6-1-2011

The Effects of Neonatal Handling on Adrenocortical Responsiveness, Morphological Development and Corticosterone Binding Globulin in Nestling American Kestrels (*Falco sparverius*)

Buddy A. Whitman
Boise State University

Creagh W. Breuner
University of Montana

Alfred M. Dufty
Boise State University

The Effects of Neonatal Handling on Adrenocortical Responsiveness, Morphological Development and Corticosterone Binding Globulin in Nestling American Kestrels (*Falco Sparverius*)

Buddy A. Whitman
Boise State University

Creagh W. Breuner
University of Montana

Alfred M. Dufty, Jr.
Boise State University

Abstract

Early developmental experiences play an important role in development of the adult phenotype. We investigated the effects of neonatal handling on the hypothalamic-pituitary-adrenal axis in a free-living avian species, the American kestrel (*Falco sparverius*). In the handled group (H), kestrel chicks were handled for 15 min/day from hatching until 26 days of age, after which time blood samples were collected for analysis of adrenal responsiveness and corticosterone binding globulin (CBG) levels. The non-handled control group (NH) was left undisturbed until 26 days of age when blood samples were collected and analyzed as above. Handled and NH kestrels did not differ in body condition index. Both total corticosterone (CORT) and CBG capacity were dampened significantly in H kestrels. However, free CORT did not differ between the two groups. In addition, hormone challenges of corticotropin releasing factor and adrenocorticotropin hormone were compared to saline injections to determine if the pituitary or the adrenal glands, respectively, were rendered more or less sensitive by handling. There was no difference in the responsiveness of H and NH kestrels to either hormone challenge. It is clear from these data that handling had an affect on fledgling phenotypic development, although whether the effects are permanent or ephemeral is unknown.

Keywords: acute stress response, corticosterone, corticosterone binding globulin, neonatal handling, phenotypic development

1. Introduction

Early life experiences can have profound and long-lasting effects on the development of organisms. Specifically, the environment to which an organism is exposed during early development can have a significant impact on a variety of physiological pathways as well as on behavior [32]. These consequences are, at least in part, due to 'environmental programming' [47]; i.e., environmental influences on gene expression that result in the observed adult phenotype. Until recently, environmental effects on developmental processes have been largely ignored; thus, the influence of early developmental environment on the inferences made in these studies is generally unknown [21]. Therefore, the potential implications of environmental influences, including those produced by investigators, on phenotypic development are vast.

Most previous studies exploring the impact of early environmental factors on phenotypic development have focused on mammals [32]. Indeed, the best known model of neonatal handling involves the rat (*Rattus domesticus*), where neonatal handling of rat pups permanently dampens responsiveness to stressors through changes in the hypothalamic-pituitary-adrenal (HPA) axis [44, 45, 46]. Although changes in maternal behavior induced by the brief periods of mother-pup separation play a major role in this phenomenon [14, 23, 25, 34], maternal behavior is not always the mediator of the phenotypic differences exhibited by handled young [19].

This study investigated the effects of a small amount (15 min) of daily investigator handling on the development of body condition, adrenal gland responsiveness, and corticosterone binding globulin (CBG) capacity in nestling American kestrels (*Falco sparverius*). Birds often are used to model the effects of environmental and anthropogenic factors on phenotypic development in free-ranging organisms, resulting in the frequent handling of young birds throughout the nestling period. However, very little is known about whether handling itself affects avian phenotypic development (but see [53]). Love et al. [36] examined handling in American kestrels in the sense that they explored the effect on corticosterone (CORT) secretion of three applications of a standard capture and handling protocol [65], a procedure that involves collecting blood samples over a prescribed period of time with the birds typically placed in cloth bags in between sampling periods. They found that repeated experience with this paradigm results in lower corticosterone (CORT) levels than in birds sampled for the first time, probably due to familiarity with the procedure. Similarly, Lynn et al. [39] found that a single previous exposure to the capture and handling protocol resulted in attenuated adrenocortical responsiveness in adult eastern bluebirds (*Sialia sialia*). Our study focused on a qualitatively different type of handling, designed to mimic what a nestling might experience during the collection of routine morphological measurements throughout the nestling period and is more similar to the type of handling that produces permanent, developmental effects on the HPA axis in rats (e.g., [44, 45, 46]).

Most investigations of developmental plasticity in the avian stress response have focused on changes in corticosterone titers, although recent studies have shown that CBG levels also are affected by environmental conditions [2, 8, 38, 40, 49, 51]. CBG is a globular protein that binds CORT with high affinity and transports it throughout the blood system, facilitating the movement of the lipid-based CORT molecules in an aqueous environment. One way that CBG may help regulate stress responsiveness is with its buffering capacity, explained by the "free hormone hypothesis." The free hormone hypothesis holds that only CORT that is not bound to a CBG molecule is "free" and accessible to target tissues and, thus, is biologically relevant [48]. According to this argument, modification of CBG levels alters the amount of free CORT that is available to target cells [7], even in the absence of changes in total corticosterone levels.

Several recent studies have demonstrated that CBG helps to modulate the stress response. For example, Lynn et al. [38] showed that after 18 hr of fasting in white-crowned sparrows (*Zonotrichia leucophrys gambelii*), CBG capacity drops, suggesting a regulatory role for CBG in the response to acute stress. Additionally, Breuner et al. [7] showed dampening of CBG in a period as short as 30 min of capture and handling stress in several avian species, although this does not occur in all species [22].

The impetus for this study was three-fold: 1) nestlings are routinely handled with little or no regard for the influence of investigator-subject interactions on phenotypic development, 2) kestrels could serve as a model of phenotypic engineering for other raptor species, and 3) no study has yet examined the effects of neonatal handling on the multidimensional stress response in an avian species. We hypothesized that daily neonatal handling would have a dampening effect on the HPA axis as measured by CORT, the primary glucocorticoid in birds [43], despite the likelihood of little to no alteration of maternal care towards handled individuals. In addition, we hypothesized that handling would regulate CBG capacity such that free CORT levels would remain unchanged even if total CORT titers were modified. Further, we employed hormone challenges of corticotropin releasing factor (CRF) and adrenocorticotropin hormone (ACTH) to a subset of handled and non-handled kestrels to test the sensitivity of the pituitary and adrenal glands, respectively. Exogenous CRF acts on the pituitary and stimulates the release of ACTH, whereas exogenous ACTH acts on the adrenal cortex and causes the release of CORT [15, 55]. Finally, to determine if routine handling affects growth, we compared body condition of handled and non-handled nestling kestrels a few days prior to fledging.

2. Materials and Methods

All methods used in this study were approved by the Boise State University Animal Care and Use Committee under protocols 006-02-013 and 006-02-014 issued to AMD. Radioimmunoassays were performed in AMD's lab whereas CBG analyses were performed in CWB's lab.

2.1. Field protocol

We studied 82 nestling American kestrels (*Falco sparverius*), 35 males and 47 females, from an established nest box breeding population in Ada and Canyon Counties (43° 33' 40" N 116° 28' 25" W), Idaho. The nest boxes were located approximately 2.5 meters high on power poles along roadsides, and were monitored during two consecutive breeding seasons beginning in March of each year. Laying date was noted for eggs within each box, and estimated hatch date was calculated from Smallwood and Bird [59]. We returned to the nest boxes beginning a few days prior to the estimated hatch date to ensure consistency in starting the handling protocol. Because kestrels often hatch asynchronously, birds entered the experiment as they hatched. The treatment of the first nest box with hatchlings was randomly assigned via a coin flip to the handled (H) group, with subsequent boxes assigned in an alternating pattern of non-handled (NH) and H. The H group consisted of 15 males and 20 females, while the NH group consisted of 20 males and 27 females. Handling consisted of removing all the nestlings from the box each day and holding them in hand for 15 min. Once old enough, nestlings were allowed to perch in hand and were not restrained.

To control for the fact that H young were not fed during the 15 min they were handled, we also prevented adults from feeding NH young for 15 min per day. We parked our vehicle near the control boxes and remained inside and quiet for the 15 min visit. Parents did not attempt to enter control boxes while the vehicle was parked nearby.

2.2. Capture and handling stress

Kestrels fledge at approximately 30 days of age [59]. A few days prior to fledging, when the kestrels were 26 days old, we performed a standard capture and handling stress series [65]. From each next box we collected baseline blood samples from one randomly selected nestling within 3 min of opening the box. Additional blood samples were collected at 5, 15, 30, and 60 min with the birds kept in cloth bags between bleeds. Morphological measurements then were taken, after which the birds were returned to their nest boxes. Blood samples were not collected from other birds in the box; however, morphological measurements were collected from all individuals. All blood samples were collected between 0900 and 1200 hours through venipuncture of the alar vein with a 26 gauge 5/8" needle. Blood (≈100-200 µl whole blood) was collected into heparinized microcapillary tubes, and stored on ice until returned to the lab. No samples were kept on ice longer than three hours. In the laboratory, samples were immediately centrifuged and plasma was removed and stored in micro-centrifuge tubes at -20°C until assayed.

2.3. Body condition

A body condition index (BCI) was developed based on Dawson and Bortolotti [18], Green [27], and Love et al. [36]. First, a principal components analysis was performed separately for males and females using the following measurements taken on day 26 post-hatch: wing chord, tarsus length, and culmen length. Next, principal components analysis factor one scores (PC1) were regressed on mass, with mass as the dependant variable and PC1 scores the independent variable. The residuals from the regression analysis were used as the BCI. Body condition index was used as a cofactor in the total and free CORT analyses.

2.4. Radioimmunoassay

Total plasma CORT levels were determined by direct radioimmunoassay (RIA) following Wingfield et al. [66]. Samples were assayed in duplicate. Briefly, recovery values were determined through the addition of approximately 2,000 counts per min (cpm) radiologically labeled CORT (^{3}H-CORT) to the plasma. CORT was extracted from 26-50 µl of plasma following incubation overnight in test tubes with freshly distilled methylene chloride. The lower phase of this mixture was then transferred to additional test tubes and the methylene chloride evaporated in a water bath under nitrogen gas. The samples were immediately resuspended in 550 µl buffer and allowed to equilibrate overnight at 4°C. One-hundred microliter subsamples were transferred into scintillation vials and 4 ml of scintillant was added for use in determining recovery counts.

For the RIA, competitive binding was established by adding 100 µl ^{3}H-CORT and CORT A$_b$ to each sample. A$_b$-bound CORT was then separated from unbound CORT by adding 500 µl Dextran-coated charcoal and incubating for 10 min before centrifugation. Samples were centrifuged at 2,000 RPM for 10 min and immediately decanted into

scintillation vials. Toluene-based scintillation fluid was added and the vials were briefly shaken and then incubated at room temperature overnight. Both recovery and RIA vials were counted for 10 min or to 2% accuracy per sample on a LS6500 Beckman scintillation counter (Beckman Coulter, Inc., Fullerton, CA). All kestrel CORT samples were run in a single assay with an intra-assay coefficient of variation of 8.6% for two standards containing a known amount of CORT.

2.5. CBG assay

CBG capacity was determined using radioligand procedures outlined by Breuner and Orchinik [9]. Maximum specific binding was optimized for kestrels with 9 ml rinse buffer (ice-cold), a final plasma dilution of 1:180 and 2 hrs of incubation at $4^{o}C$. All samples were run in triplicate, with nonspecific binding tubes containing 50 µl unlabeled CORT, 50 µl ^{3}H-CORT, and 50 µl charcoal-stripped plasma, and specific binding tubes containing 50 µl buffer, 50 µl ^{3}H-CORT, and 50 µl charcoal-stripped plasma. Bound and free radioligands were separated using rapid vacuum filtration (Brandel Harvester) over glass fiber filters (#FPD136) soaked for 1 hr prior to filtration in 25 mM Tris with 0.3% PEI. Radioligands bound to filter paper were measured using liquid scintillation spectroscopy after 12 hr equilibration in scintillation fluid. Equilibrium saturation binding ($[^{3}H]$-CORT = 0.23 – 12 nM) was determined using separate plasma pools from H and NH birds. Estimates of affinity (K_d) were 3.03 ± 0.54 nM for NH and 2.91 ± 0.46 for H kestrels and were not significantly different ($t_{16}=1.38$ $P = 0.87$) (Fig. 1). Individual CBG capacity was determined by incubating samples with 20 nM $[^{3}H]$-CORT. All CBG samples were run in a single assay using three filters. Quail plasma samples were included on each filter as a standard with a coefficient of variation of 4.8%. Point sample estimates represented approximately 87% of total binding (B_{max}). Capacity values were recalculated to 100% and the mean K_d of 3.0 nM was used for free CORT estimates.

2.6. Free CORT analysis

Free CORT titers were estimated for each of the five points of the stress series using the equation derived from the law of mass action for interactions of steroid hormones and proteins outlined in Barsano and Baumann [4]. All blood samples for CBG analysis were collected at the 60 min stress series point. Breuner et al. [7] found that CBG capacity in kestrels and other avian species drops over the 60 min serial sampling period. Therefore, we assumed that free CORT estimates for points other than the 60 min samples may be slightly overestimated. We assumed that interpretation would not be altered, as all blood samples for CBG analysis were collected at the same time (60 min).

2.7. Hormone challenges

Hormone challenge experiments were performed to determine differential sensitivity of the pituitary gland and the adrenal glands to CRF and ACTH challenges, respectively. A subset of kestrels (H = 6, NH = 7) was brought into the lab immediately following blood sample collection on day 26. These birds were housed singly or in pairs in 1m x 1m x 1m wire cages, and provided food (day old cockerels) and water *ad libitum*. Birds were in captivity three days prior to the first hormonal challenge and were kept for 2 – 3 weeks during challenges. For birds housed in pairs, only one individual was tested per day. All 13 birds were exposed randomly to each of all three of the challenges (saline, CRF, ACTH) with a washout period of 3 – 4 days between challenges. Afterwards they were released in their natal territories.

While in captivity, each bird was challenged via intraperitoneal injection as described in Wilson and Holberton [64]. Hormone treatments were assigned at random, and a 3 – 4 day washout period occurred between treatments. Saline, porcine ACTH (0.35 iu/g), and porcine CRF (0.06 µg/g) were used for the challenges. An initial baseline blood sample of ≈100 µl was taken within 3 min of removal of birds from their cage. Injections followed immediately, and after 30 min a second blood sample was taken. Blood samples were immediately centrifuged, and plasma was remove and stored until assayed for CORT as described above.

2.8. Statistics

All statistical analyses were conducted using SAS 9.1 [57]. CBG binding characteristics were determined using non-linear curve-fitting functions of Graph Pad Prism 4.0 [26]. Body condition and CBG capacity were analyzed using an ANOVA. Stress series and hormone challenge data were analyzed using a mixed model repeated measures

4

ANOVA. Body condition index was used as a covariate in total and free CORT series analyses. All data are presented as mean ± SEM and Least Significant Differences (LSD) were used for all post-hoc analyses.

3. Results

3.1. Body condition

The body condition index did not differ between H and NH kestrels ($F_{1,78} = 0.55$, $P = 0.46$). In addition, sex ($F_{1,78} = 0.02$, $P = 0.90$) and treatment × sex interaction ($F_{1,78} = 0.83$, $P = 0.37$) did not differ. H females had -2.35 ± 2.85 body condition units: H males: 0.24 ± 2.47: NH females: 1.76 ± 2.47: and NH males: -3.35 ± 2.85.

3.2. Total CORT

Routine handling reduced total stress-induced CORT titers ($F_{1,22} = 6.14$, $P = 0.021$; Fig. 2) with significant interactions of time ($F_{4,92} = 44.7$, $P < 0.0001$) and status × time ($F_{4,92} = 3.17$, $P = 0.02$). Post-hoc LSD tests revealed that 0 and 5 min total CORT titers did not differ ($t_{92} = 0.54$, $P = 0.59$ and $t_{92} = 1.09$, $P = 0.28$, respectively). However, total CORT titers at 15, 30 and 60 min were significantly lower in H kestrels ($t_{92} = 2.61$, $P = 0.01$, $t_{92} = 3.57$, $P = 0.0006$ and $t_{92} = 2.05$, $P = 0.04$, respectively). Handled kestrels had lower CORT titers at 15, 30, and 60 min, respectively than NH kestrels. Body condition did not co-vary with any factor in the model ($F_{1,92} = 0.42$, $P = 0.52$), and no other interactions were significant (all $P > 0.19$).

3.3. CBG capacity

CBG binding capacity estimates were significantly lower in H (36.8 ± 13.6 nM) compared to NH kestrels (77.2 ± 12.9), ($F_{1,14} = 4.65$, $P = 0.05$; Fig. 3). Binding capacity did not differ between the sexes ($F_{1,14} = 0.70$, $P = 0.42$) or treatment × sex ($F_{1,14} = 0.01$, $P = 0.94$). Body condition did not co-vary with CBG ($F_{1,14} = 0.373$, $P = 0.55$).

3.4. Free CORT

The lowered total CORT titers of H kestrels was not reflected in free CORT estimates, in that the secretion pattern of free CORT was similar in H and NH kestrels ($F_{1,13} = 0.01$, $P = 0.92$; Fig. 4). In addition, the time × status interaction was not significant ($F_{4,56} = 0.07$, $P = 0.99$), and all post-hoc comparison P-values were > 0.73. Body condition did not co-vary with any factor in the model ($F_{1,56} = 0.61$, $P = 0.44$).

3.5 Hormone challenges

Captivity increased baseline total CORT titers for both handled and non-handled kestrels (two-way ANOVA, $F_{1,59} = 41.17$, $P < 0.0001$). However, there was no difference between handling status ($F_{1,59} = 0.013$, $P = 0.91$) and no interaction between handling status x captivity status ($F_{1,59} = 0.159$, $P = 0.69$). Analysis of hormone challenge data revealed no significant differences in CORT secretions between H and NH kestrels ($F_{1,10} = 1.15$, $P = 0.31$; Fig. 5). Additionally, type of challenge had no effect on CORT output ($F_{2,46} = 1.28$, $P = 0.28$); nor did the treatment × challenge × time interaction ($F_{2,46} = 0.44$, $P = 0.64$).

4. Discussion

4.1. Investigator handling, baseline CORT, and adrenoresponsiveness

This study explored the influence of 15 min of daily investigator handling on adrenocortical stress reactivity and development by examining the pattern of stress-induced CORT levels, CBG levels, body condition near the age of fledging, and responses to hormone challenges. Only a few previous studies have examined the effects of handling on CORT secretion in non-domesticated birds [16, 36, 1], and none of those studies simulated short periods of investigator handling during development. However, despite significant methodological differences with our study, both Love et al. [36] and Adams et al. [1] also found that previously handled young exhibited dampened adrenoresponsiveness compared to non-handled birds.

Routine neonatal handling dampened the adrenocortical stress response in American kestrel nestlings and the effect was similar in males and females. Adrenoresponsiveness, as represented by total CORT levels, differed significantly between handled and non-handled birds at three of the five time points (15, 30, and 60 min) sampled during the stress protocol, but not at baseline or 5 min after onset of the protocol. Little is known about the relationship between baseline CORT and phenotypic development in birds, although one current idea, the CORT – Fitness Hypothesis, holds that elevated baseline CORT levels reflect individuals in poor condition and with low fitness prospects (reviewed in Bonier et al. [6]). Both body condition and baseline CORT levels were similar in our two treatment groups, suggesting that handled birds were not under chronic stress as a result of our daily manipulations.

The two groups did differ in the strength of their adrenocortical response and this, too, may have fitness consequences, although we did not measure fitness and, indeed, the relationship between adrenoresponsiveness and fitness is far from clear [11]. Blas et al. [5] demonstrated that elevated responsiveness to a standardized stress protocol is negatively correlated with survival and recruitment in the European white stork (*Ciconia ciconia)*. Other studies have demonstrated that environmental conditions and phenotypic manipulations affect the adrenocortical response to stress in developing birds. For example, Almasi et al. [2] found that environmental conditions (seasonal precipitation levels), availability of food, and exposure to exogenous corticosterone affect adrenoresponsiveness. Similarly, manipulation of nestling phenotype results in elevated adrenoresponsiveness in poor quality individuals [37]. In contrast, in American kestrels low quality juveniles exhibit a slower initial adrenocortical response than do high quality individuals, but they maintain elevated plasma corticosterone levels longer [29]. Similarly, in the congeneric Eurasian kestrel (*Falco tinnunculus*), young nestlings in the poorest condition (i.e., had the lowest furcular fat scores) mounted the weakest adrenocortical stress response [51].

4.2. Possible phenotypic consequences

The extent to which the dampened adrenocortical response in the handled nestlings affects phenotypic quality is unclear, although a reduction in phenotypic quality could, in turn, affect fitness. Total CORT titers were reduced in handled kestrels, but the same was not true for free CORT because CBG capacity likewise was lower, resulting in similar free CORT levels in handled and non-handled birds. The free hormone hypothesis postulates that it is the unbound fraction of steroid hormones in the systemic circulation that is biologically active [48]. Following this argument, our data suggest that handling may not affect the ability of birds to mount appropriate adrenocortical responses, to the extent that an appropriate adrenocortical response is determined by levels of free CORT rather than total CORT. However, CBG-bound CORT may be important in its own right, independent of free CORT titers, as there is evidence for a functional role of the CBG-CORT complex. For example, in mammals, a mechanism exists to cleave CBG molecules at the site of inflammation to increase local concentrations of free CORT [52, 28, 33]. If a similar phenomenon occurs in birds, then handling could interfere with the ability of handled birds to control inflammation. Butler and Dufty [12] demonstrated that 15 min of daily handling of nestling American kestrels and European starlings (*Sturnus vulgaris*) had no effect on the amount of swelling induced by the mitogen phytohemagglutinin (PHA). PHA, which has been thought to reflect the robustness of cell-mediated immunity, may instead stimulate a complex response involving multiple components of the immune system [42, 62]. Thus, handling nestlings does not appear to affect immunoresponsiveness. However, if the CBG-CORT complex is involved in regulating inflammatory responses, then the lower total CORT levels of handled birds could put them at a disadvantage in this regard. Finally, the absence of an effect of handling on body condition suggests that handling does not chronically elevate CORT in the nestlings because even a few days of a chronic, low-level increase in CORT can affect development of morphological features (and immune responses) in nestling kestrels [13].

The failure of daily handling to enhance adrenoresponsiveness could be due, in part, to mechanisms that minimize stress-induced CORT secretion in nestlings until individuals are independent enough to respond behaviorally, thus protecting young altricial and semi-altricial birds from suffering the deleterious effects of CORT [61, 58]. Indeed, adrenoresponsiveness in nestling kestrels approaches adult levels only late in the nestling period [60, 35]. Even so, it is unclear why daily handling would diminish adrenoresponsiveness to a novel stressor (i.e., the capture and handling stress series), although perhaps daily handling enhanced the negative feedback mechanism, possibly through up-regulation of CORT receptors.

Another consideration is that the effect of daily handling on adrenoresponsiveness may or may not be permanent. Others have shown that effects of early environmental conditions on adrenoresponsiveness may be long-lasting or may disappear in adulthood [63, 31, 50].

4.3. CBG capacity

Corticosterone binding globulins appear to regulate the availability of free CORT to its receptors on and in target tissues [10]. The relatively rapid modulation of CBG levels in response to environmental stimuli in both nestlings and adults [7, 40] offers a mechanism whereby physiological and behavioral responses to CORT can occur, even in the absence of changes in the total amount of CORT secreted. CBG capacity differed between handled and unhandled kestrels after the 60 min stress protocol. Serial blood samples collected from birds in the same population the following year showed that CBG capacity is similar to baseline 30 min after the onset of a stress protocol in kestrels, but that it declines after 60 min [7]. Baseline CBG capacity may have differed between the two groups, especially if they differed in body condition [2]. However, both handled and non-handled kestrels were similar in body condition, so this factor was unlikely to play a role. Because CBG capacity declines over time in kestrels, use of the CBG capacity found at 60 min probably underestimated the CBG capacity earlier in the stress series and, therefore, overestimated calculations of the amount of free CORT in the earlier samples. Nonetheless, the results represent real differences at the 60-min point and the pattern for the earlier points is accurate to the extent that baseline CBG capacity and the rate of the stress-induced change in CBG capacity over time were similar in the two groups.

4.4. Habituation

One could argue that the reduced stress response exhibited by handled birds is the result of habituation; that is, that handled birds became familiar with being manipulated daily and did not perceive the subsequent capture and handling stress protocol as a stressor, resulting in reduced CORT secretions compared to unhandled birds. Indeed, Adams et al. [1] felt that the reduced stress response they observed in grey-faced petrels was due to habituation. However, while we cannot rule out habituation as an explanation for our results, we believe that our findings (and possibly those of Adams et al. [1] may reflect developmental differences between the handled and unhandled birds because the blood sampling technique used to induce the adrenocortical response [66] differs markedly from the daily handling procedures to which the birds were exposed. That is, during blood sampling the birds were immobilized, serially bled from a wing vein, and held in cloth bags in between sampling events, none of which the birds experienced during the daily handling protocol. Following a similar argument made by Cyr and Romero [17] in their review of hormonal habituation, we believe that the stress protocol, which involves restraint, pain (the prick of the needle), and periods of physical isolation in an opaque bag, represented a novel stressor for the birds. Therefore, even if the kestrels did acclimate to the daily handling routine (and we suspect they did), then their failure to dishabituate when faced with the novel stressor violates one of the criteria for hormonal habituation in field studies [17] and suggests that the attenuation of the HPA axis in handled nestlings reflects differences in the underlying physiological mechanisms between the handled and unhandled birds, not habituation. While as few as one previous bout of capture and bleeding may result in habituation to the sampling protocol in nestlings or adults [36, 39], our birds were not previously bled, so habituation to the blood sampling protocol itself could not have occurred. Nonetheless, our data do not allow us to reject the possibility that it is the initial step of capturing and handling the birds, and not the subsequent blood sampling and confinement, that elicits a stress response to which the birds could habituate over time. Resolution of this question awaits further investigation.

4.5 Hormone challenges

The hormone challenge was designed to determine where in the HPA axis a change in sensitivity may have occurred. Transferring the birds into captivity resulted in elevated baseline CORT levels, as has been noted previously [41, 56, 20]. However, the effect was similar in both handled and non-handled birds.

Many studies have challenged birds with one or more pituitary and/or hypothalamic hormones (i.e., ACTH, CRF, and arginine vasotocin) to elicit downstream endocrine responses [3, 55, 64, 54, 20]. In our study, neither CRF nor ACTH challenges elicited any greater CORT release than did saline in handled or non-handled nestling kestrels. Additionally, handled and non-handled kestrels did not respond differently regardless of challenge or time. This finding is surprising given that H birds had a dampened response to capture and handling stress and it suggests that

any physiological differences in the HPA axis between the two groups may be centrally located, above the level of the hypothalamus. We consider this a tentative conclusion and one that will require additional testing to confirm. Challenges were conducted on a subset of the birds and it is possible that with a more robust sample size we would have uncovered subtle but biologically meaningful differences between the treatment groups.

5. Conclusions

Investigator handling of nestling birds is quite common, and it is clear from this study that a short period of daily handling had a dampening effect on adrenocortical responsiveness in developing kestrels, although the amount of biologically active CORT did not differ between handled and non-handled nestlings. While handling modified phenotypic development, we do not know if the effect is permanent or disappears over time, if it alters biological fitness, if reducing the frequency of handling (e.g., every other day instead of daily or minimizing handling during developmentally sensitive periods) ameliorates the effect, or if short periods of daily handling influence the development of other physiological mechanisms. Nor should we assume that any long-term effects will all be negative. That is, handling may be a useful tool in the engineering of phenotypes better suited to a particular set of conditions (e.g. captive breeding programs) [30], and the resulting phenotypes may be transferred across generation through non-genomic mechanisms [24]. The complex interactions among environmental cues, genetics, and the endocrine system, specifically the pleiotropic nature of CORT as it relates to developmental trajectories, warrants a multidisciplinary approach to fully understand their complex interplay [21]. But until more is known about the effects of investigator handling we suggest that unnecessary handling of birds during development be avoided.

Figure Legends

Figure 1: Equilibrium saturation binding curve (mean ± SEM) for non-handled (squares) and handled (triangles) kestrels showing specific binding of ^{3}H-CORT to kestrel plasma (4 ^{o}C) in response to increasing concentrations of radiolabeled CORT. Plasma was from blood samples collected at the 60 min stress series point. Estimates of affinity (K_d) for non-handled (3.03 ± 0.54 nM) and handled kestrels (2.91 ± 0.46 nM) did not significantly differ (P = 0.86). Inset is a Scatchard-Rosenthal replot of the data.

Figure 2: Total CORT titers (mean ± SEM) for non-handled (n = 13, dashed line) and handled kestrels (n = 14, solid line) showing significantly higher titers in non-handled kestrels (P = 0.02); * = P < 0.05, ** = P < 0.01 for LSD post-hoc analyses between handled and non-handled birds at a given sampling time.

Figure 3: Corticosterone binding globulin (CBG) capacity (mean ± SEM) of non-handled (n = 9, black bar) and handled kestrels (n = 9, white bar). Blood samples for CBG analysis were collected at the 60 min stress series point. CBG capacity means were different with non-handled kestrels having higher capacity (P = 0.05).

Figure 4: Free CORT titers (mean + SEM) for non-handled (n = 13, solid black line) and handled kestrels (n = 14, dashed black line) superimposed on total CORT titers in gray. Free CORT estimates did not differ between treatments (P = 0.92), or at any time point; LSD post-hoc for all time points P > 0.70.

Figure 5: Adrenocortical responses to ACTH (A), CRF (B) and Saline (C) challenges (mean + SEM) for non-handled (black histograms) and handled (white histograms) American kestrels. Sample sizes for non-handled and handled birds were 7 and 6, respectively, for each type of challenge (see text for details).

References

[1] N.J. Adams, J.F. Cockrem, G.A. Taylor, E.J. Candy, J. Bridges, Corticosterone responses of hand-reared and parent-reared grey-faced petrel chicks (*Pterodroma macroptera gouldi*), Zoo Biol. 24 (2005) 283-290.

[2] B. Almasi, A. Roulin, S. Jenni-Eiermann, C.W. Breuner, L. Jenni, Regulation of free corticosterone and CBG capacity under different environmental conditions in altricial birds, Gen. Comp. Endocrinol. 164 (2009) 117-124.

[3] L.B. Astheimer, W.A. Buttemer, J.C. Wingfield, Gender and seasonal differences in the adrenocortical response to ACTH challenge in an arctic passerine, *Zonotrichia leucophrys gambelii*, Gen. Comp. Endocrinol. 94 (1994) 33-43.

[4] C.P. Barsano, G. Baumann, Editorial: simple algebraic and graphic methods for the apportionment of hormone (and receptor) into bound and free fractions in binding equilibria; or how to calculate bound and free hormone? Endocrinology 124 (1989) 1101-1106.

[5] J. Blas, G.R. Bortolotti, J.L. Tella, R Baos, T.A. Marchant, Stress response during development predicts fitness in a wild, long lived vertebrate, Proc. Natl. Acad. Sci. U.S.A. 104 (2007) 8880-8884.

[6] F. Bonier, P.R. Martin, I.T. Moore, J.C. Wingfield, Do baseline glucocorticoids predict fitness? Trends Ecol. Evol. 24 (2009) 634-642.

[7] C.W. Breuner, S.E. Lynn, G.E. Julian, J.M. Cornelius, B.J. Heidinger, O.P. Love, R.S. Sprague, H. Wada, B.A. Whitman, Plasma binding globulins and the acute stress response, Horm. Metab. Res. 38 (2006) 260-268.

[8] C. Breuner, M. Orchinik, Downstream from corticosterone: seasonality of binding globulins, receptors and behavior in the avian stress response, in: A. Dawson, C. Chaturvedi (Eds.), Avian Endocrinology, Narosa Publishing House, Naroda, India, 2000, pp. 1-12.

[9] C.W. Breuner, M. Orchinik, Seasonal regulation of membrane and intracellular corticosteroid receptors in the house sparrow brain, J. Neuroendocrinol. 13 (2001) 412-420.

[10] C.W. Breuner, M. Orchinik, Pharmacological characterization of intracellular, membrane, and plasma binding sites for corticosterone in house sparrows, Gen. Comp. Endocrinol. 163 (2009) 214-224.

[11] C.W. Breuner, S.H. Patterson, T.P. Hahn, In search of relationships between the acute adrenocortical response and fitness, Gen. Comp. Endocrinol. 157 (2008) 288-295.

[12] M.W. Butler, A.M. Dufty, Jr., Nestling immunocompetence is affected by captivity but not investigator handling, Condor 109 (2007) 920-928.

[13] M.W. Butler, L.L Leppert, A.M. Dufty, Jr., Effects of small increases in corticosterone levels on morphology, immune function, and feather development, Physiol. Biochem. Zool. 83 (2010) 78-86.

[14] C. Caldji, B. Tannenbaum, S. Sharma, D. Francis, P.M. Plotsky, M.J. Meaney, Maternal care during infancy regulates the development of neural systems mediating the expression of fearfulness in the rat, Proc. Natl. Acad. Sci. U.S.A. 95 (1998) 5335-5340.

[15] G.P. Chrousos, J.R. Calabrese, P. Avgerinos, M.A. Kling, D. Rubinow, E.H. Oldfield, T. Schuermeyer, C.H. Kellner, G.B. Cutler, Jr., D.L. Loriaux, P.W. Gold, Corticotropin releasing factor: Basic studies and clinical applications, Prog. Neuro-Psychopharmacol. Biol. Psychiatry 9 (1985) 349-359.

[16] J.C. Collette, J.R. Millam, K.C. Klasing, P.S. Wakenell, Neonatal handling of Amazon parrots alters the stress response and immune function, Appl. Anim. Behav. Sci. 66 (2000) 335-349.

[17] N.E. Cyr, L.M. Romero, Identifying hormonal habituation in field studies of stress, Gen. Comp. Endocrinol. 161 (2009) 295-303.

[18] R.D. Dawson, G.R. Bortolotti, Are avian hematocrits indicative of condition? American kestrels as a model, J. Wildl. Manag. 61 (1997) 1297-1306.

[19] V.H. Denenberg, Commentary: Is maternal stimulation the mediator of the handling effect in infancy? Dev. Psychobiol. 34 (1999) 1-3.

[20] M.J. Dickens, K.A. Earle, L.M. Romero, Initial transference of wild birds to captivity alters stress physiology, Gen. Comp. Endocrinol. 160 (2009) 76-83.

[21] A.M. Dufty, Jr., J. Clobert, A.P. Møller, Hormones, developmental plasticity and adaptation, Trends Ecol. Evol. 17 (2002) 190-196.

[22] H.B. Fokidis, M. Orchinik, P. Deviche, Corticosterone and corticosteroid binding globulin in birds: Relation to urbanization in a desert city, Gen. Comp. Endocrinol. 160 (2009) 259-270.

[23] D. Francis, J. Diorio, P. LaPlante, S. Weaver, J.R. Seckl, M.J. Meaney, The role of early environmental events in regulating neuroendocrine development - Moms, pups, stress, and glucocorticoid receptors, Ann. N. Y. Acad. Sci. 794 (1996) 136-152.

[24] D. Francis, J. Diorio, D. Liu, M.J. Meaney, Nongenomic transmission across generations of maternal behavior and stress responses in the rat, Science 286 (1999) 1155-1158.

[25] D.D. Francis, M.J. Meaney, Maternal care and the development of stress responses, Curr. Opin. Neurobiol. 9 (1999) 128-134.

[26] Graph Pad Software, Graph Pad Prism 4.0, San Diego, CA, 2003.

[27] A.J. Green, Mass/length residuals: measures of body condition or generators of spurious results? Ecology 82 (2001) 1473-1483.

[28] G.L. Hammond, C.L. Smith, N.A. Paterson, W.J. Sibbald, A role for corticosteroid-binding globulin in delivery of cortisol to activated neutrophils, J. Clin. Endocrinol. Metab. 71 (1990) 34-39.

[29] J.A. Heath, A.M. Dufty, Jr., Body condition and the adrenal stress response in captive American kestrel juveniles, Physiol. Zool. 71 (1998) 67-73.

[30] E.D. Ketterson, V. Nolan, Jr., M.J. Cawthorn, P.G. Parker, C. Ziegenfus, Phenotypic engineering: using hormones to explore the mechanisms and functional bases of phenotypic variation in nature, Ibis 138 (1996) 70-86.

[31] Á.Z. Lendvai, C. Loiseau, G. Sorci, O. Chastel, Early developmental conditions affect stress response in juvenile but not in adult house sparrows (*Passer domesticus*), Gen. Comp. Endocrinol. 160 (2009) 30-35.

[32] S. Levine, Enduring effects of early experience on adult behavior, in: D.W. Pfaff, A.P. Arnold, A.M. Etgen, S.E. Fahrbach, R.T. Rubin (Eds.), Hormones, Brain and Behavior, Academic Press, Burlington, MA, 2002, pp. 535-542.

[33] H.-Y. Lin, Y.A. Muller, G.L. Hammond, Molecular and structural basis of steroid hormone binding and release from corticosteroid-binding globulin, Mol. Cell. Endocrinol. 316 (2010) 3-12.

[34] D. Liu, J. Diorio, B. Tannenbaum, C. Caldji, D. Francis, A. Freedman, S. Sharma, D. Pearson, P.M. Plotsky, M.J. Meaney, Maternal care, hippocampal glucocorticoid receptors, and hypothalamic-pituitary-adrenal responses to stress, Science 277 (1997) 1659-1662.

[35] O.P. Love, D.M Bird, L.J. Shutt, Corticosterone levels during post-natal development in captive American kestrels (*Falco sparverius*), Gen. Comp. Endocrinol. 130 (2003a) 135-141.

[36] O.P. Love, L.J. Shutt, J.S. Silfies, D.M. Bird, Repeated restraint and sampling results in reduced corticosterone levels in developing and adult captive American kestrels (*Falco sparverius*), Physiol. Biochem. Zool. 76 (2003b) 753-761.

[37] O.P. Love, T.D. Williams, Plasticity in the adrenocortical response of a free-living vertebrate: The role of pre- and post-natal developmental stress, Horm. Behav. 54 (2008) 496-505.

[38] S.E. Lynn, C.W. Breuner, J.C. Wingfield, Short-term fasting affects locomotor activity, corticosterone, and corticosterone binding globulin in a migratory songbird, Horm. Behav. 43 (2003) 150-157.

[39] S.E. Lynn, L.E. Prince, M.M. Phillips, A single exposure to an acute stressor has lasting consequences for the hypothalamo–pituitary–adrenal response to stress in free-living birds, Gen. Comp. Endocrinol. 165 (2010a) 337-344.

[40] S.E. Lynn, T.B. Stamplis, W.T. Barrington, N. Weida, C.A. Hudak, Food, stress, and reproduction: Short-term fasting alters endocrine physiology and reproductive behavior in the zebra finch, Horm. Behav. 58 (2010b) 214-222.

[41] P.P. Marra, K.T. Lampe, B.L. Tedford, Plasma corticosterone levels in two species of *Zonotrichia* sparrows under captive and free-living conditions, Wils. Bull. 107 (1995) 296–305.

[42] L.B. Martin II, P. Han, J. Lewittes, J.R. Kuhlman, K.C. Klasing, M. Wikelski, Phytohemagglutinin-induced skin swelling in birds: histological support for a classic immunoecological technique, Funct. Ecol. 20 (2006) 290-299.

[43] M.M. Mashaly, Effects of exogenous corticosterone on chicken embryonic development, Poult. Sci. 70 (1991) 371-374.

[44] M.J. Meaney, D.H. Aitken, S. Bhatnagar, C. van Berkel, R.M. Sapolsky, Effects of neonatal handling on age-related impairments associated with the hippocampus, Science 239 (1988) 766-768.

[45] M.J. Meaney, D.H. Aitken, V. Viau, S. Sharma, A. Sarrieau, Neonatal handling alters adrenocortical negative feedback sensitivity and hippocampal type II glucocorticoid receptor binding in the rat, Neuroendocrinol. 50 (1989) 597-604.

[46] M.J. Meaney, J.B. Mitchell, D.H. Aitken, S. Bhatnagar, S.R. Bodnoff, L.J. Iny, A. Sarrieau, The effects of neonatal handling on the development of the adrenocortical-response to stress: implications for neuropathology and cognitive deficits in later life, Psychoneuroendocrinol. 16 (1991) 85-103.

[47] M.J. Meaney, M. Szyf, Maternal care as a model for experience-dependent chromatin plasticity? Trends Neurosci. 28 (2005) 456-463.

[48] C.M. Mendel, The free hormone hypothesis: a physiologically based mathematical model, Endocrine Rev. 10 (1989) 232-274.

[49] C. Müller, B. Almasi, A. Roulin, C.W. Breuner, S. Jenni-Eiermann, L. Jenni, Effects of corticosterone pellets on baseline and stress-induced corticosterone and corticosteroid-binding-globulin, Gen. Comp. Endocrinol. 160 (2009a) 59-66.

[50] C. Müller, S. Jenni-Eiermann, L. Jenni, Effects of a short period of elevated circulating corticosterone on postnatal growth in free-living Eurasian kestrels *Falco tinnunculus*, J. Exp. Biol. 212 (2009b) 1405-1412.

[51] C. Müller, S. Jenni-Eiermann, L. Jenni, Development of the adrenocortical response to stress in Eurasian kestrel nestlings: Defence ability, age, brood hierarchy and condition, Gen. Comp. Endocrinol. 168 (2010) 474-483.

[52] P.A. Pemberton, P.E. Stein, M.B. Pepys, J.M. Potter, R.W. Carrell, Hormone binding globulins undergo serpin conformational change in inflammation, Nature 336 (1998) 257-258.

[53] P. Quillfeldt, M. Poisbleau, O. Chastel, J.F. Masello, Acute stress hyporesponsive period in nestling thin-billed prions *Pachyptila belcheri*, J. Comp. Physiol. A 195 (2009) 91-98.

[54] E.L. Rich, L.M. Romero, Exposure to chronic stress down regulates corticosterone responses to acute stressors, Amer. J. Physiol. 288 (2005) R1628-R1636.

[55] L.M. Romero, K.K. Soma, J.C. Wingfield, Hypothalamic-pituitary-adrenal axis changes allow seasonal modulation of corticosterone in a bird, Amer. J. Physiol. 274 (1998) R1338-R1344.

[56] L.M. Romero, J.C. Wingfield, Alterations in hypothalamic–pituitary–adrenal function associated with captivity in Gambel's white-crowned sparrows (*Zonotrichia leucophrys gambelii*), Comp. Biochem. Physiol. B 122 (1999) 13-20.

[57] SAS Institute, SAS 9.1, SAS Institute, Cary, NC, 2003.

[58] C.G. Sims, R.L. Holberton, Development of the corticosterone stress response in young Northern mockingbirds (*Mimus polyglottos*), Gen. Comp. Endocrinol. 119 (2000) 193-201.

[59] J.A. Smallwood, D.M. Bird, American Kestrel (*Falco sparverius*), in: A. Poole, F. Gill (Eds.), The Birds of North America, No. 602, The Birds of North America Inc., Philadelphia, PA, 2002.

[60] K.W. Sockman, H. Schwabl, Plasma corticosterone in nestling American kestrels: Effects of age, handling stress, yolk androgens, and body condition, Gen. Comp. Endocrinol. 122 (2001) 205-212.

[61] J.M. Starck, Evolution of avian ontogenies, in: D.M. Power (Ed.), Current Ornithology, Plenum Press, New York, 1993, pp. 275-366.

[62] M. Vinkler, H. Bainová, T. Albrecht, Functional analysis of the skin-swelling response to phytohaemagglutinin, Funct. Ecol. 24 (2010) 1081-1086.

[63] H. Wada, T.P. Hahn, C.W. Breuner, Development of stress reactivity in white-crowned sparrow nestlings: Total corticosterone response increases with age, while free corticosterone response remains low, Gen. Comp. Endocrinol. 150 (2007) 405-413.

[64] C.M. Wilson, R.L. Holberton, An alternative method for delivering adrenocorticotropin hormone in birds, Gen. Comp. Endocrinol. 122 (2001) 349-353.

[65] J.C. Wingfield, Modulation of the adrenocortical response to stress in birds, in: K.G. Davey, R.E. Peter, S.S. Tobe (Eds.), Perspectives in Comparative Endocrinology, National Research Council of Canada, Ottawa, 1994, pp. 520-528.

[66] J.C. Wingfield, C.M. Vleck, M.C. Moore, Seasonal changes in the adrenocortical response to stress in birds of the Sonoran desert, J. Exp. Zool. 264 (1992) 419–428.