intimacy between mother and offspring constrains the possibility to control, and therefore manipulate, maternal pre- and post-natal influences. Here, using the Japanese quail (*Coturnix coturnix japonica*) as our model, we elevated levels of the glucocorticoid stress hormone corticosterone *in ovo* and/or in the endogenous circulation of hatchlings. We examined the effects of treatments on corticosterone and glucose stress responses at two different ages, in juvenile and adult quail. In juveniles, corticosterone data revealed a sex-specific effect of post-natal treatment regardless of the previous pre-natal protocol, with post-natally treated females showing shorter stress responses in comparison with the other groups, while no differences were observed among males. In adulthood, birds previously stressed as embryos showed higher corticosterone concentrations over the stress response compared with controls. This effect was not evident in birds subjected to either post-natal treatment or the combined treatments. There were no effects on glucose in the juveniles. However, adult birds previously stressed *in ovo* showed opposite sex-specific basal glucose patterns compared with the other groups. Our results demonstrate that (1) early glucocorticoid exposure can have both transient and long-term effects on the HPA axis, depending upon the developmental stage and sex and (2) post-natal stress can modulate the effects of pre-natal stress on HPA activity.**

Key words: early life stress, vertebrate stress response, glucocorticoid, corticosterone, growth, glucose balance, HPA axis responsiveness, short-term effect, long-term effect, glucocorticoid programming.

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## INTRODUCTION

Environmental cues during the sensitive periods of early life can shape developmental trajectories and influence a wide range of phenotypic traits later in life (Mousseau and Fox, 1998; Gilbert, 2005; Monaghan, 2008). A considerable number of studies have investigated the extent to which developmental stress can modulate endocrine systems and influence adult health (Ward, 1972; Barker, 1990; Gluckman et al., 2007). In vertebrates, environmental stressors such as food shortages or extreme weather can activate the hypothalamic–pituitary–adrenal axis (HPA axis) (Romero, 2004; Wingfield, 1994). This activation leads to a short-term surge of glucocorticoids, which mobilise glucose stores to increase energy and divert behaviour to life-saving strategies (Munck et al., 1984; Wingfield et al., 1998). In the long-term, however, elevated glucocorticoids can compromise HPA axis functioning and can have negative implications for the nervous and immune systems, body energy balance and redox physiology (McEwen and Stellar, 1993; de Kloet et al., 2005; Sapolsky, 2000; Costantini et al., 2011). There is increasing evidence to suggest that if laying/gravid females experience stressful stimuli, which elevate endogenous glucocorticoids (e.g. predation pressure, social instability, unpredictable feeding or direct glucocorticoid exposure), their embryos can also be exposed to these circulating stress hormones

through the placenta (Seckl, 2004; Kaiser and Sachser, 2005) or their presence in the egg [fish (McCormick, 1999); reptiles (De Fraipont et al., 2000; Meylan et al., 2002); birds (Hayward and Wingfield, 2004; Hayward et al., 2005; Love et al., 2005; Saino et al., 2005)]. Similarly, after birth, post-natal stressors such as sibling competition, low food provisioning, maternal deprivation or direct glucocorticoid administration can lead to an increase in endogenous glucocorticoids in the offspring [reptiles (Meylan et al., 2002); birds (Kitaysky et al., 1999; Love et al., 2003; Spencer et al., 2003; Spencer et al., 2009; Banerjee et al., 2011); mammals (Rosenfeld et al., 1992; Fey and Trillmich, 2008)]. Pre- or post-natal glucocorticoid manipulations have been shown to influence a wide range of phenotypic traits, with possible implications for organismal fitness [fish (Sloman, 2010); reptiles (De Fraipont et al., 2000; Meylan et al., 2002); birds (Spencer et al., 2003; Rubolini et al., 2005; Spencer and Verhulst, 2007; Spencer et al., 2009; Monaghan et al., 2011); mammals (Vallée et al., 1997; Vallée et al., 1999)].

It has been suggested that the organisational role of early glucocorticoid exposure on the phenotype is likely to be caused by changes in HPA axis activity that modulate sensitivity to environmental stressors later in life (for reviews, see Levine, 2002; Harris and Seckl, 2011). Pioneering studies in mammalian models (primarily rodents) suggest that the effects of pre-natal stress on the

offspring HPA axis may be different from those caused by post-natal stress. While maternal pre-natal stress often results in HPA hyper-responsiveness, with pre-natally stressed offspring exhibiting enhanced and prolonged stress hormone release in response to stress (Henry et al., 1994; Barbazanges et al., 1996; Kapoor et al., 2006), post-natal exposure to stressors, such as 'neonatal handling', can produce dampened stress responsiveness (Levine, 1967; Meaney and Aitken, 1985; Vallée et al., 1996; Liu et al., 1997; Macrì et al., 2004). Importantly, several post-natal manipulations in rat pups are known to cause changes in the amount of maternal care provided by the dams, which, to a certain degree, can buffer or counteract the effects of previous pre- and post-natal stressors (Maccari et al., 1995; see also review by Macrì and Würbel, 2006). On the one hand, these data raise the question, surprisingly understudied, of interactive influences between pre- and post-natal experiences. On the other hand, they also draw attention to the difficulties in determining whether the observed effects are mediated by an altered maternal HPA axis, direct changes in the offspring HPA reactivity or an interaction of both, as has been recently proposed (Macrì and Würbel, 2006). Birds offer advantages over mammalian species in terms of experimentally manipulating pre- and post-natal environments, minimising interactions with the mother's physiology (see reviews by Henriksen et al., 2011; Schoech et al., 2011; see also Spencer et al., 2009; Love and Williams, 2008). Precocial birds in captive conditions can be reared without post-natal maternal contact, thereby excluding the potential confounding factor of maternal care. Furthermore, avian and mammalian neuroendocrine systems are highly conserved (Wingfield, 2005a), facilitating comparative approaches in a more evolutionary framework (Groothuis et al., 2005). The few studies conducted in birds to date have demonstrated that pre- (Hayward and Wingfield, 2004; Hayward et al., 2006; Haussmann et al., 2012; Love and Williams, 2008) or post-natal stress (Spencer et al., 2009; Love and Williams, 2008) can lead to long-term effects on HPA axis physiology. However, we need more studies to fully understand the directions of these modifications, as they are likely to differ across bird species and life stages. To this end, it is important to consider both pre- and post-natal contexts (Love and Williams, 2008; Monaghan, 2008). Furthermore, we have very little information about the link between early life stress and changes in metabolic energy expenditure in birds (Spencer and Verhulst, 2008), and to what extent they are involved in HPA axis modifications. High glucocorticoid concentrations in early life may induce changes in stress and metabolic responses that can help developing individuals to deal with stressful circumstances in the short-term, but can have costs later in life (Gluckman et al., 2007; Cottrell and Seckl, 2009). Glucose may be a good candidate for investigating the above links as (1) several vertebrates, including birds, can increase circulating glucose levels in response to acute stress (Oller Do Nascimento Curi et al., 1990; Widmaier and Kunz, 1993; Carragher and Rees, 1994; Remage-Healey and Romero, 2000; Remage-Healey and Romero, 2001); and (2) long-term elevation of glucocorticoids is involved in glucose mobilisation and basal metabolism (Norris, 1997).

In the present study, we mimicked prolonged exposure to stress through direct corticosterone (B) manipulations during the pre- and/or post-natal development of Japanese quail, *Coturnix coturnix japonica* L. Our main aim was to determine whether exposure to B in early life would alter HPA axis activity in the short- and long-term. Therefore, we measured B and glucose stress responses during two different post-natal stages: in juvenile and adult quail. We also monitored growth rates to assess direct *versus* indirect effects of developmental B on the stress responses (Metcalfe and Monaghan,

2001; Spencer et al., 2009). As precocial birds hatch from the egg fully developed, they may be more affected by constraints experienced *in ovo* than after hatching, during post-natal growth (Metcalfe and Monaghan, 2001). Therefore, we expected that pre-natal B exposure would produce a stronger and longer-lasting impact on the offspring physiology than post-natal B exposure. As a certain degree of development occurs during post-natal growth, variation in post-natal environmental conditions may mediate the effects induced by pre-natal stress. We tested these predictions by comparing the effects of pre-natal B, post-natal B and their combined effect on the HPA axis physiology.

## MATERIALS AND METHODS
### Experimental design
The study was conducted using a captive population of outbred Japanese quail at the University of Glasgow (Cochno Research Centre and Farm, Scotland). All indoor rooms were climate controlled at 19°C on a 12h:12h light:dark cycle. Eggs used in this experiment were obtained from this breeding stock. Breeding quail (20 females, 10 males) were housed in groups of three (two females:one male) in $79\times48\times58$ cm enclosures that were maintained throughout the experimental period (September 2010–March 2011). Fresh-laid eggs were collected, identified by colour and pattern, and marked according to maternal identity. Four groups of experimental birds were established and treated as follows: (1) pre-natal and post-natal untreated birds (CC); (2) pre-natal B-treated and post-natal untreated birds (BC); (3) pre-natal untreated and post-natal B-treated birds (CB); and (4) pre-natal B-treated and post-natal B-treated birds (BB). Treatment order was counterbalanced across females. The experiment was repeated twice (batch 1: September 2010–December 2010; batch 2: December 2010–March 2011). All procedures were carried out under Home Office Project Licence 60/4068 and Personal Licence 60/12436, and were approved by the Welfare and Ethics Committee of the University of Glasgow.

### Pre-natal environment and pre-natal hormonal B manipulation
Eggs were incubated at 37.5°C and 55% humidity while being turned twice hourly (incubator Ova-Easy 190A, Brinsea Products, Sandford, UK). The day on which incubation started was designated as embryonic day 0 (E0). At day E5, fertility was established using a bright light source. Fertile eggs were injected at the conical tip with 10μl of a sterile solution of B (Sigma-Aldrich, Poole, UK; concentration B: $850\,ng\,ml^{-1}$) dissolved in peanut oil (B-eggs; $N=74$) or with 10μl of sterile peanut oil alone (C-eggs; $N=74$) and punctures were sealed with a transparent and breathable wound dressing (Germolene New Skin, Devon, UK) before the egg was returned to the incubator. Pilot dye studies were carried out prior to the experiment to determine the depth of injection required to place the hormone into the yolk (K.A.S., unpublished). Day 5 of incubation was chosen as this is the point at which we can reliably determine egg fertility in the Japanese quail. Injection prior to this point would mean that we might inject non-fertile eggs, which will not hatch or develop and hence artificially inflate the perceived number of animals used in this study. Bird egg yolks are stratified in layers at laying and these layers break down after a few days incubation with the yolk becoming mixed. Our injection at day 5 therefore ensured that corticosterone levels were elevated once yolk layers had ceased to exist. Our injections gave a dose of 8.5 ng of B, which had been shown to elevate endogenous B concentrations within the yolk by $1.8\times$ standard deviation (s.d.) of the mean above control eggs taken from a previous generation of our breeding

population (K.A.S., unpublished). This concentration increase is comparable with previous studies in birds that aimed to manipulate stress levels during embryogenesis within the physiological range (e.g. Rubolini et al., 2005; Saino et al., 2005; Hayward et al., 2006; Love and Williams, 2008). At day E14, eggs were transferred into hatchers within the same incubator, and humidity was increased to 70–75%. In each hatcher, eggs were separated according to maternal identity with plastic dividers so that the identity of the chicks could be determined post-hatching.

Post-natal environment and post-natal hormonal B manipulation
Upon hatching (between days E17 and E19, hatch rates averaged 61.12%), chicks were labelled with unique colour combinations using nail varnish, weighed to the nearest 0.01 g (hatching mass, day PN0) and placed back into the hatcher to allow the plumage of the chicks to dry. Subsequently, chicks hatched from B-eggs were assigned to either the BC treatment (final $N$: female=10, male=6) or the BB treatment (final $N$: female=9, male=9), and chicks hatched from C-eggs were assigned to either the CC treatment (final $N$: female=9, male=14) or the CB treatment (final $N$: female=10, male=10). After 24–36 h post-hatching (day PN1), chicks were weighed again and housed in four different treatment-specific enclosures in a single room (in the second batch, treatment-specific enclosure positions were reversed to control for an enclosure effect). Food (turkey starter crumbs, Dodson and Horrell, Kettering, Northamptonshire, UK) and water were available *ad libitum*. A brooding lamp was placed over each enclosure to ensure an initial brooding temperature of 35.5°C for the first 3 days of age (from day PN3, temperature declined daily by 1–1.5°C until day PN19, when warming bulbs were switched off and chicks were subjected to the ambient temperature of 19°C). Enclosures were each divided into two or three compartments with cardboard dividers so that chicks of the same age were housed in the same compartment ($N$=2 to 7).

Birds in the CB and BB treatments were subjected to oral supplementation with B between days PN5 and PN19; birds in the CC and BC treatments were given carrier alone. To ascertain that our post-natal manipulation was physiological and mimicked a standardised acute stressor (Wingfield et al., 1982), two pilot studies were performed prior to the start of the experiment. First, we determined the natural variation of the adrenocortical response in a sample of chicks from the same population that was not used in the main study at day PN8 ($N$=12) and PN16 ($N$=12) using a standardised capture-restraint protocol as described below. As shown in other precocial birds (Holmes et al., 1989; Dickens and Romero, 2010), HPA responsiveness declined with age, with the younger chicks showing a higher 10-min B peak (i.e. maximal adrenocortical response) than the older chicks [mean ± s.e.m.=43.14±6.06 and 23.57±2.72 ng ml$^{-1}$, respectively (V.M., J.R., P.H., K.A.S., unpublished)]. In the second pilot, we determined the B dosage to be used in the main study (Hull et al., 2007; Spencer and Verhulst, 2007; Spencer et al., 2009; Wall and Cockrem, 2009). We tested two different doses, a high-B dose of 0.45 mg and a low-B dose of 0.045 mg, in four independent groups of chicks at days PN8 and PN16 (high-B, $N$=5; low-B, $N$=4 at both ages). We administered a single oral dose to each chick and took a blood sample 10 min post-B supplementation. The low-B dose at day PN8 elevated plasma B levels by 1.8× s.d. of the mean above the 10-min B peak determined in the first pilot, while at day PN16 there was no significant change in plasma B levels. The high-B dose was supra-physiological at both ages. We therefore scaled the dose for the main study to produce a daily physiological 10-min peak for each age interval (e.g. Spencer et al., 2009): 0.045 mg day$^{-1}$ between days PN5 and PN15, and an intermediate B dose of 0.09 mg day$^{-1}$ between days PN16 and PN19. During the present experiment, we tested that the B dose of 0.09 mg day$^{-1}$ was biologically relevant by sampling a sub-sample of chicks at day PN16 as described above. Plasma B levels in CB and BB chicks were found to be similar to the 10 min-B peak observed in our first pilot study ($N$=11, pooled data: mean ± s.e.m.,18.77±4.55 ng ml$^{-1}$).

Doses were delivered using mealworms (*Tenebrio molitor*, 13–18 mm) injected with B (Breuner et al., 1998). To ensure that chicks would ingest mealworms, they were provided with un-injected mealworms for 3 days prior to the experimental manipulations. During the oral B manipulation period, mealworms were removed from the fridge and injected with 10 μl of B solution dissolved in peanut oil (concentration B: 4.5 mg ml$^{-1}$ between days PN5 and PN15, and 9 mg ml$^{-1}$ between days PN16 and PN19) or 10 μl of peanut oil using a Hamilton syringe (Sigma-Aldrich, Gillingham, UK). To confirm that each chick was eating a single mealworm per day, chicks within the same brooder compartment were separated with transparent dividers during feeding. Generally, mealworms were fully ingested within the first 5–10 min (and always within 18 min). At day PN19, chicks were sexed by sexual dimorphic plumage and singly housed in 61×46×51 cm enclosures, in visual and auditory contact with conspecifics. Post-natal mortality rates averaged 8.08%.

## Analysing the short- and long-term effects of pre- and post-natal B exposure
### Growth
Body mass was measured to the nearest 0.01 g using an electronic balance at regular intervals until day PN64; from day PN3 onwards, tarsus length and head plus bill length were also measured to the nearest 0.1 mm with a digital calliper.

### HPA axis responsiveness and glucose metabolism during acute stress
Acute stress responses were measured using a standardised capture-restraint protocol on days PN22 and PN64. We chose days PN22 and PN64 because we wanted to test the effects of early glucocorticoid treatment in the short-term at the peak of the growth curve and in the long-term just prior to maturity, respectively. Birds were removed from their cages between 09:15 and 12:40 h and a basal blood sample (T0) was collected within ~1.5 min (mean ± s.e.m., 1.43±0.04) of opening the cage (Wingfield et al., 1982). Each chick was then placed into an opaque box and further stress-induced blood samples were taken after ~10 min (mean ± s.e.m., 10.52±0.08 min) and ~30 min (mean ± s.e.m., 30.31±0.06 min) of opening the cage (T10 and T30, respectively). Twenty microlitres of T0 and T30 samples were immediately used to measure glucose concentrations using a glucose meter (GlucoMen Visio, Manarini Diagnostics Srl, Florence, Italy). T0 and T30 glucose samples were chosen to represent basal and stress-induced glucose levels, respectively (e.g. Oller Do Nascimento Curi et al., 1990; Carragher and Rees, 1994; Remage-Healey and Romero, 2000; Remage-Healey and Romero, 2001). For each glucose meter barcode used, we estimated the intra- and inter-assay coefficients of variation by measuring normal and high quality control solutions provided by the manufacturer. The intra-assay variation for normal and high quality controls was 3.25 and 3.18%, respectively. The inter-assay variation for normal and high quality controls was 4.34 and 1.81%, respectively.

Once the stress response protocol was concluded, body mass, tarsus length and head plus bill measurements were taken for each bird. The remaining blood samples were kept on ice for up to 4 h before being centrifuged, and plasma aliquots were withdrawn and stored at –20°C.

### Radioimmunoassay

B was extracted in 1 ml diethyl ether (Rathburn Chemicals, Walkerburn, UK) from each aliquot of 10 to 30 μl of plasma (mean ± s.e.m., 21.81±0.15 μl plasma). Tracer amounts ($\approx$4000 c.p.m.) of [1, 2, 6, 7-$^3$H] B label (NET 399, PerkinElmer, Waltham, MA, USA) were added to each sample to estimate extraction efficiencies. After extraction, B concentrations (ng ml$^{-1}$) were measured using anti-B antiserum code B3-163 (1:100 dilution in assay buffer: 0.01 mol l$^{-1}$ PBS, pH 7.4, 0.25% BSA; Esoterix, Austin, TX, USA) and B label, as described in Spencer et al., (Spencer et al., 2009). Extraction efficiencies were calculated for each individual sample and averaged 93±0.003%. B samples from the same individuals were analysed in the same assay and samples from different treatments were randomised among the assays ($N$=3). The mean assay sensitivity for all assays was 0.12 ng ml$^{-1}$. Chicken plasma and two B-spiked plasma pools that gave approximately 80, 70 and 50% binding on the standard curve, respectively, were included as quality controls in each assay. The intra-assay coefficients of variation were 10, 20 and 23%, while the inter-assay coefficients of variation at 80, 70 and 50% binding were 17, 18 and 9%, respectively.

Unexpectedly, concentrations of B in 53.7% of the samples were undetectable. Preliminary chi-square tests showed that the likelihood of encountering undetectable values differed significantly across the stress responses. At both days PN22 and PN64, the highest percentage of undetectable values was observed in the T0 samples (day PN22: $\chi^2$=38.53, d.f.=2, $P$<0.0001; T0=83.1%, T10=33.8%, T30=55.8%; day PN64: $\chi^2$=16.76, d.f.=2, $P$<0.0001; T0=68.8%, T10=40.3%, T30=40.3%). We then performed additional chi-square tests at each time of sampling to test for a potential treatment effect. At day PN22, there was no effect of treatment in the T0 and T30 samples (T0: $\chi^2$=5.07, d.f.=3, $P$=0.17; T30: $\chi^2$=3.07, d.f.=3, $P$=0.38), whereas there was a tendency towards a treatment effect in the T10 samples ($\chi^2$=7.47, d.f.=3, $P$=0.06) due to a lower percentage of undetectable levels in the BC and BB groups (Table 1). Similarly, at day PN64, there was no effect of treatment in the T0 and T30 samples (T0: $\chi^2$=1.57, d.f.=3, $P$=0.67; T30: $\chi^2$=5.90, d.f.=3, $P$=0.12). At T10, we found a significant treatment effect ($\chi^2$=8.59, d.f.=3, $P$=0.03) due to a lower percentage of undetectable samples in the BC adult quail (Table 1). To investigate in a further statistical model these potential treatment differences (see below), we set undetectable B concentrations to the individual detection limits of each sample, calculated according to the individual extraction efficiencies and plasma volumes (mean ± s.e.m.=1.64±0.29 ng ml$^{-1}$), as shown in Landys et al. (Landys et al., 2010). This approach provides the most conservative estimate for statistical comparisons.

### Statistical analysis

Data analysis was performed in PASW statistics 18.0.0 (SPSS, Chicago, IL, USA) using linear mixed effect models (LMEs) fitted by restricted maximum likelihood. To meet the assumptions of the LME, response variables were transformed for normality when needed; all model residuals were normally distributed. Fixed factors were treatment, sex and their interaction, while batch and maternal identity were entered as random factors.

The growth curve between days PN1 and PN36 was split into three discrete age intervals: days PN1–3, days PN8–19 and days

Table 1. Percentage of undetectable corticosterone (B) samples in *Coturnix coturnix japonica* across the treatment groups at post-natal day (PN) 22 and 64 during a standardised capture–restraint protocol within 2, 10 and 30 min (T0, T10 and T30, respectively) of opening the cage

| | Treatment | | | |
|---|---|---|---|---|
| Time | CC | BC | CB | BB |
| PN22 | | | | |
| T0 | 95.7 | 68.8 | 80.0 | 83.3 |
| T10 | 47.8 | 12.5 | 45.0 | 22.2 |
| T30 | 56.5 | 37.5 | 65.0 | 61.1 |
| PN64 | | | | |
| T0 | 73.9 | 53.3 | 70.0 | 72.2 |
| T10 | 56.5 | 12.5 | 35.0 | 50.0 |
| T30 | 34.8 | 18.8 | 50.0 | 55.6 |

CC, pre-natal and post-natal untreated birds; BC, pre-natal B-treated and post-natal untreated birds; CB, pre-natal untreated and post-natal B-treated birds; BB, pre-natal B-treated and post-natal B-treated birds.

PN22–36, which corresponded to periods before, during and after the post-natal B treatment, respectively. For each interval, we estimated individual body mass growth rates by calculating the slope of a linear regression fitted for each chick. Likewise, we determined tarsus and head plus bill growth rates between days PN8 and PN19 and between days PN22 and PN36. These two measures of skeletal growth were transformed into a unique body size growth index by extracting the first component scores from a principal component analysis (PCA) in each age interval [PCA days PN8–19 (PCA1): eigenvalue=1.27, total variance=63.44%; PCA days PN22–36 (PCA2): eigenvalue=1.26, total variance=63.24%]. Similarly, the first component scores from a PCA on tarsus and head plus bill absolute values measured at day PN64 [PCA day PN64 (PCA3): eigenvalue=1.38; total variance=69.15%] gave a body size index. Hatching mass, body mass growth rates and body mass at day PN64 were analysed in separate LMEs to disentangle potential short-and long-term effects of developmental B exposure. When needed, hatching mass values or the appropriate PCA was added into the LME as a covariate (see Table 2).

For glucose and B analysis, data were split by age (PN22 and PN64). Glucose concentrations were log$_{10}$-transformed. Basal glucose levels and delta glucose (the change in glucose between T0 and T30) were analysed in separate LMEs. The glucose barcode was included as an additional random factor. B concentrations were inverse-transformed and the HPA responsiveness was analysed using similar LMEs as for glucose, with the addition of a repeated-measure approach to examine changes in B levels over the time of sampling (i.e. T0, T10 and T30). We included the interactions that were biologically meaningful to our study design: treatment × time of sampling, treatment × sex and treatment × time of sampling × sex.

In all LMEs, non-significant effects ($P$>0.05) were dropped using a backward procedure following Crawley (Crawley, 1993). *Post hoc* analyses for main effects were performed using the available Bonferroni method in PASW, which applies an adjustment to $P$-values to account for multiple comparisons. Significant interactions were further investigated in separate LMEs using pre- and post-natal treatment as two distinctive fixed factors, each of them with two levels. Unless otherwise specified, data are presented as means ± s.e.m.

Table 2. Results of linear mixed effect (LME) modelling of potential short- and long-term effects of treatment, sex and their interaction on measures of post-natal (PN) body mass or growth rates at day PN0, days PN1–3, days PN8–19, days PN22–36 and day PN64

| | d.f. | F | P |
|---|---|---|---|
| Day PN0: hatching mass | | | |
| Treatment | 3,58.05 | 1.54 | 0.21 |
| Sex*,2 | 1,59.66 | 0.21 | 0.65 |
| Treatment × sex*,1 | 3,54.78 | 1.29 | 0.29 |
| Days PN1–3: body mass growth | | | |
| Treatment | 3,66.64 | 0.22 | 0.88 |
| Sex*,5 | 1,69.24 | 0.47 | 0.49 |
| Hatching mass*,6 | 1,44.65 | 0.97 | 0.33 |
| Treatment × sex*,3 | 3,60.08 | 0.68 | 0.68 |
| Treatment × hatching mass*,2 | 3,57.48 | 0.19 | 0.90 |
| Sex × hatching mass*,4 | 1,65.07 | 2.19 | 0.14 |
| Treatment × sex × hatching mass*,1 | 3,56.40 | 0.84 | 0.48 |
| Days PN1–19: body mass growth | | | |
| Treatment | 3,58.38 | 2.28 | 0.09 |
| **PCA1** | 1,65.44 | 19.29 | <0.0001 |
| Sex*,5 | 1,62.15 | 0.53 | 0.47 |
| Treatment × sex*,3 | 3,54.09 | 0.79 | 0.51 |
| Treatment × PCA1*,2 | 3,55.64 | 0.37 | 0.77 |
| Sex × PCA1*,4 | 1,60.89 | 0.67 | 0.41 |
| Treatment × sex × PCA1*,1 | 3,51.24 | 0.61 | 0.61 |
| Days PN22–36: body mass growth | | | |
| Treatment | 3,60.85 | 2.88 | 0.14 |
| **Sex** | 1,64.94 | 5.23 | 0.02 |
| **PCA2** | 1,69.55 | 18.28 | <0.0001 |
| Treatment × sex*,3 | 3,54.31 | 1.13 | 0.34 |
| Treatment × PCA2*,4 | 3,60.23 | 1.74 | 0.17 |
| Sex × PCA2*,2 | 1,61.57 | 0.012 | 0.91 |
| Treatment × sex × PCA2*,1 | 3,52.31 | 1.56 | 0.21 |
| Day PN64: body mass | | | |
| Treatment | 3,62.51 | 0.44 | 0.72 |
| **Sex** | 1,70.51 | 79.21 | <0.0001 |
| **PCA3** | 1,69.58 | 57.30 | <0.0001 |
| Treatment × sex*,3 | 3,61.12 | 0.68 | 0.57 |
| Treatment × PCA3*,2 | 3,62.69 | 0.47 | 0.70 |
| Sex × PCA3*,4 | 1,57.12 | 2.94 | 0.09 |
| Treatment × sex × PCA3*,1 | 3,57.38 | 0.12 | 0.95 |

At days PN1–3, days PN8–19, days PN22–36 and day PN64, the appropriate covariate (hatching mass, PCA1, PCA2 and PCA3, respectively) and its interactions with treatment and sex were included to control for body size (see text for details).
Asterisks indicate excluded factors during the stepdown procedure; superscripted numbers refer to the order of removal.
Significant factors are in bold (P<0.05).

## RESULTS
### Effects of developmental B exposure on growth
Hatching mass did not differ across treatments, sex or their interaction (Table 2). Similarly, there were no treatment differences in body mass growth up to day PN3; there was no effect of sex or hatching mass on growth (Table 2). During the post-natal corticosterone manipulation (days PN5–19), we found no differences in growth between B-treated and control birds. There was a significant positive co-variation between body mass growth rates and PCA1, but the slopes did not differ across groups (Table 2). Once post-natal B exposure had ceased, neither treatment nor its interaction with sex were significant for growth rates between days PN22 and PN36 or for body mass at day PN64 (Table 2); there was a significant effect of sex on both variables (Table 2), with females showing larger growth and body mass than males. Also, growth between days PN22 and PN36 and body mass at day PN64 co-varied positively with PCA2 and PCA3, respectively, but we found no other effects of these variables (Table 2).

Table 3. Results of LME modelling of potential short- and long-term effects of treatment, sex and their interaction on HPA axis responsiveness at post-natal day (PN) 22 and 64

| | d.f. | F | P |
|---|---|---|---|
| Day PN22 | | | |
| Treatment | 3,66.05 | 2.38 | 0.08 |
| **Time** | 2,99.71 | 27.09 | <0.0001 |
| Sex | 1,66.05 | 1.48 | 0.23 |
| Treatment × sex | 3,66.05 | 0.07 | 0.98 |
| Treatment × time | 6,99.71 | 0.49 | 0.82 |
| **Treatment × sex × time** | 8,99.71 | 2.38 | 0.02 |
| Day PN64 | | | |
| Treatment | 3,70.79 | 3.25 | 0.03 |
| Time | 2,99.47 | 10.71 | <0.0001 |
| Sex*,4 | 1,69.02 | 3.17 | 0.08 |
| Treatment × sex*,3 | 3,64.09 | 0.24 | 0.87 |
| Treatment × time*,2 | 6,96.09 | 0.31 | 0.93 |
| Treatment × sex × time*,1 | 8,89.54 | 0.67 | 0.72 |

Asterisks indicate excluded factors during the stepdown procedure; superscripted numbers refers to the order of removal.
Significant factors are in bold (P<0.05).

### Effects of developmental B exposure on the HPA axis and glucose balance
#### Day PN22
As expected, B levels during the capture–restraint protocol were significantly affected by sampling interval (Table 3). Overall, baselines were lower than both stress-induced B levels (*post hoc*, T0 values *versus* T10 and T30 values: P<0.0001 in both pair-wise comparisons), whereas there were no differences between B levels at T10 and T30 (*post hoc*: P=0.49). There were no effects of sex on B levels, nor any treatment effects (Table 3). However, there was a significant interaction between treatment and sex in terms of the shape of the stress response (Table 3, Fig. 1). In females, stress response patterns in groups that experienced post-natal B, regardless of pre-natal experience, peaked at T10 and then decreased between T10 and T30, while in groups that did not experience post-natal B, stress levels peaked at T10 and tended, on average, to remain stable until T30 (Fig. 1A). In males, B levels peaked at T10 in all groups, apart from the BC males, in which stress levels tended to increase until T30, although individual variation was high (Fig. 1B). *Post hoc* analysis confirmed that the post-natal treatment was driving the observed sex-specific differences over the stress response in females (post-natal B × sex × time: $F_{4,101.66}$=2.79, P=0.03; P>0.54 for all other interactions; Fig. 1A).

We found no effect of treatment, sex or their interaction in basal glucose concentrations (CC: 13.80±0.44 mmol l$^{-1}$; BC: 13.20±0.57 mmol l$^{-1}$; CB: 13.50±0.45 mmol l$^{-1}$; BB: 13.30±0.60 mmol l$^{-1}$). In all juvenile quail, acute stress raised glucose concentrations (delta glucose, CC: 1.60±0.41 mmol l$^{-1}$; BC: 1.00±0.36 mmol l$^{-1}$; CB: 2.40±0.53 mmol l$^{-1}$; BB: 1.20±0.43 mmol l$^{-1}$); however, the increase did not differ across treatments or between males and females (Table 4).

#### Day PN64
Once again, B concentrations during the capture–restraint protocol changed significantly over the stress response (*post hoc*, T0 values *versus* T10 and T30 min values: P<0.0001 in both pair-wise comparisons; T10 values *versus* T30 values: P=1.00; Table 3, Fig. 2). However, there were no significant interactive effects (Table 3). We found an overall effect of treatment (Table 3) due to significantly
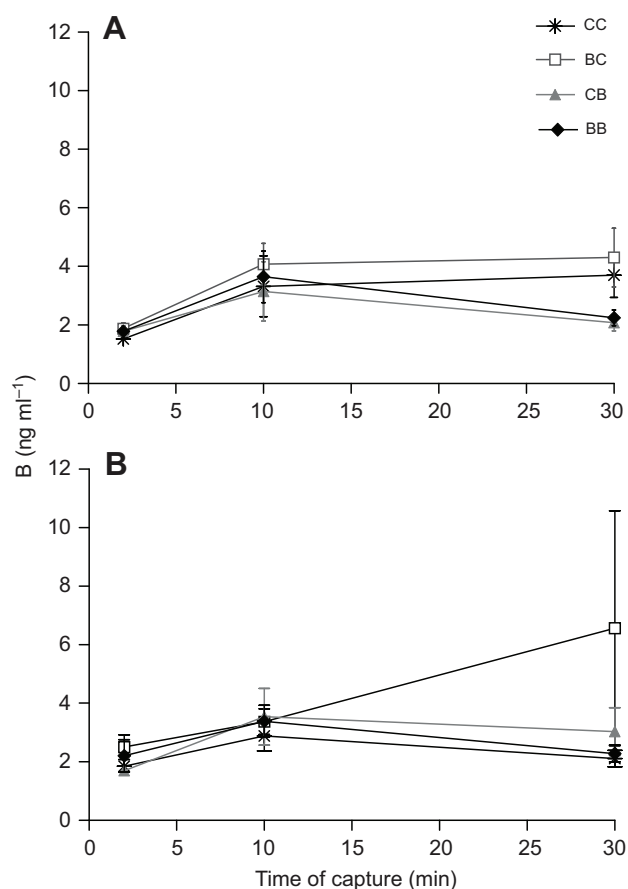
Fig. 1. Corticosterone (B) temporal responses to acute stress (standardised capture–restraint protocol) across treatment groups in (A) females and (B) males at post-natal day 22. CB and BB females exhibited shorter stress responses in comparison with BC and CC females (*P*=0.03), while no significant differences were observed among males. Sample sizes: CC female=9, male=14; BC female=10, male=6; CB female=10, male=10; BB female=9, male=9. Data represent un-transformed means ± s.e.m. and include undetectable samples that were assigned individual detection limits. CC, pre-natal and post-natal untreated birds; BC, pre-natal B-treated and post-natal untreated birds; CB, pre-natal untreated and post-natal B-treated birds; BB, pre-natal B-treated and post-natal B-treated birds.

higher hormone concentrations in BC birds compared with CC birds (*post hoc*: *P*=0.03; for all other pair-wise comparisons: *P*>0.12).

We also found a sex-specific treatment effect on basal glucose concentrations, but no main effects of treatment and sex (Table 4, Fig. 3). *Post hoc* analysis revealed that the significant interaction was driven by pre-natal B (pre-natal B × sex: $F_{1,62.43}$=10.70, *P*=0.002; post-natal B × sex interaction: $F_{1,67.95}$=0.07, *P*=0.79). In fact, males and females that experienced pre-natal B exhibited reversed basal glucose patterns compared with males and females that did not experience pre-natal B, with increased levels in BC and BB males compared with CC and CB males, and decreased levels in BC and BB females compared with CC and CB females (*post hoc* statistics in Fig. 3). Also, the combined early B treatments (BB group) tended to affect basal glucose levels (interaction: $F_{1,65.56}$=3.70, *P*=0.06), with no differences between the sexes (pre-natal B × post-natal B × sex: $F_{1,67.82}$=0.25, *P*=0.62). Contrary to what was observed early in life, glucose concentrations remained on average stable between T0 and T30, with no significant differences across treatments and sexes (delta

**Table 4. Results of LME modelling of potential short- and long-term effects of treatment, sex and their interaction on basal and delta glucose concentrations (i.e. difference between basal and stress-induced glucose concentrations) at post-natal day (PN) 22 and 64**

| | d.f. | *F* | *P* |
|---|---|---|---|
| **Day PN22** | | | |
| Basal glucose | | | |
| Treatment | 3,65.61 | 0.22 | 0.88 |
| Sex*,2 | 1,68.94 | 0.07 | 0.93 |
| Treatment × sex*,1 | 3,66.27 | 1.93 | 0.13 |
| Delta glucose | | | |
| Treatment | 3,66.14 | 1.26 | 0.29 |
| Sex*,2 | 1,70.35 | 0.20 | 0.66 |
| Treatment × sex*,1 | 3,63.05 | 1.77 | 0.16 |
| **Day PN64** | | | |
| Basal glucose | | | |
| Treatment | 3,66.76 | 1.68 | 0.18 |
| Sex | 1,67.43 | 2.06 | 0.16 |
| **Treatment × sex** | 3,67.14 | 3.48 | 0.02 |
| Delta glucose | | | |
| Treatment | 3,60.21 | 0.45 | 0.72 |
| Sex*,2 | 1,68.06 | 0.00 | 0.96 |
| Treatment × sex*,1 | 3,60.95 | 0.65 | 0.58 |

Asterisks indicate excluded factors during the stepdown procedure; superscripted numbers refers to the order of removal. Significant factors are in bold (*P*<0.05).

glucose: CC: 0.50±0.63 mmol l$^{-1}$; BC: −1.00±1.02 mmol l$^{-1}$; CB: −0.20±0.66 mmol l$^{-1}$; BB: 0.10±0.81 mmol l$^{-1}$; Table 4).

## DISCUSSION
Overall, our data suggest that exposure to elevated stress hormones during development can influence post-natal stress physiology, revealing sex-specific responses at certain post-natal stages. Our experimental protocol involved a direct physiological manipulation of corticosterone exposure during pre- and post-natal development. Therefore, we were able to attribute the effects induced by exogenous corticosterone to one (or both) of these early life stages and to analyse their potential interactions. This is the first avian study that attempts to quantify both the short- and long-term effects of developmental glucocorticoids on the HPA system and glucose metabolism during acute stress.

### Pre- and post-natal effects of corticosterone in HPA axis responsiveness
At day PN22, prolonged exposure to post-natal B mediated HPA responsiveness in females but not in males, regardless of previous pre-natal manipulations. Therefore, contrary to our predictions, elevated yolk corticosterone levels modified neither HPA axis function nor the short-term effects of post-natal treatment on the stress system. These results were unexpected, as previous studies using similar manipulations in the egg have found significant effects on post-natal growth, immunity or behaviours in juveniles of other bird species (Love et al., 2005; Rubolini et al., 2005; Saino et al., 2005; Davis et al., 2008). These studies were conducted in field conditions where a number of environmental confounding factors cannot be completely excluded. Also, variation seen across studies may also be explained by changes in HPA axis sensitivity across an individual's life cycle, especially during post-natal growth (Holmes and Kelly, 1976; Schwabl, 1999; Sims and Holberton, 2000; Wada et al., 2008). Interestingly, the effect of glucocorticoid exposure *in ovo* became evident only later in life. At day PN64, birds that had been exposed to corticosterone only during their pre-
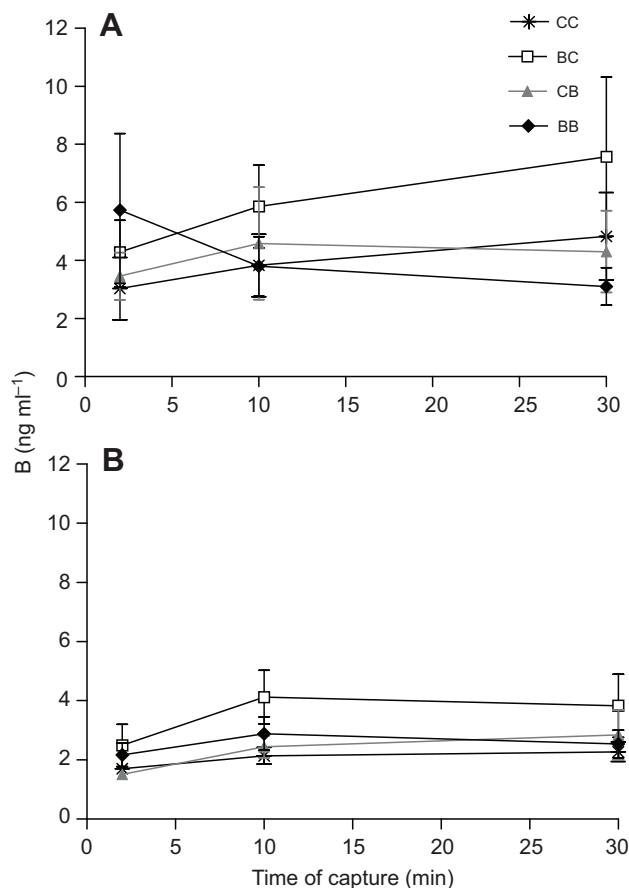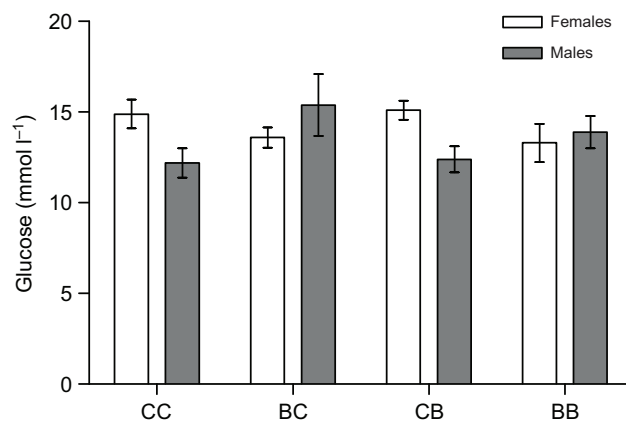
Fig. 3. The interaction between corticosterone exposure and sex in relation to basal glucose concentrations at post-natal day 64. Glucose concentrations were higher in BC and BB males compared with CC and CB males, and lower in BC and BB females compared with CC and CB females ($P$=0.002). Sample sizes: CC female=9, male=14; BC female=10, male=6; CB female=10, male=10; BB female=9, male=9. Data represent un-transformed means ± s.e.m.

Fig. 2. Corticosterone (B) temporal responses to acute stress (standardised capture–restraint protocol) across treatment groups in (A) females and (B) males at post-natal day 64. Regardless of sex, hormone concentrations were overall higher in BC birds compared with CC birds ($P$=0.03). Sample sizes: CC female=9, male=14; BC female=10, male=6; CB female=10, male=10; BB female=9, male=9. Data represent un-transformed means ± s.e.m. and include undetectable samples that were assigned individual detection limits.

natal development (BC) experienced a higher total exposure to circulating concentrations of corticosterone during acute stress compared with controls, indicating that HPA responsiveness in such treated birds was hyper-regulated. Remarkably, adult stress responses in birds that experienced the combined treatments (BB) were similar to those observed in control (CC) and post-natally stressed birds (CB). Taken together, our results suggest that prolonged post-natal stress may have somehow 'mitigated' the long-lasting impact produced by pre-natal stress and reinforced the importance of interactive influences between these two time windows, as shown in previous mammalian studies (Maccari et al., 1995; Vallée et al., 1997; Vallée et al., 1999). Pre-natal stress may have modified HPA axis function through an elevation of basal circulating corticosterone levels (Coe et al., 2003; Gutteling et al., 2005). The undetectable hormone concentrations in certain samples, especially at T0, constrained our ability to test for potential treatment differences in baselines. Similar low corticosterone levels during restraint have been reported in the same species and are likely to be the result of frequent handling for morphological measurements (Hayward and Wingfield, 2004; Hayward et al., 2006; see also Smith, 2003). In our experiment, morphological measurements were

taken in all the individuals at a specific age. The effect of handling, therefore, was standardised across all birds. We doubt that the low hormone concentrations could be a sign of incomplete maturation of the HPA axis as birds from previous generations (the same breeding population used in this experiment) were able to mount a corticosterone stress response at least from day 5 post-hatching.

There have been few studies in birds that have explored the effects of pre-natal glucocorticoid exposure on post-natal HPA function, and the results are mixed. Studies during early post-natal stages found dampened HPA responsiveness in starlings *Sturnus vulgaris* at fledging (Love and Williams, 2008) and hyper-responsiveness (Haussmann et al., 2012) or no effects (Lay and Wilson, 2002) in juvenile chickens *Gallus gallus*. Long-term studies in Japanese quail have found HPA hyper-responsiveness in adults born from corticosterone-implanted mothers (Hayward and Wingfield, 2004) and, in contrast, HPA hypo-responsiveness in females, but not in males, when the hormone was injected directly in the yolk (Hayward et al., 2006). This discrepancy has been explained by differences in the distribution of corticosterone in the egg when injected, as opposed to when deposited by the mother (Hayward et al., 2006). However, because our corticosterone dose raised yolk corticosterone levels to a similar physiological range as found in Hayward et al. (Hayward et al., 2006), other factors may be involved. For example, the timing of egg injection differed: in our study, eggs were injected at day E5, whereas in Hayward et al. (Hayward et al., 2006), injection was performed at day E0. In mammals, the effects of pre-natal stress can change depending on the duration of early stress exposure (Kapoor and Matthews, 2008). Sensitive windows might also occur in birds, and we need more comparative work to investigate this hypothesis.

The sex-specific effect produced by our post-natal manipulation suggests that growing females are more susceptible than males to alterations in their stress responses after facing prolonged environmental perturbations. Studies in zebra finches (*Taeniopygia guttata*) suggest that females may be more sensitive than males to early post-natal stress, exhibiting lower growth patterns, reduced incubation effort and decreased survival (Martins, 2004; Verhulst

et al., 2006; Spencer et al., 2010; but see Spencer and Verhulst, 2007). However, the short-term effects of post-natal stress on HPA activity have hardly been explored. Previous work has demonstrated that reduced food provisioning in nestling starlings induced an exaggerated peak in corticosterone release in response to acute stress at fledging, with smaller females showing the largest increase (Love and Williams, 2008). In our study, we measured the dynamics of the stress response, including the peak response, but also the change over time (between T10 and T30). Our data suggest that, in post-natally stressed females, hormone concentrations returned to baseline more quickly than in females that did not experience post-natal stress. This is indicative of changes in the duration, rather than the magnitude, of adrenocortical corticosterone secretion. It has been proposed that post-natal stress may produce adaptive responses in the short-term, helping the juveniles to maximise their immediate chance of survival in low-quality environments (Love and Williams, 2008; see also Meaney, 2001). Our data supports this idea as truncated stress responses could be an adaptive strategy to avoid the costs associated with high glucocorticoid concentrations (Wingfield, 2005b). Recent work in zebra finches has shown that prolonged exogenous corticosterone in nestlings produced HPA hyper-responsiveness in adulthood and decreased survival (Spencer et al., 2009; Monaghan et al., 2011); similar HPA alterations were observed in maternally deprived individuals into adulthood (Banerjee et al., 2011). This discrepancy reinforces the importance of considering the developmental strategy (precocial *versus* altricial), as well as the specific life stage, when investigating phenotypic effects of early life stress.

The mechanism underlying the short-term shift in HPA physiology produced by our post-natal protocol remains unresolved. Similar short-term alterations have been reported in rat pups subjected to daily handling during the first 21 days of post-natal life (Meaney and Aitken, 1985). Such changes have been linked with enhanced concentrations of type II corticosteroid receptors in the hippocampus (Meaney and Aitken, 1985; for a review, see Meaney, 2001), which are known to increase efficiency of glucocorticoid negative feedback (Sapolsky et al., 2000). Therefore, modifications in the density of type II corticosteroid receptors may explain the effects of post-natal stress in females observed in our study. Although research on these systems in young birds is lacking to date, studies in adult birds showed that chronic stress can sensitise the HPA axis by altering central corticosteroid receptors (Hodgson et al., 2007; Dickens et al., 2009). More work is required to test the biological relevance of such factors in this model species.

### Pre- and post-natal effects of corticosterone on basal and stress-induced glucose

Basal glucose concentrations were affected in adults, but not in juveniles in this study. At day PN64 we found a sex-specific effect of pre-natal stress, but not post-natal stress. Contrary to what was observed earlier in life, this finding did not mean that one sex was more sensitive than the other, but rather that both sexes were influenced by pre-natal glucocorticoid exposure in opposite directions. The reversed patterns were more evident in birds treated only during their pre-natal development. This metabolic alteration appears in line with the long-term effect induced by pre-natal stress on adrenocortical activity and supports the hypothesis of links between basal corticosterone and basal glucose levels, as shown in other bird species (Remage-Healey and Romero, 2000). Furthermore, our data support findings in mammals, where pre-natal stress has been shown to induce persistent changes in glucose metabolism (Hales et al., 1991; Vallée et al., 1996; Nyirenda et al.,

1998; Lesage et al., 2004; Benyshek et al., 2006; but see D'mello and Lin, 2006), which can be sex-linked (Franko et al., 2010). There are indications that these alterations may compromise adult health and increase vulnerability to metabolic diseases (Cottrell and Seckl, 2009). Although these data are interesting, they remain speculative as we still know little about how glucocorticoids influence glucose metabolism in birds. We are currently exploring gene expression differences within the HPA axis in a subset of adult quail used in the current experiment and we hope that these data may help us to define a clearer picture of the mechanisms of action.

We did not find any treatment differences in glucose stress responses at any life stage. Based on our findings on HPA responsiveness, we would have expected differences in glucose stress responses in post-natally treated females. However, other systems or hormones, such as the autonomic nervous system or insulin, may co-operate with the HPA axis in the control of blood glucose release in response to stress (Havel and Taborsky, 1989; Remage-Healey and Romero, 2001). Finally, we pointed out that at day PN22 glucose increased at the end of the restraint in all birds, regardless of treatment. In contrast, this stress-induced hyper-glycaemia did not appear in the adults, in line with what has been reported in adult starlings during the active photophase (Remage-Healey and Romero, 2000). It is possible that birds use different energy sources between early post-natal development and adulthood. Work is underway to further investigate this hypothesis.

### Pre- and post-natal effects of corticosterone on growth

Developmental corticosterone did not induce any significant effects on growth over the short- or long-term. This result was unexpected as stressful experiences often depress growth in the short-term (Eriksen et al., 2003; Hayward and Wingfield, 2004; Saino et al., 2005; Janczak et al., 2006; Spencer and Verhulst, 2007; Wada and Breuner, 2008; Müller et al., 2009; Spencer et al., 2009). Studies of pre-natal stress have also suggested that males are more affected than females (Love et al., 2005; Hayward et al., 2006; Love and Williams, 2008). Again, we did not find sex-specific patterns. Our study adds to the few studies in birds that deviate from the above trends, finding no effects of developmental exposure to glucocorticoids on growth (Kitaysky et al., 2003; Rubolini et al., 2005; Haussmann et al., 2012). In the long-term, the lack of treatment differences were expected as the growth decline in response to developmental glucocorticoid often disappears over the longer period (Hayward and Wingfield, 2004; Spencer and Verhulst, 2007; Spencer et al., 2009). We therefore conclude that the effects of developmental corticosterone observed on the HPA axis physiology and glucose metabolism are direct effects of early life glucocorticoid exposure and not indirect effects due to altered growth trajectories.

### Conclusions

In conclusion, our study supports the hypothesis that early life exposure to glucocorticoids can induce both transient and permanent changes to the HPA axis and related metabolic pathways, which depend upon the developmental stage and sex. We showed that the impact of pre-natal stress on adult stress responses may be modulated by post-natal stressful environmental conditions experienced during growth. Although the underlying mechanisms of such shifts in the stress system are currently unknown in birds, our results corroborate findings in many mammalian models, suggesting that glucocorticoid programming on the phenotype is a widespread phenomenon among vertebrates. We, therefore, emphasise the use of avian models in developmental research as they have the potential to tease apart

indirect maternal and direct environmental stimuli acting on early life phenotypic plasticity. Finally, the high degree of variation in developmental strategies and life histories in birds offers an excellent opportunity to undertake comparative approaches to further our understanding of potential ultimate costs and benefits of early life stress on young and aging phenotypes.

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