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Abstract

I compared the effects on plasma corticosterone levels of two methods of collecting blood samples during standardized capture and handling stress protocols. In one method, individual nestling American Kestrels (*Falco sparverius*) were bled at three time periods: when initially removed from the nest, and 15 and 30 min later. In the other method, siblings removed from a nest were bled once each, either at the time of removal, or 15 or 30 min later. I found that there was no difference between the two groups in plasma corticosterone levels at the first sampling period, but 15 and 30 min after capture the singly-bled birds had significantly higher plasma corticosterone levels than the multiply-bled nestlings. The results suggest that data from multiply-bled birds underestimate actual circulating hormone levels. The underlying mechanism for this phenomenon is unknown, although it may involve hemodilution.

Key words: American Kestrel, stress, corticosterone, hemodilution, nestlings

Organisms experiencing stressors typically secrete glucocorticoids to maintain or restore homeostasis (Wingfield and Romero 2001). The pattern of corticosterone secretion in response to a standardized stressor frequently is used to compare how different organisms respond to disruptive events. For birds, the most common technique is to use capture and handling stress to activate the hypothalamic-pituitary-adrenal (HPA) axis, with the sensitivity of the axis to the stressor determined through repeated blood sampling followed by radioimmunoassay (RIA) of the blood samples for corticosterone. Birds are held in a cloth bag between sampling sessions. This protocol facilitates comparison of adrenal responsiveness within and among species, and the results of such studies have enhanced our understanding of how ecological factors modulate stress responses (Wingfield et al. 1998, Wingfield and Romero 2001).

The importance of a standardized approach in collecting blood samples to measure adrenoresponsiveness has been acknowledged, either explicitly or implicitly, in myriad papers. However, subtle, but possibly important, procedural differences occur among studies that use this protocol. For example, in many studies morphological measurements are taken after the last sampling period, so that the only time birds are handled is when blood samples are taken (Wingfield et al. 1992, Silverin et al. 1997, Blas et al. 2005). In contrast, sometimes morphological measurements are taken in between blood samples, when the birds would otherwise be in a cloth bag (Meddle et al. 2003, Adams et al. 2005). Similarly, occasionally birds are held in small cages in between sampling periods (e.g., Love et al. 2005) rather than in cloth bags. Furthermore, different trapping methods may be utilized in the same investigation (e.g., Lynn et al. 2003). In addition, in most studies similar amounts of blood are removed at each sampling period, but in others the initial blood sample may be larger, if additional hematological measurements are performed (e.g., Clinchy et al. 2004). Finally, the same individual is sampled repeatedly in many studies (Wingfield et al. 1992, Silverin et al. 1997, Love et al. 2003), while, in others, each bird is bled only once during the capture and handling period (Sockman and Schwabl 2001, Quillfeldt et al. 2004, Brown et al. 2005).

The possible effects of methodological differences in the standardized protocol have rarely been examined directly, yet there is evidence that they may be significant. For example, Canoine et al. (2002) found that the stress response induced by restraint in a cage is greater than that induced by restraint in a cloth bag. Romero and Romero (2002) found that onset of the stress response in birds is influenced by trapping technique. Kannan and Mench (1996) noted that how a bird is handled prior to blood sampling affects corticosterone levels. Since the point of using a standardized capture and handling protocol is that it allows comparisons to be made among investigations, it is important to know which aspects of the protocol can or cannot be modified without affecting the results.

My study was conducted to investigate whether one of these factors, the number of times a bird is sampled during the capture and handling protocol, affects plasma corticosterone levels. I compared the adrenal responses of singly- versus multiply-bled nestling American Kestrels (*Falco sparverius*) to a standardized capture and handling protocol. If the process of blood sampling itself modifies the stress response, then a nestling taken from its nest and bled only once (e.g., 10 or 20 min after removal from the nest) may exhibit a different trajectory of corticosterone secretion than a bird taken from its nest and handled and bled repeatedly.

Methods

Study area and species.—This investigation was performed with the approval of the Boise State University Institutional Animal Care and Use Committee. The study population consisted of American Kestrels breeding in nest boxes affixed to telephone poles on roadsides between Meridian and Kuna, ID. Nest boxes (N = 48; inside dimensions: width x depth x height = 19.1 cm x 18.1 cm x 43.8 cm) were 2-3 m off the ground and most were 0.8-1.6 km from the nearest neighboring box. The study area (43° 5' N, 116° 4' W) is primarily agricultural land and pastures, with occasional housing clusters.

Nest boxes were checked irregularly for occupancy, eggs, or nestlings beginning in April, and almost daily starting on 9 May. Nestlings discovered post-hatch were aged using a photographic guide (Griggs and Steenhof 1993). American Kestrel clutches hatch asynchronously (Smallwood and Bird 2002), and the hatch date for a nest was defined as the hatch date of the oldest nestling.

Blood sampling procedure.—Blood samples were collected when nestlings were 10-12 days old, a time when the American Kestrel HPA axis is functional, if not fully mature (Sockman and Schwabl 2001, Love et al. 2003). Nestlings cannot be sexed at this age, so for this and all subsequent analyses the sexes were combined. To produce the proposed comparison between multiply-bled and singly bled birds, one nestling from each clutch was handled and bled three times, while all others were handled and bled only once, as follows: During a test, all nestlings were taken from the nest box and placed in a white, plastic, 5-gallon pail. One bird, selected at random, was bled multiple times: immediately after removal from the nest (referred to as Time 0) and again 15 and 30 minutes later (Time 15 and Time 30, respectively). All other young from the same nest were bled once, as follows: if two additional young were present, then one was bled at Time 0 and the other at Time 15. If there were three additional young, then one each was sampled at Times 0, 15, and 30, respectively. If there were four additional nestlings, then birds were sampled at Times 0, 15, 25 (data not shown), and 30. One clutch contained only one additional bird; it was bled at Time 15. Finally, body mass measurements were collected from all birds after all blood samples had been collected.

The order of bleeds was initially determined by a coin toss and alternated thereafter. For example, if the multiply-bled bird in a nest was the first individual sampled at Time 0, then it was sampled second at Time 15 and first again at Time 30. All blood samples were collected within five min (mean \pm SE = 2.2 min \pm 0.1) of the designated time period and were collected between 1000 h and 1430 h. The samples likely reflect baseline and stress-induced plasma corticosterone levels, even if somewhat imprecisely (Romero and Reed 2005). However, because the order of bleeding alternated from one sample to the next, variability within a time period was distributed equally between the two groups. Each sample was collected after puncturing the wing vein with a 26-gauge needle. I halted the flow of blood by applying pressure to the wound with a small piece of cotton. Samples were collected in 2 - 6 heparinized microhematocrit tubes (mean \pm SE = 3.9 tubes \pm 0.15) that were sealed with clay and stored on ice until I returned to the laboratory, where the tubes were centrifuged and the plasma harvested and stored in microcentrifuge tubes at -20° C until assayed.

Corticosterone radioimmunoassay.—Corticosterone was measured directly from plasma in a single radioimmunoassay, after Wingfield et al. (1992). In brief, to sample test tubes containing approximately 50 μ l plasma (mean \pm SE = 49.3 μ l \pm 0.35; range 29 - 50 μ l) I added 20 μ l of labeled corticosterone. I also added labeled corticosterone to each of two test tubes containing 1000 pg corticosterone. Corticosterone was extracted in 4 ml of freshly distilled dichloromethane, dried under nitrogen gas, and resuspended in 550 μ l of buffer. Two-hundred microliters of this solution was added to each of two assay tubes and

another 100 µl was added to a scintillation vial. Scintillant (4 ml) was added to the latter vials, which provided an estimate of the percentage of steroid recovered after extraction. Mean recovery was 88%. A standard curve (range: 2000 pg to 7.8 pg) was prepared in duplicate assay tubes. To each sample and standard curve tube I added 100 µl of tritiated corticosterone and 100 µl of corticosterone antibody (Endocrine Sciences). This equilibrated overnight at 4° C. I added Dextran-coated charcoal (500 µl) to all samples and to the standard curve. Ten minutes later, tubes were centrifuged at 2000 rpm for an additional 10 minutes. The supernatant was decanted into scintillation vials, scintillant was added, and the vials were vortexed. The vials were counted on a Beckman LS-6800 scintillation counter the following day. Nonspecific binding of the antibody was less than 5%. Two water blanks measured undetectable levels of corticosterone. The two 1000 pg corticosterone standards averaged 960 pg ± 17.5 SE.

Hematocrit values.—Eighteen birds from 10 nests used in the first part of this study were bled again about two weeks later, at approximately 27 days of age. Values for birds from the same nest were averaged. All birds were bled three times, at T0, T15, and T30, with blood collected in 2-6 heparinized microhematocrit tubes. Tubes were centrifuged for 60 sec in a hematocrit centrifuge that also was used to measure hematocrit levels from one microhematocrit tube for each bird at each collection time. Hematocrit measurements for singly bled birds were obtained from the heparinized microhematocrit tubes collected from the 10 – 12 day old birds sampled for corticosterone either at T0, T15, or T30 (see above). Hematocrit levels were compared within, but not between, the two groups.

Statistical analyses.—Plasma corticosterone levels of multiply- and singly-bled birds in a given nest were compared using paired *t*-tests for each of the three time periods. That is, the plasma corticosterone level measured at T0 for each multiply-bled individual was paired with that of its singly-bled sibling that was bled at T0. Similarly, the T15 and T30 values for multiply-bled birds were paired with the values from siblings bled at T15 and T30, respectively.

Hematocrit levels measured from blood collected at T0, T15, and T30 were compared by repeated-measures ANOVA. Hematocrit levels measured from blood collected at T0, T15, or T30 were compared with a one-way ANOVA. Data are presented as means ± SE, and significance values are set at $P < 0.05$.

Results

The results of the paired *t*-tests are shown in Figure 1. Plasma corticosterone levels in singly- and multiply-bled individuals were similar at T0 (singly-bled: 5.43 ng/ml ± 1.25; multiply-bled: 5.34 ng/ml ± 1.52; paired $t = 0.06$, 14 df, $P > 0.4$). At T15 there was a significant difference in plasma corticosterone titers between singly- and multiply-bled kestrel nestlings, with singly-bled birds exhibiting higher levels than multiply-bled birds (singly-bled: 12.71 ng/ml ± 1.41; multiply-bled: 10.22 ng/ml ± 1.51; paired $t = 2.152$, 15 df, $P < 0.025$). This same pattern continued at T30 (singly-bled: 11.39 ng/ml ± 2.16; multiply-bled: 8.91 ng/ml ± 2.19; paired $t = 2.569$, 12 df, $P < 0.025$).

Hematocrit values changed significantly over time in multiply-bled birds ($F_{2,18} = 23.90$, $P < 0.0001$). Mean ± SE hematocrit levels declined over the three time periods, from 42.9% ± 1.1 at T0 to 39.6% ± 0.7 at T15 and to 38.1% ± 0.9 at T30 (Figure 2A). There were no differences among hematocrit levels in birds bled once, either at T0 (39.73% ± 0.83), T15 (40.4% ± 0.93), or T30 (38.6% ± 1.00) ($F_{2,42} = 0.97$, $P = 0.386$; Figure 2B).

Discussion

The handling-induced corticosterone titers I report are lower than those commonly found in adult birds, but adrenoresponsiveness in nestlings is less than that of adults (e.g., Sims and Holberton 2000), and my results are consistent with values reported by others for nestling kestrels (Sockman and Schwabl 2001, Love et al. 2003). These data suggest that plasma corticosterone levels measured using the standard capture and handling protocol can be affected by the number of times a bird is sampled, with multiply-bled birds exhibiting lower corticosterone levels than singly-bled birds. Therefore, corticosterone levels measured from multiply-bled birds may underestimate the plasma hormone levels in unbled birds. Consequently, this factor must be considered when comparing data among studies. This is but one of several seemingly

innocuous variations that occur within the nominal “standard” protocol. Other variables, such as when morphological measurements are taken, how the birds are maintained between samples, and the size of the blood samples may also prove to be important and deserve to be examined systematically.

The finding that multiply-bled birds had significantly lower plasma corticosterone levels than singly-bled birds sampled 15 or 30 min after the onset of capture/handling stress is counterintuitive, because a bird handled and bled several times would be expected to experience more stress, and to secrete more corticosterone, than a bird sampled only once. The mechanism for this unexpected result is unclear and the phenomenon deserves closer examination.

One possible contributing factor is hemodilution following hemorrhage, which would reduce the plasma concentration of corticosterone in multiply-bled birds. Hemodilution occurs when transcappillary interstitial fluid reabsorption replaces fluids lost by hemorrhage, restoring blood volume and helping to maintain blood pressure. Birds can restore blood volume within a few minutes of hemorrhage (Djojogugito et al. 1968, Kovách and Bálint 1969, Radke et al. 1985). Indeed, the rapid movement of extracellular fluid into the capillaries is so rapid and strong that it can overshoot the pre-hemorrhage blood volume within a few minutes (Takei and Hatakeyama 1987). This replacement fluid results in a diluting effect that is manifest by a reduction in hematocrit values (Djojogugito et al. 1968, Kovách and Balint 1969, Ploucha et al. 1981, Gildersleeve et al. 1985, Dressen et al. 1999, Totzke et al. 1999, Piersma et al. 2000).

Although many early studies of hemorrhage involved the removal of a large percentage of the total blood volume and were not designed for the birds to survive, the hematocrit results presented in Figure 2A suggest that hemodilution also occurs when small amounts of blood are collected repeatedly over 30 min. In contrast, hematocrit levels did not differ among singly-bled birds sampled during that time frame (Fig. 2B).

Although the hematocrit patterns suggest that hemodilution may, indeed, play a role in explaining the corticosterone results, they must be viewed with caution. For example, the multiply-bled birds were two weeks older than the singly-bled birds when blood samples were collected. Hematocrit levels change during development in kestrels (Dawson and Bortolotti 1997), and it is possible that the response to hemorrhage changes as nestlings develop. Furthermore, body size plays a role in the effects of hemorrhage, and it would have been useful to know what percentage of a nestling’s blood volume was removed in each blood sample for multiply-bled and singly-bled birds. However, I did not attempt these calculations because the blood sampling technique (i.e., venipuncture) estimates blood loss imprecisely. That is, it does not account for blood lost in the form of hematomas or for residual bleeding following the end of blood sampling. Lastly, the effects of hemorrhage on corticosterone and hematocrit levels are difficult to predict because the physiological response to hemorrhage is complex (Schadt and Ludbrook 1991) and probably involve more than just hemodilution. For example, Dressen et al. (1999) also found that repeated blood sampling reduces hematocrit levels in kestrels; however, after correcting for hemodilution, they concluded that other, unknown factors also contribute. Clearly, there is need for additional work in this area.

In summary, my results demonstrate that corticosterone levels differ in multiply- and singly-bled birds sampled at the same times after the onset of capture/handling stress. The most common technique, in which multiple samples are collected from individual birds, underestimates corticosterone values. The underlying mechanism for this is unknown. Although it may involve hemodilution, establishing such a link will require rigorous experimentation. It is the pattern of corticosterone secretion, rather than the absolute corticosterone values, which usually is important in interpreting the effects of stressors on organisms. Nonetheless, it will be important to consider effects of such procedural differences when comparing results among studies.

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Figure legends

Fig. 1. Mean + SE plasma corticosterone values for nestling American Kestrels sampled at one of three time periods after removal from the nest (black histograms) or at each of those same three time periods (white histograms). Data from singly-bled and multiply-bled birds at each time period were compared with paired *t*-tests, and an asterisk indicates significant differences ($P < 0.025$). Numbers within histograms are sample sizes.

Fig. 2. Mean \pm SE hematocrit levels A) for birds sampled during each of three time periods: at capture, 15 min after capture, and 30 min after capture, and B) for birds sampled once, either at capture or 15 min or 30 min after capture. Numbers below SE bars are sample sizes.

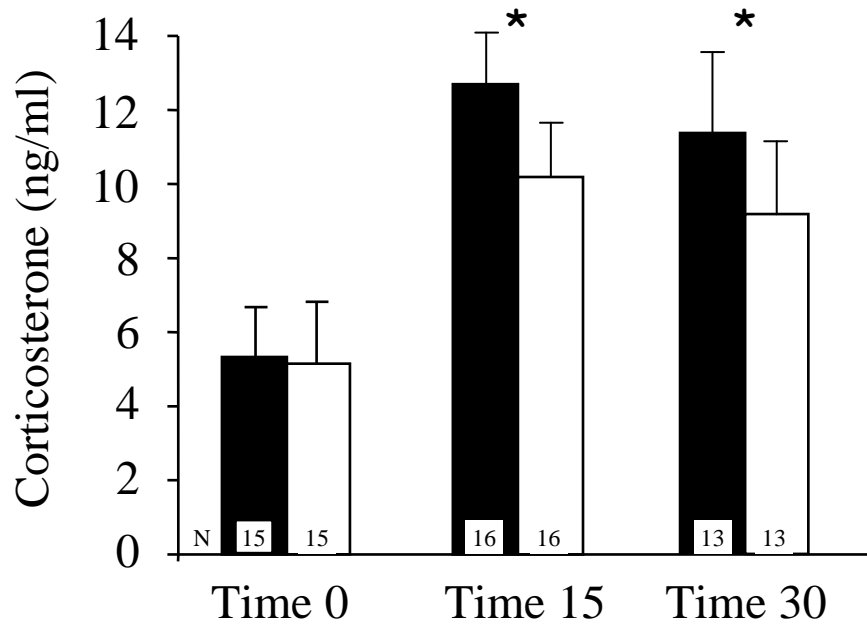


Fig. 1

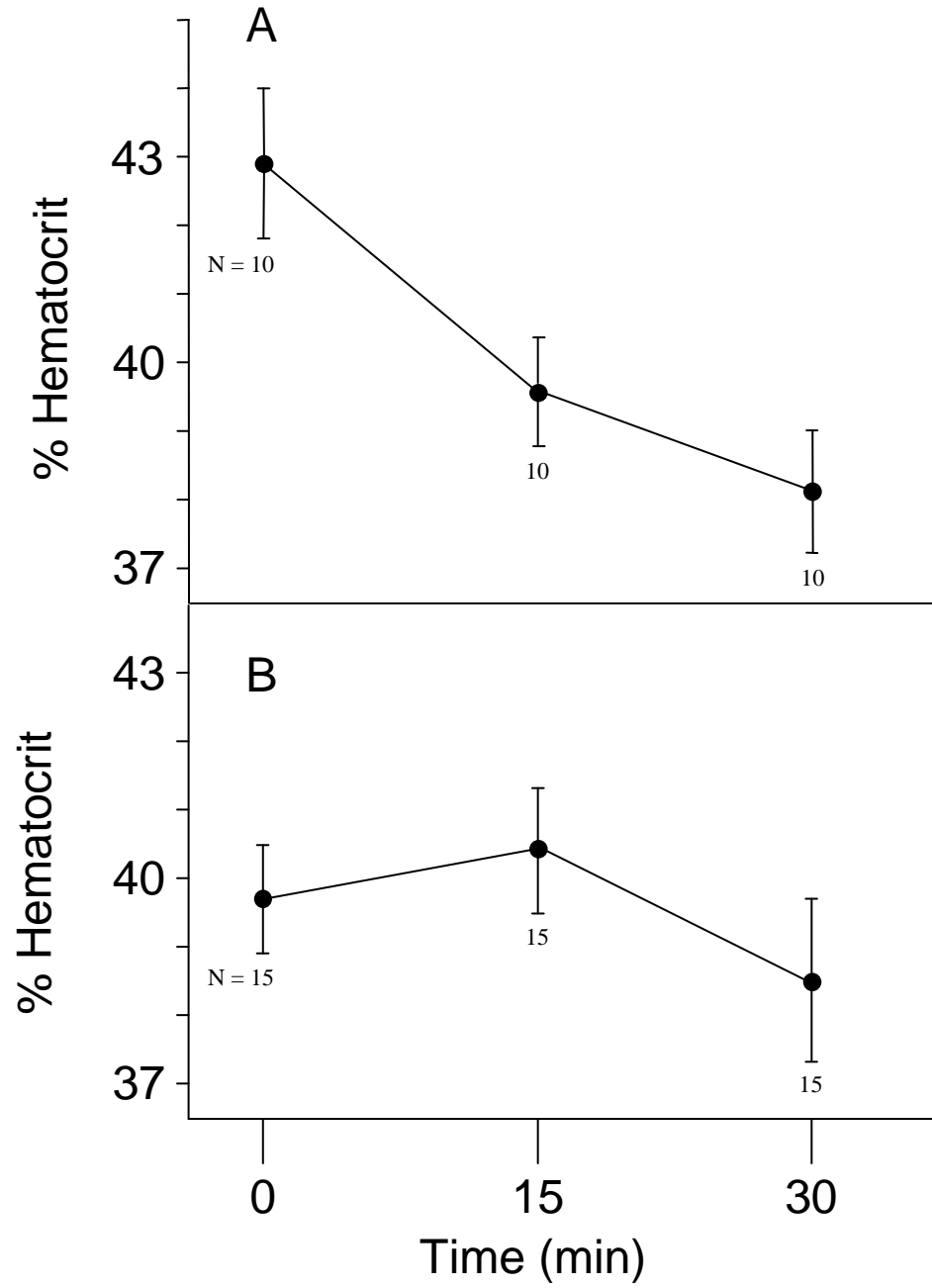


Fig. 2