Plasma Corticosterone in Nestling American Kestrels: Effects of Age, Handling Stress, Yolk Androgens, and Body Condition

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The effects of age, handling-induced stress, yolk androgens, and body condition on plasma corticosterone levels were investigated in free-living nestling American kestrels, Falco sparverius, a semialtricial falcon species. In an observational study, corticosterone levels varied with age and handling time. Specifically, corticosterone was low until age 15 days and then rose from age 20 through 25 days. Nestlings as young as age 10 days showed a handling-induced rise in corticosterone. Neither sex nor hatching order of the nestling affected corticosterone levels. Concentrations of maternally derived yolk androgens have previously been shown to be lower in first-laid than in later-laid eggs in the clutch. In an experimental study, androgens were injected into the yolk of the first-laid egg to elevate its levels to those of later-laid eggs, a treatment that substantially reduces nestling body condition compared with that of controls. Yolk androgen treatment elevated posthatching corticosterone levels compared with those of controls, and corticosterone levels were negatively correlated with body condition. These findings indicate that even very young, developing birds can show stress-induced increases in corticosterone and that age-related changes in corticosterone secretion may be modified by body condition and maternal effects such as yolk androgen deposition. The short- and long-term consequences of high glucocorticoid levels in young, developing vertebrates are largely unknown.

Key Words: avian growth; development; Falco sparverius; glucocorticosteroids; hypothalamic-pituitary-adrenal (HPA) axis; maternal effects; sibling competition; stress response.

Elevated glucocorticosteroid secretion promotes physiological and behavioral responses that enhance an adult animal’s ability to cope with stressors and other challenges to homeostasis (Wingfield, 1994). Less well understood is whether similar glucocorticosteroid-mediated responses occur during the developmental stages of life and how maternal effects such as embryonic exposure to maternal androgens influence these responses (Schwabl, 1999). Among the most salient challenges a developing vertebrate might experience is a shortage of parentally provided food, leading to energetic stress and reduced body condition and potentially affecting growth and development. Nests of altricial bird species can be highly competitive arenas, where, in some species, sibling competition for food routinely leads to siblicide (Mock et al., 1990). Food deprivation can elevate corticosterone secretion in young birds, including domestic ducks, Anas platyrhynchos (Harvey et al., 1980), nestling blue-footed boobies, Sula nebouxii (Núñez-de la Mora et al., 1996), and nestling black-legged kittiwakes, Rissa tridactyla

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Kitaysky et al. (1999) proposed that hunger-induced elevations in corticosterone secretion may enhance begging behavior for food and thereby restore body condition in altricial nestlings.

In the booby, the brood size is typically two chicks. As a result of hatching asynchrony, the older chick aggressively dominates its younger sibling, sometimes killing it when food availability is limited (Drummond et al., 1986). The subordinate chick has higher baseline levels of corticosterone, possibly because of the stress of chronic submission and the associated reduction in body condition (Nuñez-de la Mora et al., 1996). In the canary, Serinus canaria, however, the younger siblings of the asynchronously hatching brood generally have reduced baseline levels of corticosterone compared with those of their older siblings, although differences among siblings are small (Schwabl, 1999). Younger nestlings in the canary brood are exposed embryonically to higher levels of testosterone in the yolks of their eggs compared with those of their older siblings (Schwabl, 1993). This yolk testosterone increases nestling begging vigor and body condition, thus mitigating against the negative effects of hatching asynchrony (Schwabl, 1996) and possibly modifying corticosterone secretion. The purpose of this study was to investigate the effects of hatching order, body condition, and yolk androgens on basal and handling-induced stress levels of corticosterone in a semialtricial falcon species, the American kestrel, Falco sparverius.

In the kestrel, like canaries, concentrations of maternally derived yolk androgens vary among sibling yolks, with eggs laid late in the laying sequence of a clutch having significantly higher concentrations than first-laid eggs (Sockman and Schwabl, 2000). In contrast to the canary, experimental manipulations revealed that yolk androgens substantially reduce nestling body condition, as measured by age-specific changes in body mass (Sockman and Schwabl, 2000). In adult birds, both baseline and stress-induced levels of corticosterone negatively correlate with body condition (Schwabl et al., 1991; Ramenofsky et al., 1995; Schwabl, 1995; Jenni et al., 2000). Yolk androgens, which reduce body condition in nestling kestrels, may thus elevate plasma levels of corticosterone and corticosterone levels may depend, in part, on body condition.

### MATERIALS AND METHODS

#### General Field Procedures and Species

The standards of the Washington State University Institutional Animal Care and Use Committee (in accordance with the National Institutes of Health) for the humane treatment of the subjects were applied. Nest boxes (inside width × depth × height: 17.4 × 16.3 × 33.8 cm) were hung at approximately 0.5-km intervals along roadsides within a 35-km radius of the Washington State University campus (Pullman, WA, 46° 44′ N, 117° 10′ W). Originally a typical temperate steppe ecoregion (Bailey, 1998), this area has since undergone widespread conversion mainly to wheat, barley, and pea farming. Boxes were hung approximately 2–3 m high on trees or posts and provided with pine shavings to protect eggs from the hard floor. Boxes were checked every 3–4 days for signs of occupancy. During laying, boxes were checked every 1–3 days, and new eggs were marked to determine laying order. American kestrels usually lay every 2 days until the clutch is complete at 4–6 eggs, but laying intervals of 1 and 3 days sometimes occur. Incubation and nestling periods, each approximately 30 days, follow, after which nestlings fledge (Balgooyen, 1976; Sockman and Schwabl, 1998).

#### Observational Study

In spring and summer 1998, data were collected on the natural variation in plasma levels of corticosterone for each nestling over the course of its development. Beginning approximately 25 days after clutch completion, nests were checked for hatching once or twice daily, and new hatchlings were marked by clipping talons. Within broods, the first egg typically hatched approximately 2 days before the last. If multiple nestlings hatched between nest visits, their hatching order was determined by comparing the dampness of their down with that of siblings. Nestlings were banded when they were 15 days of age and sexed based on sexually dimorphic plumage.

Between 08:00 and 11:00, 100–400 µl blood was collected from nestlings’ wing veins every 5 days of age from 5 to 25 days. Blood was stored on ice for a few hours before centrifugation, and plasma was stored at −20°. Times were recorded for removal from
the nest box (the entire brood was removed each time any nestlings were removed) and collection of blood for each nestling. For each brood, the order of bleeding nestlings was random (only those that were 5, 10, 15, 20, or 25 days old were bled). The handling-induced stress response was quantified by analyzing corticosterone levels as a function of time from removal from the box to blood collection (see below). Following a standard stress-response protocol, in which repeated blood samples are collected at set times following capture, would not have been practical for this study. This is because individual nestlings could not have been bled without precipitating stress responses in siblings and because 1 or 2 min were usually required to collect the blood from an individual nestling. Although sample collection times tended to cluster around 0–5 min following capture, variation in collection times was sufficient to characterize the stress response (see Results).

**Experimental Study with Yolk Androgen Injections**

In spring 1999, within a few days of clutch completion, either 50 μl sesame oil (control) or 0.1 μg testosterone and 4 μg androstenedione dissolved together in 50 μl sesame oil (androgen-treated) was injected into the yolk of the first-laid egg in a clutch (Schwabl, 1996), mimicking in egg 1 the high maternal doses of each of these androgens found in later-laid eggs (see Sockman and Schwabl, 2000, for the methods used to calculate these concentrations). Throughout the laying season, androgen and control injections were alternated among clutches.

Beginning approximately 25 days after clutch completion, nests were visited every 1 or 2 days until pipping occurred in eggs, at which point nests were visited twice daily to check for hatching. A small drop of food coloring was applied to the pipped area of the shell and would seep into the egg through the pip. The eggs from which nestlings hatched were determined based on coloring of the nestlings, and talons were clipped for identification until nestlings were old enough to be banded (15 days). Colors were alternated among nests and eggs to control for potential effects of color. Blood samples were collected in the same manner as described above, and nestling body mass was measured. In nestling birds, body mass is primarily a function of age and can increase 10-fold or more over the kestrel’s 4-week nestling stage. But at any specific nestling age, body mass variation is largely a result of differences in growth rates, which depend on energy intake. Therefore, an age-specific measure of mass, termed body condition, was calculated (see below).

**Measurement of Plasma Corticosterone**

Corticosterone in plasma from the 1998 observational study was measured in one radioimmunoassay (intraassay coefficient of variation, 14.7%) and from the 1999 experimental study in three radioimmunoassays (interassay coefficient of variation, 6.8%, intraassay coefficients of variation, 8.6, 9.3, 10.3%), each with a sensitivity of 3.9 pg/tube. For all assays, tritiated corticosterone (2000 cpm) was added to each plasma sample for estimation of recoveries (mean, 68.5%), and samples were allowed to equilibrate overnight at 4°. Steroids were extracted with 2 × 4 ml of petroleum ether and diethylether (3:7 by volume) using Extrelut (EM Science, Gibbstown, NJ) minicolumns, and extracts were dried over a stream of N2. For the 1998 study and assays 1 and 2 in the 1999 study, corticosterone was separated and partially purified on diatomaceous earth chromatographic columns as described by Schwabl (1992). Corticosterone fractions were dried (under N2) and redissolved in phosphate-buffered saline (pH 7.1). For assay 3 in the 1999 study, chromatography was omitted, and after extracts were dried, samples were redissolved in phosphate-buffered saline. The remainder followed a previously published radioimmunoassay protocol (e.g., Schwabl, 1995).

**Statistical Analysis**

For both the observational and experimental studies, logarithmic transformation of corticosterone levels normalized otherwise nonnormal distributions. Therefore, all statistical analyses were conducted using log-transformed corticosterone levels but non-transformed values were used in the figures. For the observational study, analysis of covariance for repeated measures was used to determine whether plasma corticosterone (dependent variable), measured serially at ages 5–25 days (independent variable), varied according to handling time (covariate). Because multiple measures per nest (i.e., multiple nestlings) were available, the nest was included as a blocking
factor in the analysis (i.e., a second factor that is not of biological interest but which may contribute to variation in corticosterone levels), allowing among-nest variation to be removed. Because handling time was a significant factor (see Results), it was retained as a covariate in two subsequent analyses, one in which sex was added as a factor and the other in which hatching order of the nestling was added as a factor. Baseline corticosterone levels were not available. Rather, samples were collected at various times after capture and the time from capture to sample collection was noted (see above). Although these methods of analysis controlled for handling time, changes in corticosterone with respect to independent variables such as age or sex may reflect changes in baseline levels, changes in the stress response, or both.

For the experimental study, analysis of variance for repeated measures was used to determine whether corticosterone levels of the nestling hatching from the treated egg 1 (dependent variable), measured serially at ages 5–20 days (independent variable), varied according to experimental treatment (independent variable) in one model and according to nestling body condition (covariate) in another. Because of the strong covariation between body mass and age, it would be inappropriate to include both as independent variables in a single model. Therefore, an analysis of variance of body mass with age as an independent variable was conducted, and the residuals of this (thus removing age-related variation in body mass) were used in the above model. Body condition was defined as these residuals. In addition to $F$ and $P$ values, for covariates $r^2$ values were also reported to indicate the magnitude of their covariation with the dependent variable.

RESULTS

Observational Study

Plasma corticosterone levels in nestling kestrels strongly covaried with handling time ($F_{1,18} = 16.66$, $P = 0.0007$, $r^2 = 0.14$). A significant effect of the interaction between age and handling time ($F_{4,53} = 2.96$, $P = 0.028$) suggested that the magnitude of the handling-induced stress response varied with age. When each age was analyzed separately by linear regression, corticosterone was positively correlated with handling time at ages 10 ($F_{1,16} = 25.85$, $P = 0.0001$, $r^2 = 0.49$) and 15 ($F_{1,15} = 28.98$, $P < 0.0001$, $r^2 = 0.41$) days but not at ages 5 ($F_{1,16} = 2.71$, $P = 0.12$), 20 ($F_{1,12} = 1.53$, $P > 0.2$), or 25 ($F_{1,12} < 0.01$, $P > 0.2$) days (Fig. 1).

Plasma corticosterone also varied with nesting age ($F_{4,53} = 4.28$, $P = 0.0045$). According to post hoc analyses, corticosterone levels did not differ among 5-, 10-, and 15-day-old nestlings. Levels rose at age 20 days and continued to rise through age 25 days (Fig. 2).
Neither sex ($F_{1,16} = 1.10, P > 0.2$) nor hatching order ($F_{5,13} = 0.72, P > 0.2$) affected corticosterone levels when each was individually and separately included in the statistical model. Interactions between sex and handling time, sex and age, hatching order and handling time, and hatching order and age were not statistically significant.

Experimental Study with Yolk Androgen Injections

In an initial model with handling time and treatment as independent variables, neither handling time nor the handling time × treatment interaction term contributed significantly. Therefore, these terms were removed from the model. Plasma corticosterone levels in nestlings that hatched from androgen-treated eggs were higher than those from control eggs ($F_{1,16} = 3.81, P = 0.069$). The effects of the treatment were large, resulting in a greater than threefold increase in corticosterone levels in nestlings 10 days of age (Fig. 3). However, error variation in the androgen group was also large. The interaction between age and treatment was not significant. Day 25 values were not included in this model because only one nestling from an androgen-treated egg survived to age 25 days. The corticosterone values of the two treatments converge probably as a result of the gradual dying-off of nestlings with high corticosterone levels and their exclusion from the model rather than because levels in androgen nestlings failed to rise at 15 and 20 days as they did in control nestlings.

The effects of age on body mass were statistically removed to estimate body condition (see Materials and Methods). Yolk treatment was excluded from this model because it strongly covaries with the independent variable body condition. This permitted the inclusion of values from age 25 days in the analysis. Plasma corticosterone levels covaried with body condition in the full model in which nestlings from each age were included ($F_{1,20} = 4.59, P = 0.045, r^2 = 0.08$). Inspection of this relationship at each nesting age using linear regression (Fig. 4) revealed a negative correlation between body condition and corticosterone at ages 5 ($F_{1,14} = 4.85, P = 0.045, r^2 = 0.26$), 10 ($F_{1,13} = 5.00, P = 0.044, r^2 = 0.28$), 15 ($F_{1,11} = 15.52, P = 0.0023, r^2 = 0.58$), and 25 ($F_{1,7} = 6.13, P = 0.042, r^2 = 0.46$) days but not at age 20 days ($F_{1,9} = 1.07, P > 0.2$).

In summary, corticosterone levels changed according to nesting age, with lower levels at ages 5, 10, and 15 days rising to higher levels at ages 20 and 25 days. Nestlings as young as 10 days of age showed a handling-induced stress response, but nestling hatching order and sex did not affect corticosterone levels. Yolk androgen treatment elevated posthatching plasma corticosterone levels compared to those of nestlings hatching from control eggs; corticosterone levels in these nestlings negatively correlated with their body condition.

![FIG. 3.](https://example.com/fig3.png) Effects of yolk androgen treatment on posthatching levels of plasma corticosterone in American kestrels from age 5 to 20 days. Values represent the mean (with standard error) of nestlings hatching from eggs in which either androgens (open circles) or sesame oil (solid circles) were injected into the yolk. The number of nestlings analyzed at each age is indicated next to each point.
DISCUSSION

Effects of Nestling Age and Handling-Induced Stress on Plasma Corticosterone

Consistent with earlier research, the present study indicates that plasma corticosterone levels change developmentally. In the canary, corticosterone levels show a general rising trend from ages 5 to 10 days but then level off by age 15 days, the point immediately before fledging (Schwabl, 1999). Fledged juveniles tend to show higher levels than nestlings. Elevated corticosterone in older nesting kestrels (Fig. 2) may be associated with increased locomotor activity (see Astheimer et al., 1992) and may prepare nestlings for fledging (Heath, 1997).

Several lines of evidence over the past 20 years suggest that the stress response, typically triggered by sudden, threatening environmental events, may suspend the life history cycle in adult vertebrates and redirect behavior and physiology toward survival (reviewed in Wingfield et al., 1998). For example, in response to an acute energetic stressor, a sudden elevation in corticosterone may induce hyperphagia; or, in response to predator- or weather-related threats, the animal may flee and seek refuge (Astheimer et al., 1992). It is not clear, however, what purposes the stress response may serve in altricial nestlings. Nest-bound and entirely dependent on parentally provided food, they have little ability to redirect behavior toward survival (Sims and Holberton, 2000). The deleterious effects of elevated corticosterone levels on immune, reproductive, and brain function in young vertebrates (Sapolsky, 1992) further underscore why the occurrence of a stress response in young nestling birds is surprising.

Romero et al. (1998) did not observe a stress response in redpolls, Carduelis flammea, that were 1–2 weeks postfledging. Using a multifactor analysis, Sims and Holberton (2000) assessed the effects of handling time (0 and 30 min) and age (birds of several age classes from 5–7 days to adults) on corticosterone levels in northern mockingbirds, Mimus polyglottos. They found that handling time, age, and their interaction have statistically significant effects on corticosterone. However, it is not clear from their analysis whether the handling-induced levels of plasma corticosterone in birds age 5–7 days were significantly higher than baseline levels. Newly hatched domestic fowl, Gallus domesticus, and ducks elevate corticosterone secretion in response to mild restraint stress (Holmes et al., 1992). These newly hatched precocial birds are fairly advanced developmentally compared to altricial or semialtricial species during much of the nestling stage. Findings that the semialtricial kestrel as young as 10 days showed a significant handling-induced stress response (Fig. 1) were surprising because 10-day-old kestrels have completed only about one-
third of their nestling development. To our knowledge, this is the earliest developmental stage at which a stress response has been shown in a bird species. The absence of a stress response at ages 20 and 25 days was puzzling. It is possible that the rate of corticosterone secretion approaches a maximum by this time (Fig. 2) and that these high levels facilitate fledging behavior (Heath, 1997). Further rises in corticosterone secretion induced by handling may not be possible.

**Effects of Yolk Androgens and Nestling Body Condition on Plasma Corticosterone**

In American kestrels, yolk androgens reduce body condition to such an extent that 15-day-old nestlings hatching from androgen-treated eggs were approximately the mass of 10-day-old controls (Sockman and Schwabl, 2000). This 5-day lag in growth, a period one-third of the nestlings life at that age, quickly gives rise to increased mortality. Results from the present study now suggest that yolk androgens elevate post-hatching plasma corticosterone levels. It is possible that the large variation in corticosterone levels in nestlings from androgen-treated eggs was due to the sex of the nestling. Only two males in the androgen group survived long enough to be sexed, precluding a statistical analysis with sex as a factor. However, corticosterone levels in these males were substantially higher than levels in females from androgen-treated eggs (data not shown). The fact that corticosterone levels negatively correlated with body condition suggests that the effects of yolk androgens on corticosterone levels may be mediated by the very strong, deleterious effects of yolk androgens on body condition shown previously. This relationship between body condition and corticosterone in nestling kestrels is similar to those of other studies in which experimental food restriction caused a reduction in body mass or fat reserves and an elevation of corticosterone levels (Nuñez-de la Mora et al., 1996; Kitaysky et al., 1999). Others have not found a relationship between baseline corticosterone levels and energetic condition in young birds (Schwabl, 1999; Sims and Holberton, 2000), including the American kestrel (Heath, 1997; Heath and Dufty, 1998). It is possible that only very large reductions in body condition, as were induced by yolk androgen treatment and experimental food manipulations, were sufficient to elevate corticosterone.

Because the relationship between body condition and corticosterone is only correlational, whether the change in corticosterone is caused by the change in body condition is not known. Indeed, the reverse may be true. Elevated glucocorticosteroids can deplete protein reserves by enhancing gluconeogenesis (Sapolsky, 1992; Wingfield, 1994). Synthetic glucocorticosteroids inhibit growth in developing domestic fowl (Leili and Scanes, 1998). Additionally, variation in levels of corticosterone-binding protein may influence the availability of corticosterone for receptors. Future studies in which either body condition or corticosterone is directly manipulated may clarify the relationship between these variables. Studies involving corticosterone-binding proteins may reveal how age, yolk androgens, and body condition interact to influence the physiological consequences of changing levels of plasma corticosterone.

Elevated levels of glucocorticosteroid hormones during development may have long-lasting effects on reproductive strategy (Moore et al., 1998), learning, and brain development (Sui et al., 1997). The fact that very young birds elicit a stress response and that age, yolk androgens, and body condition may all modulate corticosterone secretion make the short- and long-term consequences of elevated glucocorticosteroid levels in young, developing vertebrates of particular interest.

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