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ScienceDirect
Hormones and Behavior 54 (2008) 496–505
Hormones and Behavior
www.elsevier.com/locate/yhbeh

Plasticity in the adrenocortical response of a free-living vertebrate: The role of pre- and post-natal developmental stress

Oliver P. Love *, Tony D. Williams

Department of Biological Sciences, Simon Fraser University, Burnaby, British Columbia, V5A 1S6 Canada

Received 21 November 2007; revised 15 January 2008; accepted 16 January 2008
Available online 7 February 2008

Abstract

Optimal functioning of the hypothalamo–pituitary–adrenal (HPA) axis is paramount to maximizing fitness in vertebrates. Research in laboratory mammals has suggested that maternally-induced stress can cause significant variation in the responsiveness of an offspring's HPA axis involving both pre- and post-natal developmental mechanisms. However, very little is known regarding effects of maternal stress on the variability of offspring adrenocortical functioning in free-living vertebrates. Here we use an experimental approach that independently lowers the quality of both the pre- and post-natal developmental environment to examine programming and plasticity in the responsiveness of the HPA axis in fledglings of a free-living passerine, the European starling (*Sturnus vulgaris*). We found that mimicking a hormonal signal of poor maternal condition via an experimental pre-natal increase in yolk corticosterone decreased the subsequent responsiveness of the HPA axis in fledglings. Conversely, decreasing the quality of the post-natal developmental environment (by decreasing maternal provisioning capability via a maternal feather-clipping manipulation) increased subsequent responsiveness of the HPA axis in fledglings, apparently through direct effects on nestling body condition. The plasticity of these responses was sex-specific with smaller female offspring showing the largest increase in HPA reactivity. We suggest that pre-natal, corticosterone-induced, plasticity in the HPA axis may be a 'predictive adaptive response' (PAR): a form of adaptive developmental plasticity where the advantage of the induced phenotype is manifested in a future life-history stage. Further, we introduce a new term to define the condition-driven post-natal plasticity of the HPA axis to an unpredictable post-natal environment, namely a 'reactive adaptive response' (RAR). This study confirms that the quality of both the pre- and post-natal developmental environment can be a significant source of variation in the responsiveness of the HPA axis, and provides a frame-work for examining ecologically-relevant sources of stress-induced programming and plasticity in this endocrine system in a free-living vertebrate, respectively.

Keywords: Corticosterone; Stress response; Maternal effects; Yolk hormones; Body condition; Predictive adaptive responses; Developmental plasticity

Introduction

The vertebrate adrenocortical response is highly conserved across terrestrial taxa and involves the release of glucocorticoids by the hypothalamo–pituitary–adrenal (HPA) axis designed to direct energetic and behavioral modifications away from an environmental stressor (Sapolsky et al., 2000; Wingfield and Sapolsky, 2003; Wingfield, 2005). However, the functioning of the HPA axis also shows a large degree of inter-individual variation within species in both juveniles (Sockman and Schwabl, 2001; Love et al., 2003a, 2003b; Kitaysky et al., 1999a, 2001a, 2005; Wada et al., in press) and adults (Kitaysky et al., 1999b, Breuner and Hahn, 2003; Love et al., 2003a). This inter-individual variation is likely maintained through differential corticosterone-induced costs and benefits across life-history stages (e.g. Love et al., 2004; Blas et al., 2007) and effects of HPA axis function on fitness (Dufty et al., 2002; Cavigelli and McClintock, 2003; Boonstra, 2005; Wingfield, 2005; Hadany et al., 2006; Blas et al., 2007). Numerous studies have shown how variation in the responsiveness of the HPA axis plays adaptive roles in free-living vertebrates (e.g., dampened responses in severe arctic and alpine breeding birds to reduce reproductive abandonment: Wingfield and Hunt, 2002; Breuner and Hahn, 2003; reduced responsiveness during successive reproductive stages to reduce reproductive abandonment: Holberton and

* Corresponding author. Department of Biological Sciences, Simon Fraser University, 8888 University Drive, Burnaby, B.C., Canada V5A 1S6. Fax: +1 604 291 3496.
E-mail address: olovea@sfu.ca (O.P. Love).

0018-506X/$ - see front matter © 2008 Elsevier Inc. All rights reserved.
doi:10.1016/j.yhbeh.2008.01.006

Wingfield, 2003; Love et al., 2004; variation in stress-induced plasma corticosterone during development is correlated with survival and recruitment: Cavigelli and McClintock, 2003; Blas et al., 2007). However, other than studies of developmental changes in the HPA axis itself (Sims and Holberton, 2000; Sockman and Schwabl, 2001; Love et al., 2003a, 2003c; Blas et al., 2006; Wada et al., 2007), few studies have focused on how the quality of the pre- and post-natal developmental environment might contribute to individual variation in the adrenocortical response in free-living vertebrates (but see Blas et al., 2007; Cyr and Romero, 2007).

Variation in stress responsiveness has a strong genetic component (Solberg et al., 2006) and the response is heritable (Bartels et al., 2003; Federenko et al., 2004; Evans et al., 2006) and can be selected for under laboratory conditions (Satterlee and Johnson, 1988; Evans et al., 2006). Significant variation can also result from developmental plasticity of the HPA axis, especially since both the embryo/fetus and post-natal offspring must balance immediate physiological developmental challenges and prepare for adulthood (Seckl, 2001, 2004; Gluckman et al., 2005; Fowden et al., 2006). Research in laboratory mammals and captive birds indicates that exposure to pre-natal maternal stress (via elevated glucocorticoids, maternal malnutrition) can cause both short- and long-term effects on the responsiveness of the HPA axis (mammals, reviewed in Seckl, 2001, 2004; Roussel et al., 2005; Jarvis et al., 2006; birds, Hayward and Wingfield, 2004; Hayward et al., 2006). Likewise, the quality of the post-natal environment also influences the responsiveness of the HPA axis in offspring (mammals: e.g., Liu et al., 1997; Meaney, 2001; Holmes et al., 2005; Pryce et al., 2005; Macrì and Würbel, 2006; Parfitt et al., 2007; birds: e.g., Kitaysky et al., 1999a, 2001a, 2001b, 2006; Pravosudov and Kitaysky, 2006; Cyr and Romero, 2007; Hull et al., 2007). However, studies examining the combined effects of pre- and post-natal developmental stress have only been undertaken in laboratory or captive conditions due to the difficulty of disentangling pre- and post-natal maternal and environmental effects in free-living vertebrates.

Here we use an experimental approach to independently manipulate the quality of both the pre- and post-natal developmental environment to examine how this affects programming and plasticity of the adrenocortical response in a wild passerine, the European starling (*Sturnus vulgaris*). To manipulate the quality of the pre-natal environment we injected corticosterone into yolks of freshly-laid eggs (i.e., prior to embryonic development) at levels designed to mimic hormonal-transfer to eggs by poor condition mothers; that is, we altered a pre-natal embryonic signal of maternal quality (Love et al., 2005, 2008). At hatching, we manipulated the quality of the post-natal developmental environment using feather-clipping of mothers (Winkler and Allen, 1995; Hill, 2003; Rowland et al., 2007) reducing maternal chick-provisioning capability. Combining both the pre- and post-natal treatments resulted in four overall treatment groups: two groups of corticosterone-exposed offspring and two sham-exposed groups of offspring, each raised by either a low or high-quality mother. We hypothesized that being exposed to elevated yolk corticosterone provides developing offspring with a *predictive* signal that their mother or the post-natal environment is of poor quality. From previous work (see above) we hypothesized that exposure to pre-natal stress would therefore result in a programmed dampening in the responsiveness of the HPA axis compared with control offspring, since a maximal response may not be adaptive in a severe or unpredictable environment (Wingfield and Hunt, 2002; Breuner and Hahn, 2003; Love et al., 2004). Secondly, we hypothesized that nestlings faced with an unpredictable low-quality post-natal environment (being raised by feather-clipped mothers) would be energetically-stressed during development (sensu Kitaysky et al., 1999a, 2001a, 2001b, 2006; Pravosudov and Kitaysky, 2006) and would therefore have the lowest body condition of all treatments. The result would be a temporarily more responsive HPA axis, adrenocortical plasticity involving an immediate *reactive* response to balance the energetic deficit (Dallman et al., 1993; Kitaysky et al., 1999a, 2001a, 2001b). Finally, examining the combined effects of corticosterone exposure and reduced maternal provisioning allowed us to determine the relative influences of pre- and post-natal stress on the reactivity of the HPA axis. Although it is difficult to make predictions on whether pre- or post-natal stress has larger relative effects on the HPA axis, one might predict that corticosterone-exposed nestlings raised by feather-clipped mothers would exhibit an adrenocortical response that was intermediate between that of corticosterone-exposed offspring raised by unclipped mothers and sham-exposed offspring raised by unclipped mothers. Together with baseline and stress-induced corticosterone levels, we also report effects of the treatments on offspring body condition and brood size in an attempt to determine direct versus indirect effects of the pre- and post-natal treatments on HPA axis responsiveness.

Methods

Field site, hormonal and maternal manipulation

This study was conducted using a wild colony of European starlings (approximately 250 nest boxes) located at a large dairy farm in Langley, British Columbia, Canada (49°10′N, 122°50′W) from April to mid-May 2005 (Love et al., 2005, 2008) under a Simon Fraser University Animal Care permit (657B-96), following guidelines of the Canadian Council on Animal Care. Starlings at this site lay 5.9±0.2 (mean±sem) eggs per clutch within the synchronous peak of laying (covers 7–8 days; Love et al., 2005; Smith, 2004), incubate for 10.3±0.1 days and fledge nestlings 21±0.6 days following hatching (Love et al., 2005). Nest boxes were checked daily to determine clutch initiation, laying sequence, and clutch completion dates. For yolk hormone manipulations, 68 nests were randomly split into either sham (n=34) or corticosterone (n=34) treatment groups (controlling for laying date) within the synchronous laying peak. Within 3 h of a new egg being laid, it was removed from the nest (while females were foraging away from their nests to eliminate possible disturbance effects on yolk corticosterone levels in subsequent eggs), the injection site was cleaned with ethyl alcohol and the injection was made into the yolk. The hole was sealed using cyanoacrylate glue (Loctite® Superglue Control™ Gel, Henkel Inc.), the egg's laying order was marked and the egg was measured and returned to the nest. Treatment eggs were injected with 10 µl of a 1277 ng/ml corticosterone (Sigma C2505) dissolved in sterilized sesame oil (Sigma S3547). The treatment was designed to elevate mean yolk corticosterone concentrations of the population by 1.5 standard deviations (from 15.4 ng/g to 28.3 ng/g, Love et al., 2008) based on an average fresh yolk weight for the population of 1.012±0.008 g (n=163 eggs). Sham injected eggs received the same handling but were injected with the oil vehicle only.

Clutches were removed 0.5 days prior to hatching (once the first egg had begun pipping) and placed in an incubator for approximately 6–8 h until nestlings hatched; replacement of artificial eggs maintained maternal incubation behavior. Nestling body mass and structural size measures were taken at hatching, a small blood sample was collected for PCR (sex) analysis (placed on a sterile piece of filter paper) and nestlings were individually-marked (with order-specific feather-clipping) and returned to their natal nests. Just prior to hatching (1–1.5 days), all females in both hormone treatment groups were caught and were again split pair-wise by laying date into one of two maternal treatment groups (feather-clipped: $n=32$ females; non-feather-clipped: $n=32$ females); feather-clipping was employed to reduce maternal provisioning rates to the offspring (see Winkler and Allen, 1995; Hill, 2003; Rowland et al., 2007). The maternal treatment consisted of clipping the ninth, sixth, and third primary feathers, the sixth and third secondary feathers, and the sixth and third rectrices (tail feathers) near the base of each feather; control birds were only captured, handled (see below) and released. Prior to release, all females were measured (beak length, tarsus length, wing chord, and body mass), banded with metal and color bands and were released to return to normal activities. Females were re-caught 7–8 days following hatching during the linear phase of nestling growth to determine potential changes in body mass. Combining the hormonal and maternal treatments resulted in four overall treatments: 1) corticosterone-exposed nestlings raised by feather-clipped mothers (herein referred to as CORT-clipped or B_C), 2) corticosterone-exposed nestlings raised by non-clipped mothers (CORT-non-clipped or B_{NC}), 3) sham-exposed nestlings raised by feather-clipped mothers (sham-clipped or S_C), and 4) sham-exposed nestlings raised by non-clipped mothers (sham-non-clipped or S_{NC}).

Growth and adrenocortical response to handling of nestlings

All nestlings were weighed and measured (exposed culmen, tarsus, wing) at the ages of 5, 10, 15 and 17 days. Nestlings were banded with metal bands at 10 days of age so that we could continue to track individual nestlings. At 15 days of age, nestlings underwent a standardized stress response protocol (described in Love et al., 2004) designed to elicit a corticosterone response by the HPA axis in near-fledging nestlings (see Love et al., 2003a, 2003c; Wada et al., 2007). All birds were blood sampled (*ca*. 200 µl) from the wing vein within 2 min of the nest box being opened by the researcher (i.e., the first moment when the nestlings were aware of human presence). We found no effect of time after capture (within 0–2-min interval) on plasma corticosterone levels in initial blood samples (linear regression analysis: $r^2=0.03$, $p=0.82$) indicating that initial blood samples reflect baseline levels of corticosterone. Birds were then placed in a cloth bag and one additional blood sample was taken 30 min following capture. Prior work in our field population has revealed that juvenile and adult starlings show maximum responsiveness during a handling-induced stress response series at 30 min following initial capture (Love, Vézina and Williams, unpubl. data). As such, we blood sampled all individuals in the present study only at capture and after 30 min to minimize excessive blood loss in nestlings which may lead to anemia (see Dawson and Bortolotti, 1997; Williams et al., 2004). Blood was collected in heparinized capillary tubes and samples were centrifuged within 2 h and plasma was stored at −20 °C until further analysis. We returned the birds to their nest boxes immediately following the second sampling. To assess changes in maternal quality in relation to the feather-clipping treatment, we measured parental provisioning rates by performing a 30-min behavioral observation of each nest box, per day, over three consecutive days using spotting scopes when nestlings were aged 6–8 days. Provisioning rates were calculated as the number of feeds per chick, per hour for each parent based on the mean brood size of the nest for the three-day observation period (as in Chin et al., 2005; Rowland et al., 2007).

Plasma corticosterone determination and molecular sexing

The concentration of total corticosterone (see Breuner and Orchinik, 2002; Love et al., 2004, 2005) in non-extracted plasma was determined using a corticosterone Enzyme-linked-Immunoabsorbent Assay (EIA — Assay Designs Inc., Michigan USA, catalog # 901-097) with a 4-parameter logistic fit. Samples were run in triplicate across six assay plates at a total volume of 100 µl with 1:40 dilution and 1.5% steroid displacement buffer. As per kit instructions, plates were first incubated at 26 °C under shaking at 5000 rpm for 2 h and then at 26 °C without shaking for 1 h; the detection limit of the assay was calculated at 0.018 ng/well (0.72 ng/ml) with intra- and inter-assay variations of 6.3% and 7.4%, respectively. The small blood sample collected at hatching for PCR sexing was stored in a labeled tube and frozen at −20 °C. Nestling sex was determined using techniques reported in Love et al. (2005) via polymerase chain reaction (PCR) amplification. Briefly, DNA was isolated from the blood samples using Insta-gene matrix (Bio-Rad Laboratories, Hercules, California, Cat. No. 732-6030) following the manufacturer's protocol. PCR amplification was then carried out in a total volume of 10 µl and run using the P2 (5′-TCTGCATCGCTAAATCCTTT) and CW (5′-AGAAATCATTCCAGAAGTTCA) primers followed by digestion with HAE III Enzyme. PCR products were run on a 1.5% agarose gel containing ethidium bromide for 1.5 h before examination under ultraviolet light.

Statistical data analysis

We used Analysis of Covariance (ANCOVA) to analyze maternal treatment effects on maternal characteristics (body mass, brood size, provisioning rates); maternal treatment was included as a fixed factor and relevant covariates (all $p<0.05$ when included in the analysis) were included where necessary (i.e., original capture date was included in the analysis of pre-treatment maternal body mass, clutch size was included in the analysis of brood size and original mass

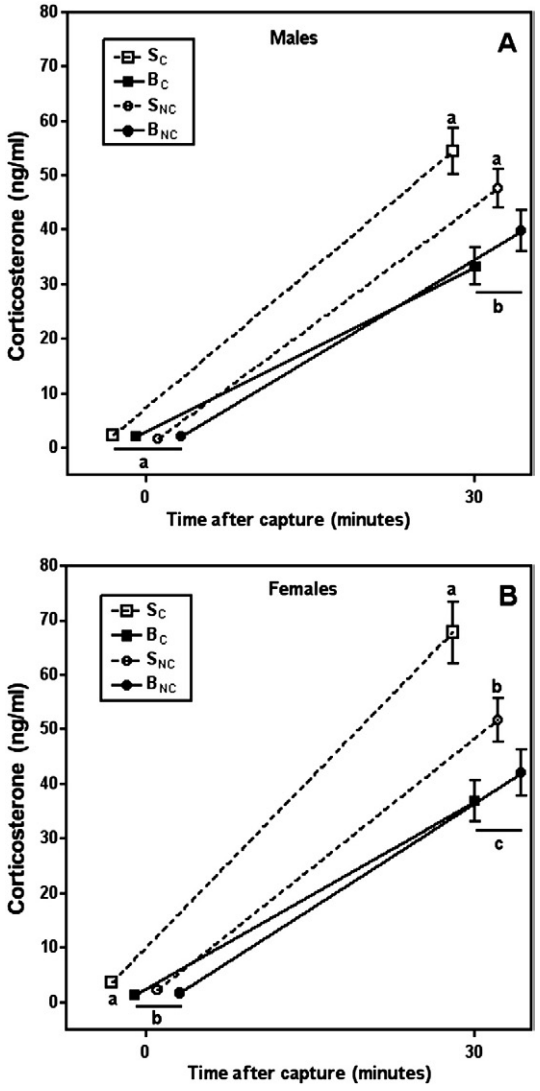

Fig. 1. Stress responses varied significantly by treatment in both (A) males and (B) females (means±sem; different letters represent a significant difference between groups as calculated for pair-wise Bonferroni comparisons; treatments: sham-clipped (S_C), CORT-clipped (B_C), sham-non-clipped (S_{NC}) and CORT-non-clipped (B_{NC}).

was included in the analysis of post-treatment body mass change). We explored the effects of the pre- and post-natal manipulations on the functioning of the HPA axis in fledglings as well as traits related to nestling quality. To investigate the activity of the HPA axis, we examined treatment effects on baseline, absolute stress-induced corticosterone and the area under each response curve (as a measure of the total exposure to corticosterone during the 30-min stress response). Area under the curve is thought to provide an integrated measure of HPA activity during the entire period of restraint (Breuner et al., 1999). Both baseline and stress-induced corticosterone were not normally distributed and as such were square-root transformed before analysis. We used GLMM to analyze sex-specific effects of the treatments on nestling traits (growth, fledgling body mass/body condition, baseline and area under the curve corticosterone levels) by including nestling sex and treatment as fixed factors; maternal identity was included as a random factor to control for non-independence due to the inclusion of siblings in the analysis. For the analysis of the adrenocortical response, we used a similar analysis, except using a repeated-measures approach, to examine changes in corticosterone levels over the course of the stress response. To calculate an estimate of body mass corrected for body size (body condition index), we used the residuals from a regression of body mass against tarsus length. Post-hoc comparisons for significant interaction terms were carried out using the adjusted Bonferroni post-hoc procedure, with the p-value corrected for the number of pair-wise comparisons made depending on the type of analysis (Rice, 1989). Sex ratio of offspring, as a function of treatment, was analyzed using GLMM with a binomial error structure (Love et al., 2005). For sex ratio analysis, maternal identity was included as a random factor. The significance of the explanatory variables was determined by their Wald statistic using the χ^2-distribution with alpha set to 0.05 in all analyses (Crawley, 1992).

Results

Treatment effects on maternal traits, offspring phenotype and brood dynamics

There was no significant difference in initial laying date (Chi-square 3,39=0.06, p=0.99), egg mass ($F_{3,58}$=0.09, p=0.96), clutch size ($F_{3,58}$=0.39, p=0.76), structural size (tarsus: $F_{3,50}$=0.25, p=0.86), body mass ($F_{3,58}$=0.46, p=0.71) or wing-loading ($F_{3,58}$=1.49, p=0.23) of the mothers assigned to the four treatments, indicating we successfully obtained a random sample of birds. There was also no effect of feather-clipping on whether mothers returned to their clutches following the treatment (χ^2_3=5.61, p=0.13). Both maternal and total parental provisioning rates (number of feeds/nestling/hour) were significantly lower in the sham-clipped group compared with all other groups (maternal: repeated-measures time×treatment: $F_{3,38}$=2.92, p<0.05; post-hoc: all groups compared to S_C p<0.02: S_C: 2.51±1.32, B_C: 5.66±1.41, S_{NC}: 5.72±1.12, B_{NC}: 5.52±1.22; parental: repeated-measures time×treatment: $F_{3,38}$=2.86, p<0.05; post-hoc: all groups compared to S_C p<0.028: S_C: 6.53±1.13, B_C: 8.93±1.23, S_{NC}: 9.56±1.01, B_{NC}: 9.10±0.92). All females regardless of treatment lost body mass during mid-chick-rearing (repeated-measures ANOVA: time effect: $F_{1,38}$=15.84, p=0.0003), although the feather-clipping manipulation resulted in a larger reduction in maternal body mass compared with unclipped mothers regardless of the hormonal treatment of their eggs (repeated-measures ANOVA: time×treatment: $F_{3,38}$=2.93, p=0.045; S_C: 13.31±1.57 g, B_C: 12.51±1.43 g, S_{NC}: 9.24±1.21 g, B_{NC}: 8.06±1.16 g).

Male nestlings hatching from eggs injected with corticosterone were significantly lighter than sham-injected counterparts; female nestlings appeared unaffected by the treatment (sex×hormone treatment: χ^2_1=4.21, p=0.04, post-hoc: male pair-wise comparison P<0.015; female pair-wise comparison P=0.42). By fledging however, female nestlings from the sham-clipped treatment were both significantly lighter and structurally smaller than females in all other groups; however, male fledglings showed no effects of either treatment combination on fledging body mass or size (repeated-measures ANOVA, time×sex×treatment−body mass: $F_{12,222}$=2.53, p=0.004, post-hoc: all <0.022; tarsus: $F_{12,222}$=1.89, p=0.04, post-hoc: all <0.025; wing cord: $F_{12,222}$=1.78, p<0.05, post-hoc: all <0.025). Although we found no significant treatment effects on hatching brood sizes (treatment: $F_{1,67}$=1.42, p=0.17, control: 5.52±0.38 nestlings, corticosterone: 5.24±0.37 nestlings), brood sizes in

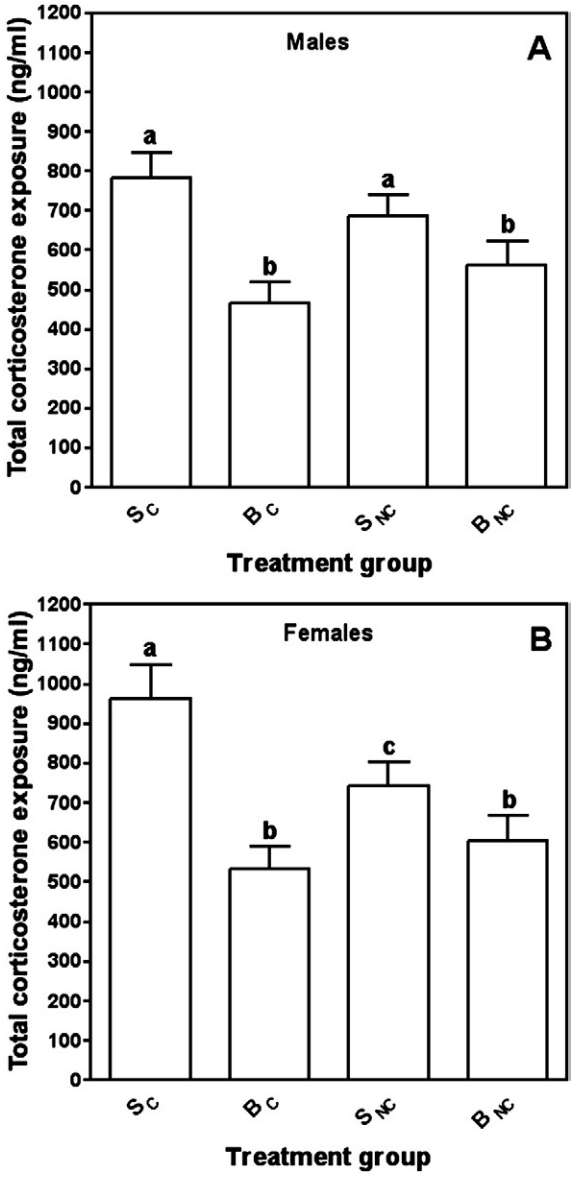

Fig. 2. Total exposure to corticosterone during the 30-min response period varied significantly by treatment: sham-clipped (S_C), CORT-clipped (B_C), sham-non-clipped (S_{NC}) and CORT-non-clipped (B_{NC}). (means±sem, different letters represent a significant difference between groups within a capture time as calculated for pair-wise Bonferroni comparisons; total corticosterone exposure calculated as area under response curve — see Methods for description).

both clipped treatments were significantly lower at fledging than the non-clipped groups (repeated-measures ANOVA, time × treatment: $F_{12,42}=2.12$, $p=0.03$, post-hoc: all <0.02; S_C: 4.45±0.23 nestlings, B_C: 3.43±0.54 nestlings, S_{NC}: 5.28±0.52 nestlings, B_{NC}: 5.03±0.39 nestlings). Moreover, although sex ratios were equal among the groups at hatching (hormone treatment: $\chi^2_1=1.3$, $p=0.25$), sex ratio became significantly female-biased by fledging in the CORT-clipped group (treatment × time interaction: $\chi^2_3=7.89$, $p<0.05$, post-hoc: <0.025; S_C: 0.52, B_C: 0.37, S_{NC}: 0.51, B_{NC}: 0.47 — male/female ratio).

Effects of pre- and post-natal treatments and brood size on fledging stress responses

We found a significant sex × treatment interaction for baseline corticosterone (baseline: $\chi^2_3=7.99$, $p<0.05$), the adrenocortical response (stress-induced: repeated-measures, sex × treatment × time: $F_{3,119}=4.55$, $p=0.005$) and the total amount of corticosterone released during the 30-min adrenocortical response (area under the curve: $\chi^2_3=10.45$, $p=0.015$). As such, we examined the sexes separately to determine the origin of treatment differences. Female nestlings in the sham-clipped treatment had the highest plasma baseline corticosterone levels of all groups (treatment: $\chi^2_3=8.85$, $p=0.03$, post-hoc: all $p<0.02$; Fig. 1), whereas male nestlings from all treatments had similar plasma baseline corticosterone levels (treatment: $\chi^2_3=3.47$, $p=0.32$; Fig. 1). Male nestlings in both corticosterone-injected groups had lower adrenocortical responses than males in the sham-injected groups (repeated-measures ANOVA, treatment × time: $F_{3,46}=3.26$, $p=0.03$, post-hoc: all $p<0.019$; Fig. 1). While we also detected a significant effect of treatment on stress-induced corticosterone levels in female nestlings, it was female nestlings in the sham-clipped group showing differences by responding with the highest adrenocortical responses (repeated-measures ANOVA, treatment × time: $F_{3,45}=4.26$, $p=0.01$, post-hoc: all $p<0.01$; Fig. 1). Finally, examining the area under the stress response curve revealed that males from the CORT-exposed groups released the lowest amount of corticosterone over the 30-min adrenocortical response compared with offspring from sham-injected eggs (treatment: $\chi^2_3=9.77$, $p=0.02$, post-hoc: all

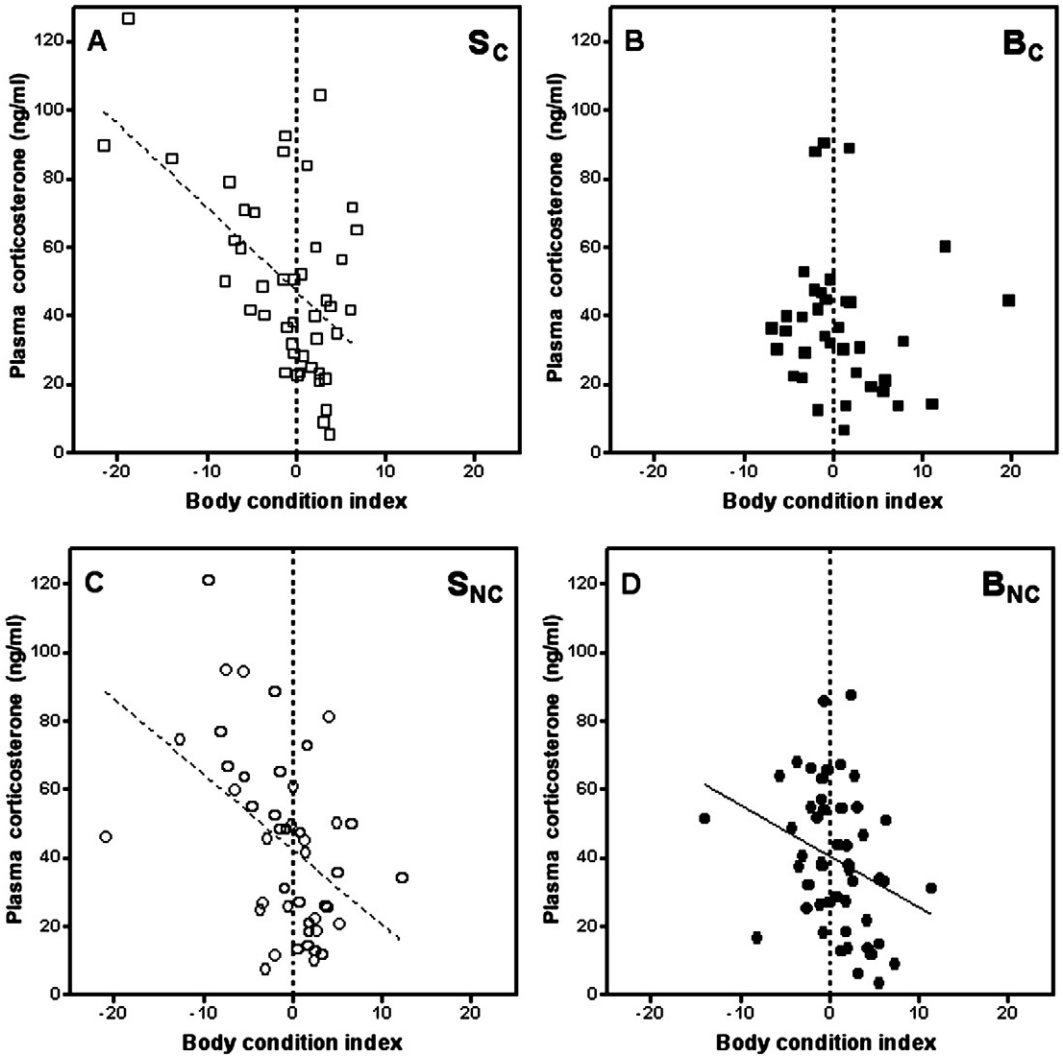

Fig. 3. Stress-induced corticosterone varied with body condition index in nestlings from three of the four treatments; (A) sham-clipped (S_C), (B) CORT-clipped (B_C), (C) sham-non-clipped (S_{NC}) and (D) CORT-non-clipped (B_{NC}).

$p<0.017$; Fig. 2). In female nestlings, not surprisingly those in the sham-clipped group have the highest corticosterone exposure over the 30-min period and this total exposure was twice that of the B_C group; again, both corticosterone-injected groups showed the lowest total exposure of the 30-min response (treatment: $\chi^2_3=9.86$, $p<0.02$, post-hoc: all $p<0.015$; Fig. 2).

In an attempt to identify the mechanism(s) driving these treatment differences in corticosterone levels, we examined relationships between 1) body mass and 2) changes in brood size (from hatching to fledging) and both baseline and stress-induced corticosterone. Fledglings from CORT-exposed groups had higher condition indices than those hatching from sham-exposed eggs (treatment: $\chi^2_3=7.91$, $p<0.05$, post-hoc: all $p<0.025$; CORT-exposed: 0.74 ± 0.59, Sham-exposed: -1.51 ± 0.61; Fig. 3) and male nestlings were in better condition than female nestlings (sex: $\chi^2_3=8.03$, $p<0.05$, all $p<0.025$); however we could not detect a treatment × sex interaction for body condition ($p=0.74$). Moreover, body condition index was negatively related to fledgling stress-induced corticosterone in all but the CORT-clipped group (treatment × body condition index: $\chi^2_3=8.16$, $p<0.05$, all $p<0.025$; Fig. 3) but not to baseline corticosterone (all $p>0.26$). Although the change in brood size could not be included in a multivariate analysis given that variance in this trait differed greatly across treatments (and as such would lead to a highly unbalanced analysis), we nonetheless examined within-sex relationships between corticosterone levels and the change in brood size for the specific treatment groups. In males, we found no significant relationships between the change in brood size and either baseline (linear regression analysis: all $p>0.59$), or stress-induced corticosterone (linear regression analyses: all $p>0.31$). Likewise, in females we found no relationship for baseline corticosterone (linear regression analysis: all $p>0.12$); however, females in the clipped groups showed significant negative relationships between the change in brood size and stress-induced corticosterone (linear regression analysis: S_{NC}: $p=0.56$; B_{NC}: $p=0.48$; S_C: $p=0.025$, $r^2=0.18$; B_C: $p=0.05$, $r^2=0.13$; Fig. 4).

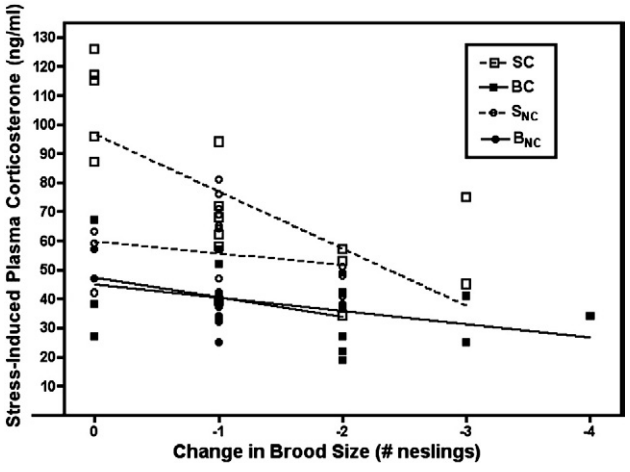

Fig. 4. Stress-induced corticosterone for starling daughters in relation to the change in brood size (from hatching to fledging) of their natal nest and treatment: sham-clipped (S_C), CORT-clipped (B_C), sham-non-clipped (S_{NC}) and CORT-non-clipped (B_{NC}); individual data points represent mean values per nest.

Discussion

We manipulated the quality of both the pre- and post-natal developmental environment to examine how this affects programming and plasticity of the HPA axis in offspring of a free-living vertebrate, respectively. Overall, we found a large degree of inter-individual variation (10-fold) in the magnitude of the fledgling adrenocortical response to a standardized stressor. We predicted that nestlings exposed to elevated maternally-derived yolk corticosterone would exhibit decreased responsiveness of the HPA axis as has been reported recently in laboratory mammals and captive birds (see Introduction). This prediction is based on the hypothesis that pre-natal exposure to elevated yolk corticosterone would provide embryos with a prediction of the low-quality of their future post-natal environment (Love et al., 2005, 2008). This hypothesis was supported in that offspring exposed to elevated yolk corticosterone, regardless of the post-natal environment they were later exposed to, had lower adrenocortical responses at fledging than offspring from sham-injected eggs. Our second prediction was based on the hypothesis that an unpredicted decrease in the quality of the post-natal developmental environment would result in increased responsiveness of the HPA axis mediated through decreased nestling condition (i.e., increased energetic demand). This hypothesis was also confirmed: nestlings raised in a low-quality post-natal environment (by feather-clipped mothers) had the lowest body condition and the highest adrenocortical responses at fledging. Moreover, daughters from the larger sham-clipped broods had the highest HPA axis responsiveness and were exposed to twice as much corticosterone during the response as CORT-clipped daughters. Our data therefore indicate that both pre- and post-natal effects of the quality of the developmental environment lead to modulations of the adrenocortical response designed to cope with both predictable and unpredictable perturbations in the post-natal environment, respectively. It should be appreciated that although we based sample collection times on our prior work indicating that starlings showed maximum responsiveness at 30 min following initial capture, there remains the possibility of plasticity in the shape of the response and therefore future studies may wish to include more sampling times to address this issue.

Effects of pre-natal corticosterone exposure on HPA axis responsiveness

Our results indicate that pre-natal exposure to elevated maternally-derived corticosterone results in a dampening of the adrenocortical response to stress in pre-fledging starlings, regardless of the offspring's sex. If transfer of elevated levels of corticosterone to the yolk serves as a cue to embryos of low maternal or future environmental quality (Love et al., 2005; Saino et al., 2005; Love et al., 2008), is there an adaptive function of having a less responsive HPA axis under these conditions? That is, do these corticosterone-mediated organizational effects allow nestlings to physiologically respond optimally as fledglings? In adults, species breeding in severe (arctic or alpine) habitats appear to have evolved dampened

adrenocortical responses designed to decrease the chance of making an inappropriate decision (reproductive abandonment) in the face of unpredictable, but short-term, bad weather conditions (Wingfield and Hunt, 2002; Holberton and Wingfield, 2003; Breuner and Hahn, 2003). Similarly, Kitaysky et al. (2005) have shown that the adrenocortical responses of seabird chicks in species with unpredictable food delivery rates do not increase in response to food restriction, whereas in species that deliver food at predictable rates the adrenocortical response increases significantly. Therefore, offspring expecting a poor or unpredictable post-natal environment (be it seabird chicks whose parents deliver food unpredictably or starling embryos programmed by yolk corticosterone to expect the poor provisioning of a low-quality mother) would benefit from having a dampened adrenocortical response to avoid maladaptive increases in begging behavior and energy mobilization that are associated with elevated plasma corticosterone (Wingfield et al., 1998; Kitaysky et al., 2001b; Breuner and Hahn, 2003). This suggests that if an embryo has access to a predictive cue of the severity or unpredictability of its post-natal environment, natural selection will favor a dampened adrenocortical response thereby reducing an offspring's chance of depleting energetic stores through maladaptive increases in offspring demand from a low-quality mother or low-quality environment. This conclusion is supported by work in laboratory mammals (Weinstock et al., 1992; McCormick et al., 1995; Szuran et al., 2000) and by the limited amount of work undertaken thus far in domesticated birds (Hayward et al., 2006; although see Hayward and Wingfield, 2004).

Pre-natally-induced reactions to maternal cues have recently been termed 'Predictive Adaptive Responses' (PARs) (Gluckman and Hanson, 2004; Gluckman et al., 2005). These responses are a form of developmental plasticity thought to have evolved as an adaptation to environmental cues acting during the pre-natal stage of life, but where the advantage of the induced phenotype is manifested in a future life-history stage. The induced change in the developmental trajectory of form and function presets the offspring's physiology in 'expectation' of that physiology matching its future environment; the cue therefore acts as a predictor of the nature of the neonate's environment (Gluckman et al., 2005). Unfortunately, almost all of the work on PARs to date is in clinical and laboratory systems and as such, the true adaptive function of these predictive responses has not yet been tested. Although outside of the scope of the current study (since starlings are not natally philopatric), being able to follow juveniles across life-history stages and varying habitats would allow researchers to test whether the potentially adaptive predictive programming of the HPA axis in embryos provides delayed benefits or costs to these individuals when they are adults.

Effects of the quality of the post-natal environment on HPA axis responsiveness

When faced with an unpredictable decrease in the quality of the post-natal environment, it appears that smaller daughters can be out-competed by their larger brothers, something that has recently been discussed for species with small to moderate levels of sexual size-dimorphism (Råberg et al., 2005; Rowland et al., 2007). Our study suggests that decreases in individual body condition in response to a poor quality post-natal environment also drive the observed increase in HPA axis responsiveness in daughters, supporting work in other avian species under captive conditions (Kitaysky et al., 1999a, 2001a, 2006; Pravosudov and Kitaysky, 2006). The fact that we detected a significant negative relationship between the change in brood size and HPA axis responsiveness in offspring raised by feather-clipped mothers reinforces the idea that sibling competition resulting from poor rearing conditions alters adrenocortical responsiveness in starling fledglings. Variation in body condition can be related to both baseline and stress-induced corticosterone levels in young and adult birds (Holberton et al., 1996; Kitaysky et al., 1999a, 1999b, 2001a, 2006; Love et al., 2005). In our study, body condition of fledgling starlings was negatively related to their stress-induced corticosterone levels in three out of the four experimental groups (all except the CORT-clipped treatments). Nestlings in the sham-clipped treatment had lower body condition indices than nestlings in both corticosterone-exposed treatments as well as the highest adrenocortical responses among all treatments. In particular, sham-clipped daughters had the highest adrenocortical responses on average being exposed to twice as much corticosterone during the response as same-sex counterparts pre-natally exposed to elevated yolk corticosterone (CORT-clipped). The hyper-responsiveness of the HPA axes in the sham-clipped daughters likely stems from being in a negative energetic state (low body condition) potentially brought about through competition with an abundance of larger faster-growing brothers. The immediate effects of poor body condition on the increased responsiveness of the adrenocortical axis may be caused by increased sibling competition given that individual adrenocortical responsiveness decreased when brood size decreased via mortality of competing brothers (Fig. 4). In contrast, while numerous studies in laboratory mammals have found that increasing stressfulness of the post-natal environment results in offspring with a more responsive HPA axis, this is largely thought to be a long-term programming effect (rev. in Meaney, 2001; Macrì and Würbel, 2006). Research into how developing birds respond to a decrease in the energetic quality of the post-natal environment indicate that the response is complex and species-specific: some species respond with increases in stress-induced corticosterone secretion (Nunez-de la Mora, 1996; Kitaysky et al., 1999a, 2001a; Pravosudov and Kitaysky, 2006), some species decrease responses (Kitaysky et al., 2005) and some do not appear to respond at all (Heath and Dufty, 1998; Romero et al., 1998; Sims and Holberton, 2000). These differences are largely thought to reflect differences in life-history, phylogenetic and ecological characteristics between species (Kitaysky et al., 2003). When an increase in corticosterone secretion in nest-bound chicks does occur, it is thought that it does so to either 1) increase begging rates if parents can provide more food or 2) mobilize energy stores if external food sources are absent in chicks that are in low energetic state (Kitaysky et al., 1999a, 2001a, 2001b; although see Kitaysky et al., 2005). We define this condition-driven

adrenocortical plasticity as a 'Reactive Adaptive Response' (RAR), since the offspring are reacting adaptively to an unpredictable change in the quality of the post-natal developmental environment. It is interesting that CORT-non-clipped nestlings had lower adrenocortical responses than sham-non-clipped nestlings despite having similar brood sizes and maternal provisioning rates (and therefore seemingly similar levels of sibling competition). These results suggest that the pre-natal programming cues of corticosterone (discussed above) may limit the degree of post-natal reactive plasticity in the responsiveness of the HPA axis. Moreover, although pre-natal exposure to corticosterone did not have obvious mortality or growth effects on offspring when they were raised under normal post-natal conditions (i.e., by non feather-clipped mothers), previous work has shown that offspring exposed to elevated glucocorticoids as embryos can show underlying physiological costs (Love et al., 2005; Rubolini et al., 2005; Saino et al., 2005). Together with previous work, our results suggest that the quality of the post-natal developmental environment is a significant source of post-natal variation in fledgling adrenocortical responses. Specifically, our results indicate that the quality of the post-natal environment (amount of parental care and/or sibling competition) influences individual body condition of offspring which can thereby influence both baseline and stress-induced corticosterone secretion.

Potential effects of individual variation on fitness

We have shown here that variation in the quality of both the pre-natal and post-natal developmental environment can result in significant variation in the responsiveness of the HPA axis. Unfortunately, long-term monitoring of individuals in many free-living passerine populations is not yet a reality and as such it is difficult to determine how much post-fledging plasticity there is in the adult HPA axis. Indeed, although work in laboratory mammals suggests that adult responses are programmed early in life (Liu et al., 1997; Seckl, 2001, 2004; Meaney, 2001), recent work in birds suggests that repeatability of stress responses from fledging to adulthood is low in birds, even under constant captive conditions (Wada et al., in press). However, it is known that embryonic or fetal exposure to maternal glucocorticoids can alter the exploratory, competitive and coping behavior of the individual in avian and mammalian species (rev. in Seckl, 2004; Jones et al., 1992, 1997; Weinstock, 1997; Janczak et al., 2006). Furthermore, pre-natal exposure to maternal stress may have long-term effects on learning behavior (Vallee et al., 1999; Weller et al., 1988) and even predator detection (Freire et al., 2006). Finally, individual variation in the responsiveness of the adrenocortical response to stress influences individual 'personalities' (exploratory activity, boldness) and coping styles that in turn affect survival (Cavigelli and McClintock, 2003). Proactive individuals show reduced glucocorticoid responses whereas shy individuals show marked increases in glucocorticoid secretion (see Blas et al., 2007). Our results would suggest that those individuals exposed to a maternal signal of environmental stress would be more proactive in exploring novel environments as independent pre-adults, which should theoretically increase their survival prospects (see Cavigelli and McClintock, 2003) and their fitness (Blas et al., 2007). Moreover, pre-natal exposure to maternal stress therefore has the potential to influence future breeding habitat choice by dispersing offspring (e.g., Stamps and Davis, 2006). Examining the link between the maternally/environmentally-induced developmental plasticity of the juvenile HPA axis and fitness is therefore a key to understanding the potential adaptive role of individual variation in adrenocortical function (Blas et al., 2007).

Acknowledgments

We are very grateful to the Davis family at Davistead Dairy Farms, Langley, British Columbia for their generous field support of our starling research. We would also like to thank E. Rowland and J. Verspoor for their countless hours and hard work both in the field and in the laboratory, and L. Sheldon and E. Chin for much-needed help in the field. Finally, we would like to thank K. Salvante and C. Semeniuk for help with the experimental design and T.G.G. Groothuis, C. Kennedy, B. Roitberg, P. Shulte, members of the Williams lab and two anonymous reviewers for invaluable comments and contributions that greatly improved this work. Research was funded by an operating Natural Sciences and Engineering Research Council of Canada (NSERC) grant to T.D.W., a PGS-A NSERC award to E. Chin and summer undergraduate NSERC awards to J. Verspoor and L. Sheldon.

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