TECHNICAL COMMENT

Repeated Restraint and Sampling Results in Reduced Corticosterone Levels in Developing and Adult Captive American Kestrels (*Falco sparverius*)

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Introduction

The study of intraspecific variation in adrenocortical function in birds through measures of glucocorticoids is an emerging and potentially useful tool in the field of behavioral and evolutionary endocrinology. However, the effects of handling of study animals on endocrine-mediated phenotypic development and endocrine phenotypic variation have rarely been considered (Dufty et al. 2002), despite the fact that handling may produce physiological effects that can bias subsequent physiological and behavioral interpretations (Clinchy et al. 2001). These potential effects increase in importance during long-term studies such as those examining endocrine-mediated life-history trade-offs (Zera and Harshman 2001) and endocrine system disruption by human-caused environmental perturbations (Dufty et al. 2002; Love et al. 2003*c*). Studies of adrenocortical function in birds through measures of the stress hormone corticosterone (B) are one such area routinely involving the recapture, rehandling, and resampling of animals, potentially affecting variation in corticosterone itself.

The adrenocortical response to stress in birds initiates several important physiological changes, including effects on intermediary metabolism and growth (Wingfield 1994) via short-term, temporary increases of the hormone messenger, corticosterone. These elevated plasma concentrations of corticosterone are known to stimulate alternative metabolic pathways and behavioral patterns, including increased or decreased locomotory activity depending on prior food availability (Astheimer et al. 1992; Breuner et al. 1998; Lynn et al. 2003), decreased nocturnal oxygen consumption (Astheimer et al. 1992), lipogenesis (Harvey et al. 1984; Gray et al. 1990), increased food intake (Wingfield et al. 1990; Bray 1993), and increased energy availability resulting from protein catabolism (Gray et al. 1990; Wingfield et al. 1995). The corticosterone response has been shown to vary both daily and seasonally (Wingfield et al. 1992; Astheimer et al. 1994; Wingfield et al. 1994; Silverin 1997, 1998; Silverin et al. 1997), and it may also vary between populations and between individuals of the same species (Wingfield et al. 1992; Astheimer et al. 1994; Schwabl 1995; Silverin et al. 1997; Silverin and Wingfield 1998). Recently, both baseline and stress-induced corticosterone levels have also been shown to vary significantly during postnatal development in altricial birds (Schwabl 1999; Sims and Holberton 2000; Sockman and Schwabl 2001; Love et al. 2003*a*).

A number of recent studies examining baseline and stress-induced corticosterone levels in birds have utilized sampling of the same birds during mutiple occasions. This phenomenon has often arisen for a priori reasons, either to reduce the need to collect and maintain large sample sizes of wild birds (Rehder et al. 1986; Dufty and Belthoff 1997; Piersma and Ramenofsky 1998; Holberton 1999; Piersma et al. 2000; Rich and Romero 2001), to increase the statistical strength of results through within-bird analysis (Rich and Romero 2001), or to examine repeatability of the corticosterone response itself (Cockrem and Silverin 2002). However, while multiple sampling can provide researchers with useful data without the need for capturing and disturbing large numbers of potentially sensitive species, it is possible that multiple sampling causes habituation to the sampling protocol. In fact, many studies of captive laboratory mammals have shown that researcher and maternal handling can significantly affect stress responses and development associated with the hormones produced during these responses (reviewed

in Meaney et al. 1988, 1991, 1993; Bhatnager et al. 1995; Liu et al. 1997; Vallée et al. 1997; Fleming et al. 1999; Francis and Meaney 1999; Francis et al. 1999; Caldji et al. 2000; Kazushige et al. 2001; Bernatova et al. 2002). Although less evidence exists at present of this phenomenon in birds, several studies have indicated that habituation to restraint and blood sampling may be an issue of concern. Species in recent studies where this possible habituation was noted or directly observed have included wild great tits (*Parus major*; Cockrem and Silverin 2002), wild red knots (*Calidris canutus*; Piersma and Ramenofsky 1998), wild screech owls (*Otus kennicottii*; Dufty and Belthoff 1997), and wild European starlings (*Sturnus vulgaris*; Romero and Remage-Healey 2000) brought into captivity, as well as domesticated chickens (*Gallus domesticus*; Freeman and Manning 1979; Freeman and Flack 1980; Webb and Marshaly 1985). In fact, decreased corticosterone levels have even been associated with habituation to the mere presence of humans in wild Magellanic penguins (*Spheniscus magellanicus*; Fowler 1999).

Given the fact that corticosterone secretion is affected by handling in small mammals, it is reasonable to hypothesize that birds may exhibit similar effects. Furthermore, since the pattern of corticosterone secretion is a potentially useful tool in the study of behavioral and evolutionary endocrinology, habituation to handling is an important issue to investigate. The purpose of this study was to experimentally evaluate the effects of repeated restraint on the ability of postnatally developing chicks and adults to respond to a repeated standardized stressor of capture and handling in a semialtricial raptorial species, the American kestrel (*Falco sparverius*). We hypothesized that baseline corticosterone levels would be largely unaffected by handling given its important role in the maintenance of homeostasis (Wingfield 1994), whereas stress-induced levels would be negatively related to the number of handling and sampling episodes due to habituation to the procedure. Postnatally developing captive chicks of this species were chosen for this experiment since (1) accurate age, and hence differential effects of handling during development, can be determined, and (2) this is a relatively large species, and hence chicks can be blood sampled on multiple occasions without harm. We chose to examine adults in this species since we had access to individuals of known age, coupled with accurate handling and sampling records for each bird.

Material and Methods

Breeding Procedures and Species

The study was conducted at the Avian Science and Conservation Centre (ASCC) of McGill University from April to July 1999 using captive birds reared from captive-raised adults. We adhered to the standards of the McGill University Institutional Animal Care and Use Committee for the humane treatment of our subjects. In the first experiment, 45 pairs of adult banded

kestrels were removed from same-sex communal flight pens and placed in outdoor breeding pens measuring 3.5 m × 5.5 m × 3.0 m (l × w × h). Pen walls were of solid plywood so that pairs could not see each other and could not see researchers until the pen door was opened. Depending on pairs, egg laying began within 10–13 d following pairing. Individual eggs were marked to document laying and hatching order of chicks, and once hatched, all chicks were individually marked with nontoxic colored felt markers every second day until they were 16 d of age, at which time they were marked with colored plastic bands. Parents were fed a daily diet of three, day-old cockerels each until hatching, at which time they were fed this plus an additional 1.5-d-old cockerels for each nestling present. Fresh water was supplied for drinking and bathing.

The second experiment involved captive banded adult male kestrels aged 1–8 yr originally reared from captive-raised adults. Twenty-four kestrels were removed from same-sex communal flight pens and placed singly in the previously described outdoor pens. Individual birds were separated from one another by six empty pens (21 m) to ensure that birds could not hear one another, and birds were allowed to habituate to the pens for 4 d before the initiation of the study. The sampling design was completely randomized, and a maximum of four birds were sampled per day, allowing all birds to be sampled within 6 d. All birds were fed a diet of day-old cockerels ad lib. during the sampling.

Blood Sampling and General Procedure

In the first experiment, a group of 10 chicks was blood sampled using a standardized method (described below) at the ages of 10, 16, and 28 d posthatching, between 1000 and 1500 hours, hereafter described as the "handled" group. Three additional groups of 10 chicks each were blood sampled from one age class only, at either 10, 16, or 28 d for comparison, hereafter described as the "unhandled" group. This study therefore involved a total of 40 chicks. In order to control for sibling variation in adrenocortical function (Schwabl 1999; Love et al. 2003b), only the first-hatched chicks of each nest were sampled during this experiment. Birds underwent a standardized handling and restraint protocol known to elicit an increase in the circulating hormone corticosterone (Wingfield et al. 1992). Briefly, chicks were removed from their nest boxes, and a stopwatch was used to record the time of first contact when the nest box door was opened. Birds were transported in 45 × 35 × 40-cm (l × d × h) insulated plastic coolers with 10-mm holes drilled for ventilation. Ten-day-old chicks were kept warm with the use of a hot-water bottle covered with a towel placed at the bottom of the cooler. From 16 d and older, opaque mesh tops were used on the coolers after chicks had been brought in from the breeding pens to facilitate capture of the bird for subsequent blood samples.

Individual birds were first weighed to determine maximal

amounts of blood for initial and subsequent samples. Between 25 and 75 μL (depending on the age of the chick) of whole blood was collected from the brachial vein as soon as possible after removal of the bird from its pen (∼1–2 min) using a heparinized 27-gauge needle and 1-cc syringe. Additional samples were collected at 5, 10, 30, and 45 min after capture from the same bird. All initial samples were taken in under 2 min following capture in this study, and linear regression analyses showed no effect of time after capture (within 0–2 min interval) on plasma levels of corticosterone in initial blood samples ($P = 0.38$). Thus, initial blood samples were considered to reflect baseline levels of corticosterone. Whole blood samples were centrifuged at 10,000 rpm for 10 min. The plasma was removed and stored frozen at $-20°C$ for radioimmunoassay analysis.

In the second experiment, adult kestrels underwent the same handling and restraint procedure as the chicks did in experiment one. Accurate records of all kestrels housed at the ASCC allowed for the precise determination of the number of times a particular bird had previously been handled and sampled. This allowed for the comparison of corticosterone levels and the number of handling episodes experienced by a particular kestrel. Blood sampling of individual birds took place on average 35 ± 4 d (mean $\pm$ SE) since the bird's last handling event. All testing took place on fasted birds between 1000 and 1300 hours. Birds were removed from their pens using large scoop nets, and a stopwatch was used to record the time of first contact when the pen door was opened. Birds were transported in 8.5-cm diameter opaque tubes closed at one end from the outdoor pens to a holding room. Individual birds were weighed, and approximately 75 μL of whole blood was collected from the brachial vein on removal of the bird from its pen (<2 min) using a heparinized 27-gauge needle and 1-mL syringe. An additional blood sample was collected at 10 min after capture. As with the first experiment, all initial samples were taken in under 2 min following capture, and linear regression analyses showed no effect of time after capture (within 0–2 min interval) on plasma levels of corticosterone in initial blood samples ($P = 0.53$). Thus, initial blood samples were considered to reflect baseline levels of corticosterone. Between blood collections, adult birds were placed individually in wooden opaque holding boxes containing a perch and wood shavings. Individual birds were unable to hear or see other test birds. Whole blood samples were centrifuged and stored as described for chicks.

Unflattened wing chord and tarsus length were measured in all birds following the sampling period and combined with mass to provide a measure of body condition. To calculate mass corrected for body size, we first calculated the scores of a principal component analysis (PCA) based on unflattened wing chord and tarsus length of all birds. The scores from the first principal component were used as an estimate of skeletal body size. We then regressed body mass against these PCA scores and used the residuals from this regression as an estimate of mass corrected for body size and included them as a covariate in an ANCOVA.

Corticosterone Radioimmunoassay Analysis

Plasma levels of corticosterone were measured using a specific radioimmunoassay (RIA) at the National Wildlife Research Centre, Hull, Quebec. Plasma samples were thawed, vortexed, and diluted at a 1 : 10 ratio of plasma to steroid diluent using RIA kits (ICN Biomedicals, Costa Mesa, Calif., cat. no. 07-120103) designed for mouse and rat corticosterone analysis. Kits were validated in-house for avian plasma using standard RIA analysis techniques described in Wingfield et al. (1982). Briefly, we used dextran-coated charcoal to strip the plasma and then determined plasma corticosterone interferences by measuring a range of plasma while (1) keeping the plasma volume constant and (2) with the plasma volume varied. We also ran range-finding experiments to determine the dilutions of stress-induced and baseline plasma, which generated corticosterone concentrations in the linear portion of the standard curve. Finally, we checked the inter- and intraassay variation using a Herring gull (*Larus argentatus*) plasma pool. Recovery values ranged from 85%–90% and were used to adjust assayed concentrations of corticosterone. Quality-control samples were run with every set, and intra- and interassay coefficients of variation were 9.4% and 11.6%, respectively.

Statistical Analysis

Due to the fact that we wished to examine the effects of multiple handling events on corticosterone secretion, the experimental design was naturally unbalanced since handled chicks were sampled multiple times through development, whereas unhandled chicks were only sampled at one developmental stage. To compare baseline (T0) and stress-induced (taken 10 min later = T10) corticosterone levels, we therefore utilized Student's *t*-tests (paired and unpaired, where appropriate) for comparisons involving two groups, ANOVA for developmental comparisons within the unhandled group and repeated-measures ANOVA for developmental comparisons within the handled group. We used the Bonferroni procedure (Rice 1989) to correct the *P* level of significance ($P = 0.013$) for post hoc comparisons within the handled and unhandled groups. In all other cases, probability values less than or equal to 0.05 were considered statistically significant. During statistical analyses, initial data were tested for homoscedasticity required by a parametric statistical test according to Sokal and Rohlf (1995). Preliminary analysis indicated that baseline and stress-induced corticosterone did not vary with time of day, sex, or condition. We therefore excluded these variables from subsequent analysis.

Table 1: Mean (±SE) plasma concentrations of baseline (T0) and stress-induced (T10) corticosterone (ng/mL) in three age classes of captive American kestrel chicks either unhandled (U) or handled (H)

	10U	10H	P	16U	16H	P	28U	28H	P
T(0)	1.24 ± .21	1.25 ± .24	.86	1.84 ± .34	1.68 ± .26	.37	3.78 ± .78	1.97 ± .51	.016
T(10)	7.42 ± .74	7.61 ± .68	.94	10.51 ± 1.11	8.21 ± .98	.006	19.25 ± 2.13	12.45 ± 1.79	<.0001

Note. The numbers 10, 16, and 28 are days posthatching, and P refers to the level of significance for age pairwise comparisons.

Results

Developing Chicks

Both handled and unhandled chicks showed increases in baseline (T0) corticosterone levels during postnatal development (repeated-measures ANOVA: $F_{2,36} = 5.50$, $P = 0.008$; Table 1; Fig. 1). There was a significant difference between treatments in T0 corticosterone levels during development ($F_{1,18} = 11.6$, $P = 0.003$; Table 1; Fig. 2A). While there was no significant difference of T0 levels between unhandled and handled chicks aged 10 and 16 matched for age (unpaired Student's t-test: $t = -1.235$, $P = 0.86$ and $t = -0.914$, $P = 0.37$, respectively; Table 1; Fig. 2A), unhandled chicks aged 28 d had significantly higher T0 levels than handled chicks of the same age (unpaired Student's t-test: $t = -1.532$, $P = 0.16$; Table 1; Fig. 2A). Fur-

thermore, whereas T0 levels of chicks 28 d old were higher than chicks at both 16 and 10 d within the unhandled group ($P = 0.01$ and $P = 0.001$, respectively; Fig. 2A), T0 levels were not significantly different in the handled groups ($P = 0.21$ and $P = 0.34$, respectively; Fig. 2A).

Both handled and unhandled chicks showed increases in stress-induced (T10) corticosterone levels during postnatal development (repeated-measures ANOVA: $F_{2,36} = 36.325$, $P < 0.0001$; Table 1; Fig. 1). There was a significant difference between treatments in T10 corticosterone levels during development ($F_{1,18} = 22.0$, $P = 0.0002$; Table 1; Fig. 2B). While there was no significant difference of T10 levels between unhandled and handled chicks aged 10 d (unpaired Student's t-test: $t = 0.341$, $P = 0.94$; Table 1; Fig. 2B), T10 levels were significantly higher in unhandled chicks aged 16 and 28 d compared with
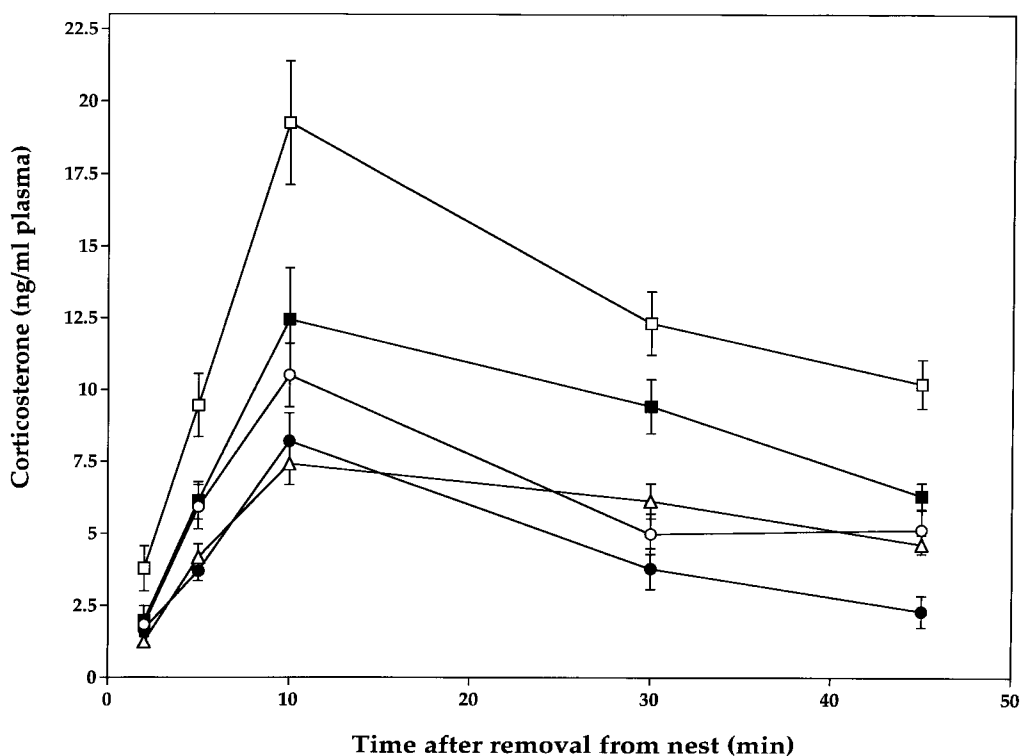

Figure 1. Plasma profiles of corticosterone secretion (mean ± SE) over time in response to restraint and sampling in captive American kestrel chicks during postnatal development, resulting from either no previous restraint and sampling (unhandled) or previous restraint and sampling (handled). Ten-day-old unhandled chicks (*open triangles*), 16-d-old unhandled chicks (*open circles*), 16-d-old handled chicks (*filled circles*), 28-d-old unhandled chicks (*open squares*), and 28-d-old handled chicks (*filled squares*).
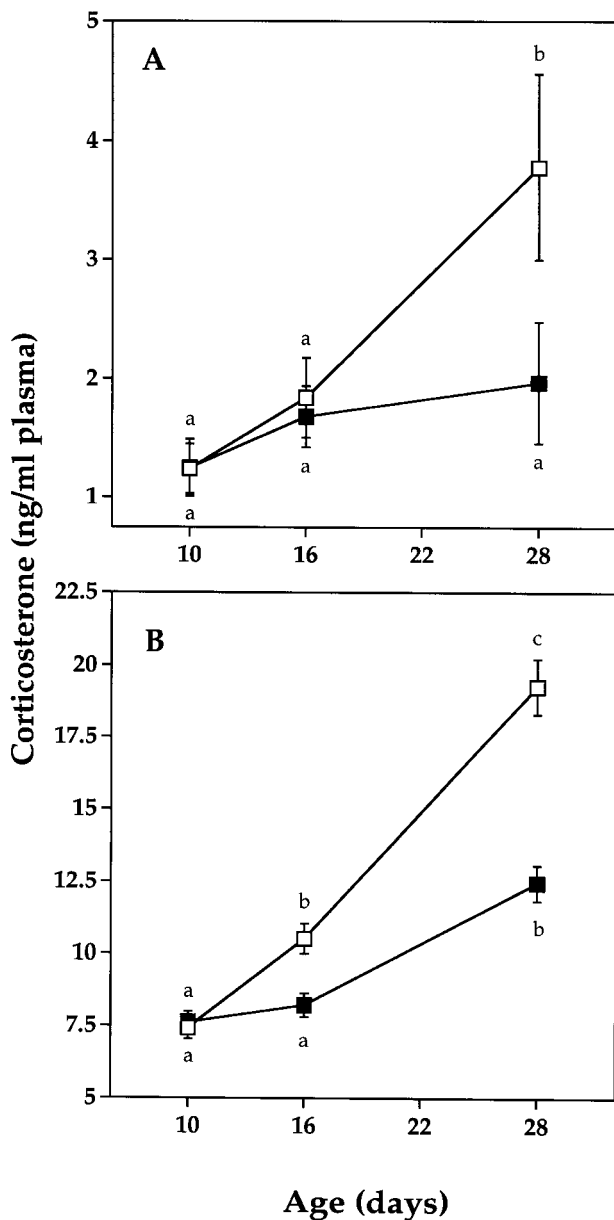
Figure 2. Baseline (T0; *A*) and stress-induced (T10; *B*) corticosterone levels in captive American kestrel chicks during postnatal development, resulting from either no previous handling (unhandled, *open squares*) or previous handling (handled, *filled squares*). Points with at least one letter in common were statistically indistinguishable (*P* > 0.05).

handled chicks of the same age (unpaired Student's *t*-test: *t* = 5.331, *P* = 0.006 and *t* = 7.224, *P* < 0.0001, respectively; Table 1; Fig. 2*B*). T10 levels of chicks 28 d old were higher than chicks 16 d of age within each group (both *P* < 0.01; Fig. 2*B*).

Adult Birds

Adult male kestrel T0 corticosterone levels showed no significant relationship with the number of capture and handling episodes birds had undergone (linear regression: *P* = 0.15; Fig. 3*A*). However, adult male stress-induced T10 corticosterone levels showed a significant negative relationship with the number of capture and handling episodes, which leveled off as the number of handling events increased (second-order polynomial regression analysis: $r^2 = 0.71$, $P < 0.0001$; Fig. 3*B*). Linear regression analysis of this relationship revealed that it did not explain the relationship as well ($r^2 = 0.62$, $P < 0.001$). Finally, regression analysis revealed that baseline and stress-induced corticosterone levels were independent of the age of the adult birds used in this study ($P = 0.35$ and $P = 0.22$, respectively).

Discussion

Our results indicate that the number of times a bird is restrained and blood sampled significantly affects baseline and stress-induced corticosterone levels in captive American kestrels. Even during relatively early postnatal development, birds sampled on only one previous occasion exhibited lower stress-induced corticosterone levels compared with previously unsampled chicks. This disparity increased when chicks were restrained and blood sampled on another occasion, exhibiting even lower than expected levels of corticosterone at 28 d of age and being comparable with those of unsampled, middevelopment chicks. Furthermore, adult male kestrels exhibited a significant negative relationship between the number of handling and blood sampling episodes and stress-induced corticosterone levels. To our knowledge, this is the first experimental study that shows that even young developing birds can show a decreased corticosterone response in relation to multiple restraint and sampling events.

Corticosterone Levels in Postnatally Developing Chicks and Adults

Our hypothesis predicted that baseline (T0) corticosterone levels would not differ significantly between handled and unhandled chicks, given the fact that baseline levels are generally responsible for the maintenance of homeostasis (Wingfield 1994) and may therefore not be affected by habituation to handling. It was therefore somewhat surprising to find that although young and middevelopment chicks were similar in both groups, T0 levels were significantly affected by researcher sampling by 28 d of age. Moreover, it is important to note that T0 levels of unhandled chicks aged 16 d and handled chicks aged 28 d showed no significant difference, with the result that chicks at fledging handled only twice previously showed developmentally stunted baseline corticosterone secretion. In contrast to our hypothesis concerning the effects of handling on T0 levels, we did expect to see the effects of habituation on
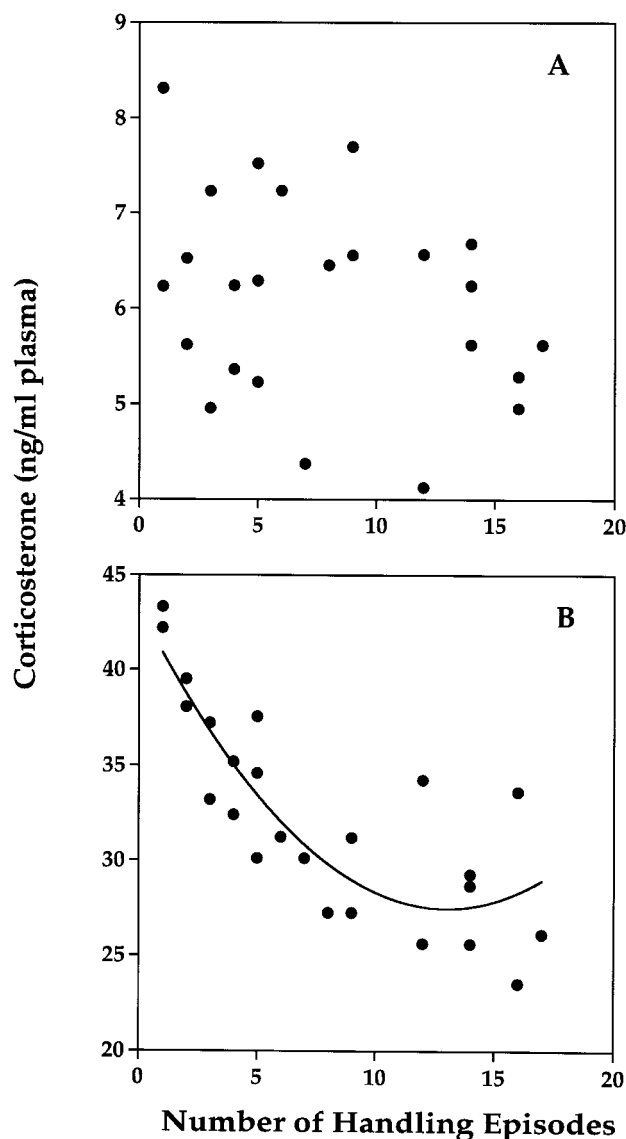
Figure 3. Baseline (T0; *A*) and stress-induced (T10; *B*) corticosterone levels in captive American kestrel adult males in relation to the number of capture, handling, and sampling episodes individual birds had undergone.

stress-induced (T10) corticosterone levels since the birds are potentially habituating to the perception of the severity of the stressful event, in this case capture and handling. Indeed, unhandled chicks aged 16 and 28 d exhibited higher T10 levels than handled chicks of the same age. This appears to show that even one prior restraint and sampling event as early as 10 d of age, when most muscular and neuromuscular development has only just begun in kestrels (Bird and Palmer 1988), causes habituation to handling in subsequent sampling periods. In conjunction with T0 levels, unhandled chicks aged 16 d showed similar T10 levels to handled chicks aged 28 d, resulting in

handled chicks at fledging exhibiting developmentally stunted stress-induced corticosterone secretion. Unlike chicks, adult male kestrels showed no significant relationship between T0 corticosterone levels and the number of restraint and sampling episodes. However, as with postnatally developing chicks, adult male kestrels showed a significant negative relationship between T10 levels and the number of restraint and sampling episodes.

Habituation to Handling in Small Mammals

It is already well established in mammalian research that habituation to handling can alter responses to stress in adult (Kazushige et al. 2001; Bernatova et al. 2002) and developing small mammals (reviewed in Meaney et al. 1993; Liu et al. 1997; Francis and Meaney 1999) and even adult large mammals (Andrade et al. 2001). Observed effects have included the reduction of adrenal corticosterone responses and reduced pituitary adrenocorticotropic hormone (ACTH) secretion of adult Norway rats (*Rattus norvegicus*) of those handled versus unhandled during early development (Bhatnager et al. 1995; Vallée et al. 1997; Francis and Meaney 1999). These differences were even apparent as late as 24–26 mo of age (Meaney et al. 1988; Meaney et al. 1991). Furthermore, handling effects have also been shown to modify the number and type of hormone receptors found in specific brain areas of offspring from handled mothers (Caldji et al. 2000), and these effects can also affect developmental trajectories of young in subsequent generations (Fleming et al. 1999; Francis et al. 1999). Based on studies of human and maternal handling of young small mammals, researchers have even hypothesized that individual differences in maternal handling of young may contribute to intraspecific variation in adrenocortical activity in small mammals (Meaney et al. 1993; Liu et al. 1997). Postnatally handled animals show enhanced glucocorticoid negative-feedback sensitivity compared with nonhandled rats (Meaney et al. 1988; Viau et al. 1993) and therefore decreased hypothalamic CRH (corticotropin-releasing hormone) and AVP (arginine vasopressin) mRNA expression, as well as lower levels of both CRH and AVP immunoreactivity (Plotsky and Meaney 1993; Francis et al. 1996). This handling effect on feedback sensitivity is mediated by an increase in glucocorticoid receptor expression in the hippocampus (Meaney et al. 1985; Sarrieau et al. 1988; O'Donnell et al. 1994), a region that has been strongly implicated in glucocorticoid negative feedback regulation in mammals (Jacobsen and Sapolsky 1991).

Habituation in Birds

The effects of handling on phenotypic variation in adrenal responsiveness are less well understood in birds, although research in lizards has indicated that corticosterone may act as a modulator of offspring phenotype (Sinervo and DeNardo 1996). However, in birds, captivity and handling are already

understood to have a profound effect on the function of the hypothalamic-pituitary-adrenal (HPA) axis (Marra et al. 1995; Romero and Wingfield 1999) and other physiological endpoints such as heart rate and core body temperature (Cabanac and Guillemette 2001). Despite the fact that handling is known to have a significant effect on adrenal responsiveness in mammals, many studies in birds utilizing multiple handling episodes of the same birds have failed to examine this potentially confounding variable. Romero and Remage-Healey (2000) noted that a decrease in corticosterone levels in molting starlings sequentially sampled in relation to season may have been a by-product of habituation to the handling procedure. Piersma and Ramenofsky (1998) noted that the longer captive red knots spent in an artificial aviary environment (where the same birds were blood sampled every 4–6 wk over a 1-yr period), the lower the concentrations of plasma corticosterone became. Cockrem and Silverin (2002), in a study examining repeatability of the corticosterone response in great tits, noted that although baseline corticosterone levels were similar on three bleeding occasions, the magnitude of the corticosterone response decreased from the first to the third sampling episode. The authors emphasized that this indicated a possible habituation to the stressor of capture and handling in the captive birds. Even more surprising is the fact that even wild Magellanic penguins in breeding colonies exposed to high levels of human visitation have been shown to exhibit decreased baseline and stress-induced corticosterone levels compared with birds breeding in isolated colonies (Fowler 1999).

Conclusion

We are aware that effects of handling are of little concern in most studies of wild birds, since these most often involve the sampling of birds during one episode only. However, in captive studies and those of wild birds where recapture is possible (such as nest-box breeding species where chicks and adults are potentially easily accessible), habituation to handling may be an important factor to consider and can potentially confound the interpretation of data. It is unlikely that short-term habituation to a restraint and sampling protocol of adult birds has any effect on the adrenocortical response to events such as severe weather or food shortages since the HPA axis is already fully developed. However, as discussed earlier, mammalian research has already indicated that corticosterone secretion in offspring is highly dependent on the rearing environment. It has therefore been proposed that the environment can have significant and long-lasting effects on the HPA axis during offspring development. Further studies of the effects of the maternal environment in birds are necessary to understand the interaction between corticosterone and offspring phenotype.

Presently at least, it is understood that captivity significantly affects seasonal patterns of both baseline and stress-induced corticosterone levels (Romero and Wingfield 1999), and ha-

bituation to capture and sampling may be one factor inducing these differences. However, captive studies are necessary for the understanding of basic physiological pathways and mechanisms, and comparisons within captive populations can be very informative in themselves. We therefore suggest that researchers minimize the use of repeated restraint and sampling procedures in studies of adrenocortical function in birds. Since this is not always possible, especially in studies of captive birds, records of handling and sampling should be kept in order to control for this possible confounding effect. We suggest that future studies involving stress hormones should be designed to take habituation to handling and sampling into account.

Acknowledgments

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