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Comparative Biochemistry and Physiology, Part A 140 (2005) 73–79

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Collecting baseline corticosterone samples in the field: is under 3 min good enough?

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Received 24 July 2004; received in revised form 1 November 2004; accepted 2 November 2004

Abstract

Evaluating corticosterone (CORT) responses to stress in free-living vertebrates requires knowing the unstressed titers prior to capture. Based upon laboratory data, the assumption has been that samples collected in less than 3 min of capture will reflect these unstressed concentrations. This assumption was tested for six species using samples collected from 945 individuals at 0–6 min after capture. Samples were from five avian species trapped at multiple times of year and one reptilian species, comprising a total of 14 different data sets for comparisons. For seven of 14 data sets, including five species, there was no significant increase in corticosterone titers within 3 min of capture. In six of the 14 data sets, corticosterone titers increased significantly after 2 min, and in one data set, the increase started at 1.5 min. In all seven of the cases showing an increase before 3 min, however, corticosterone titers from the time of increase to 3 min were significantly lower than titers after 30 min of restraint stress. These results indicate a high degree of confidence for these species that samples collected in less than 2 min reflect unstressed (baseline) concentrations, and that samples collected from 2–3 min also will likely reflect baseline concentrations but at worst are near baseline.

Keywords: Stress; Glucocorticoids; Corticosterone; Wild animals; Field endocrinology; Bird; Reptile

1. Introduction

One important question in biology is how animals cope with their environment. Recent studies have tried to shed light on this problem by assessing the stress response of vertebrate animals in their natural habitats (e.g., Wingfield et al., 1997; Wingfield and Romero, 2001), thereby applying principles of physiology in an ecological context. Work has focused on the release of glucocorticoids, the steroid hormones acutely released in response to noxious stimuli that have become characteristic of the vertebrate stress response (Selye, 1971). Measuring glucocorticoids under field conditions is difficult. Glucocorticoids are released during stress, and simply capturing the animals in order to take a blood sample is stressful. Many studies have tried to avoid this problem by focusing on fecal concentrations of glucocorticoid metabolites (e.g., Kotrschal et al., 1998; Harper and Austad, 2000; Goymann et al., 2002), which reflect blood concentrations from the period of several hours prior to capture rather than right at capture. However, measuring fecal metabolites has its own methodological and interpretation drawbacks (e.g., Khan et al., 2002; Morrow et al., 2002; Washburn and Millspaugh, 2002; Huber et al., 2003), and the understanding of blood-borne glucocorticoid physiology and release gained from over 60 years of research (Sapolsky et al., 2000) continues to make collecting blood for glucocorticoid analysis an attractive technique.

The approach field researchers have used to minimize interpretation problems resulting from capture-induced glucocorticoid release is to ensure that blood samples are collected within a few minutes of capture (Wingfield and Romero, 2001). Glucocorticoid release is under the control of the anterior pituitary hormone adrenocorticotropin (ACTH), which is in turn under the control of the hypothalamic hormones corticotropin-releasing factor (CRF) and arginine vasopressin/arginine vasotocin (AVP/AVT, depending upon the species; Dallman and Bhatnagar,

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doi:10.1016/j.cbpb.2004.11.004

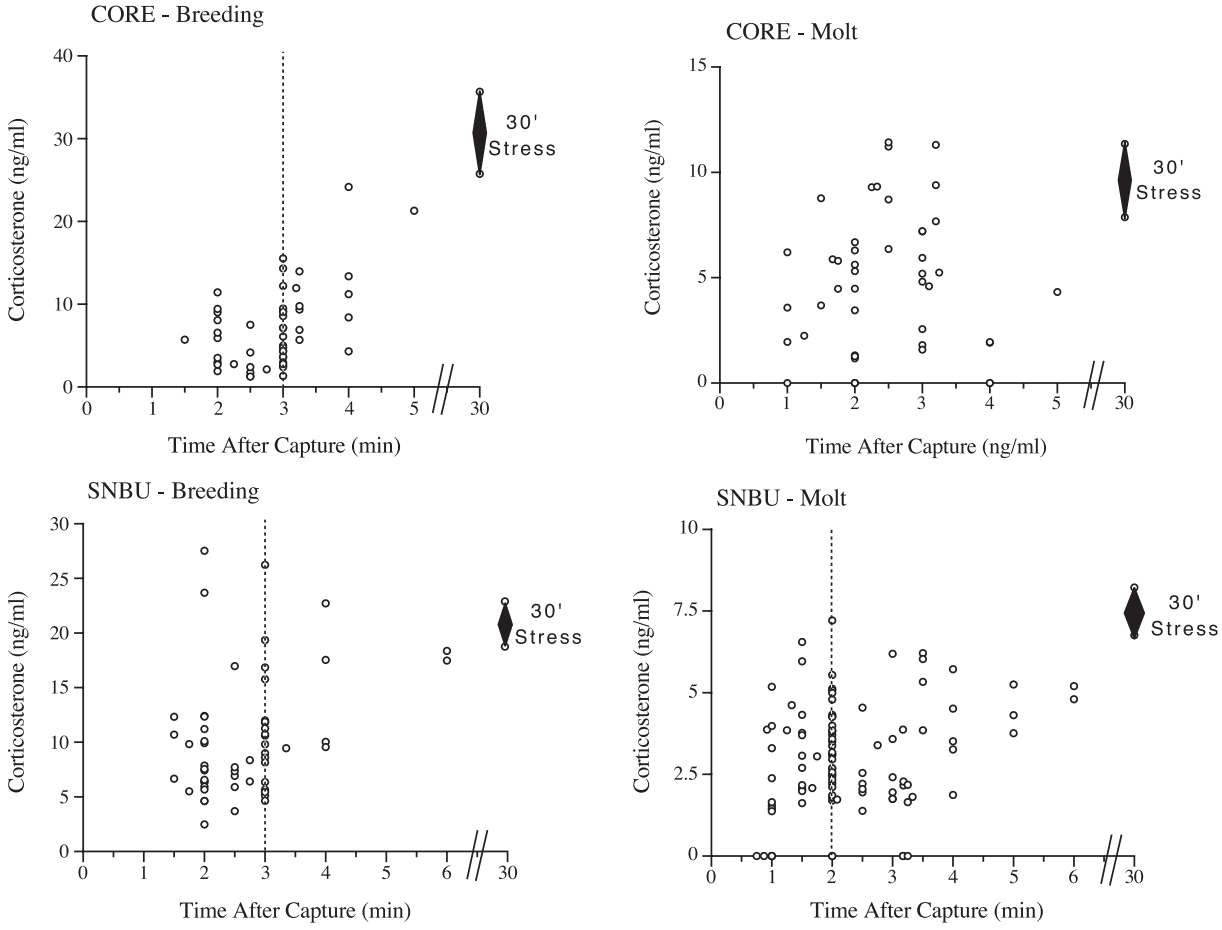


Fig. 1. Corticosterone (CORT) concentrations from initial samples taken as quickly as possible after capture and samples collected after 30 min of restraint. Stress-induced samples at 30 min represent a range of 1 standard error above and below the mean. Note differences in scale for each species at each time of year. Dashed line indicates statistically significant change point, and lack of a dashed line indicates no significant change point (Table 1). Species: CORE—common redpoll (*C. flammea*); SNBU—snow bunting (*P. nivalis*); GWCS—Gambel's white-crowned sparrow (*Z. leucophyrys gambelii*); HOSP—house sparrow (*P. domesticus*); LALO—Lapland longspur (*C. lapponicus*); iguana (*A. cristatus*).

2001). CRF and AVP/AVT release is ultimately under the control of higher brain centers that detect a stimulus, decide that the stimulus is noxious, and send neuronal signals to the CRF and AVP/AVT cell bodies in the hypothalamus (Weninger and Majzoub, 2001). This cascade of events, from the animal detecting a stressful stimulus to measurably elevated glucocorticoid concentrations in the blood, takes 3–5 min in domestic rats (Dallman and Bhatnagar, 2001). Consequently, if the animal can be removed from the trap and bled within 3 min of capture, field researchers have assumed that the glucocorticoid concentrations in that sample represent baseline, or prestressed, concentrations.

Surprisingly, this assumption has not been rigorously tested in field studies. Only a few studies have directly tested whether glucocorticoid concentrations begin to increase in the first few minutes of capture in free-living animals, and results have been equivocal. Some studies found no increase in the first few minutes (e.g., Wingfield et al., 1982; Schoech et al., 1999), in concordance with laboratory studies, but others found earlier increases (e.g., Dawson and Howe, 1983). In this study, we examined glucocorticoid concentrations from samples collected 0–6 min after capture to determine whether glucocorticoid concentrations begin to rise within this time frame. We used data from six species (five avian and one reptile) originally collected for other studies. All species in this analysis use corticosterone as the primary glucocorticoid (the primary glucocorticoid for avian and reptilian species and abbreviated as CORT; Wingfield and Romero, 2001). Our specific goal was to determine whether or not samples taken any time during the first 3 min after capture were statistically identical.

2. Materials and methods

2.1. Sample collection

The data used in this study were collected as part of different studies and most are published elsewhere as baseline means (see below). In those studies, multiple blood samples were collected from each individual to monitor the

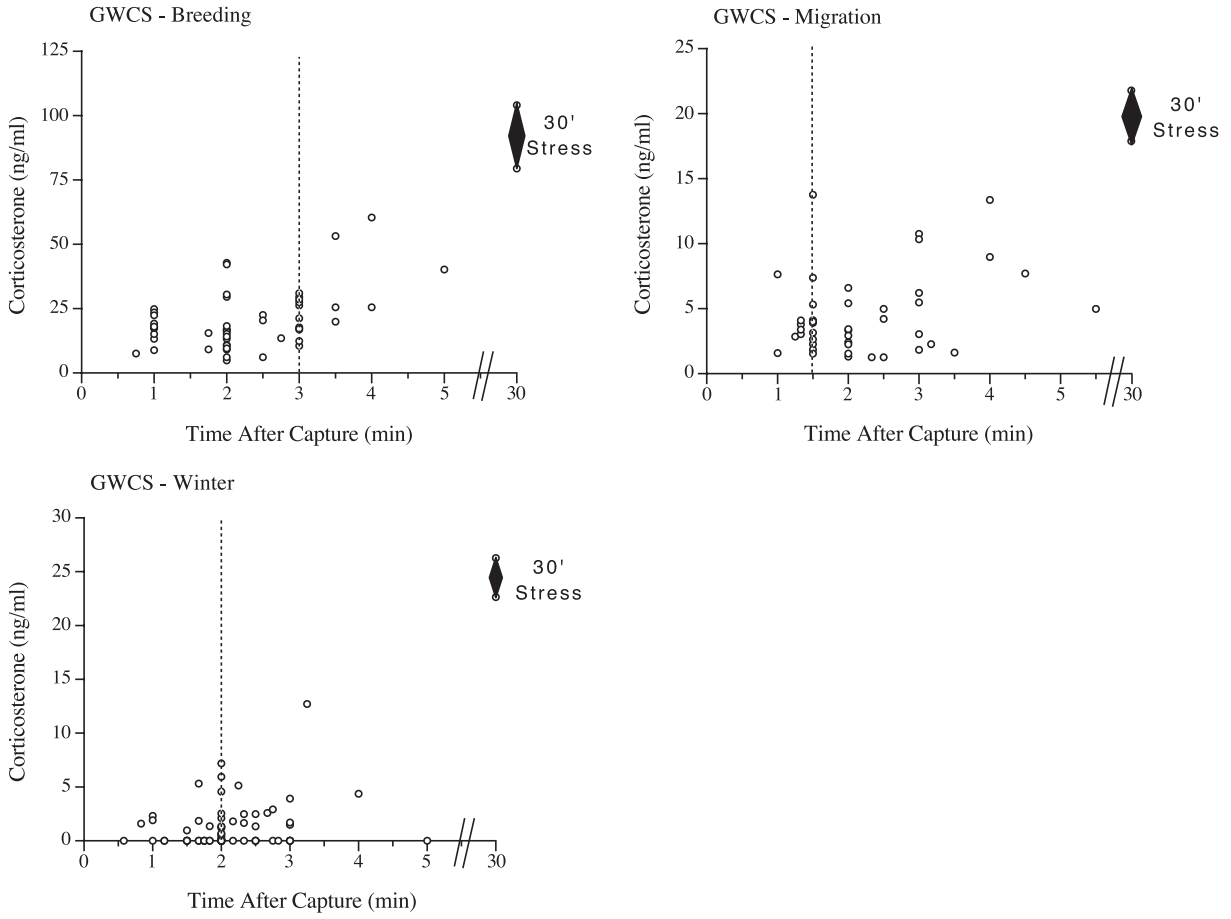
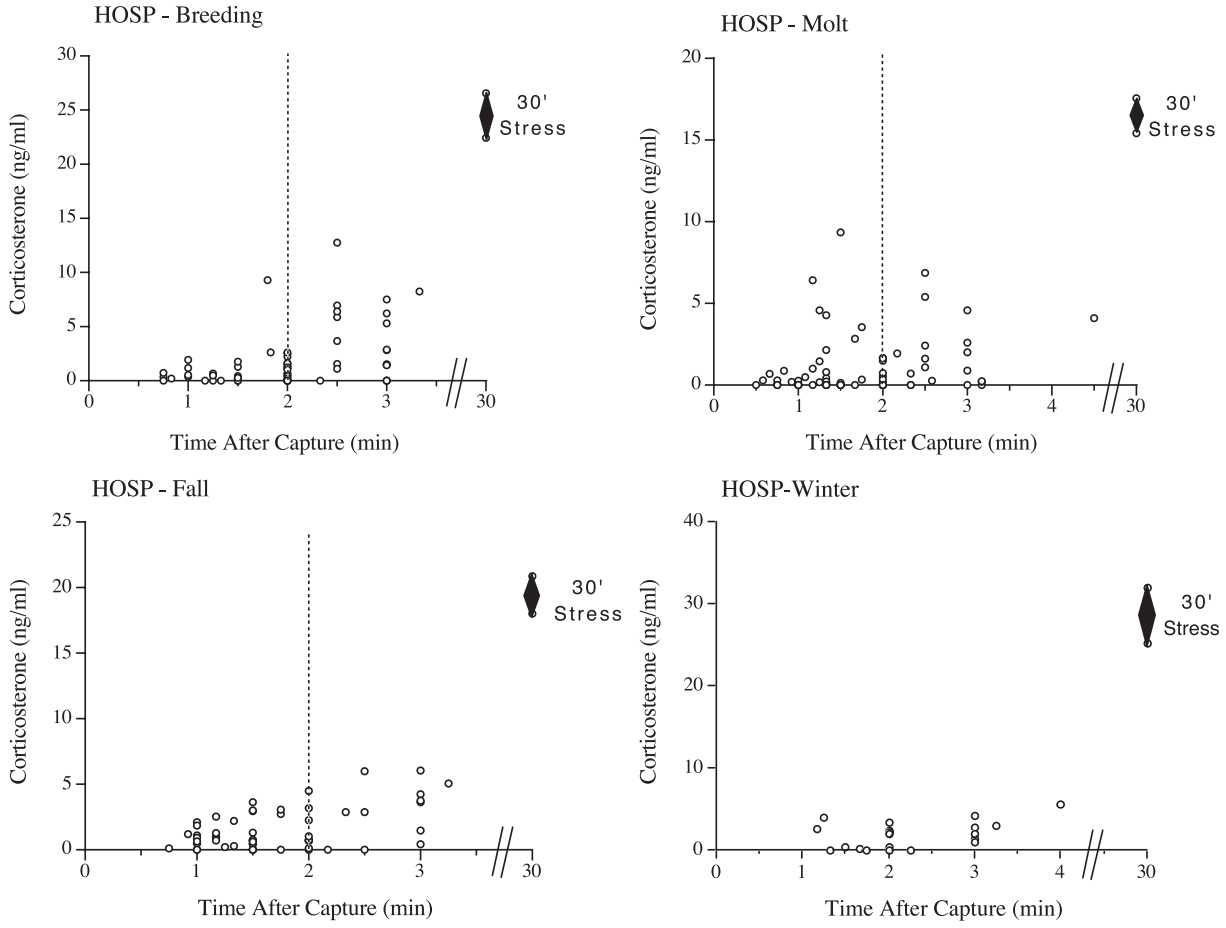


Fig. 1 (*continued*).

increase in CORT concentrations in response to the stress of capture and handling, and many individuals were injected with exogenous hormones immediately following the collection of the first sample. Only samples collected within the first 3 min after capture were included in the original publications. In this study, we included those samples and added those samples collected between 3 and 6 min. We have few samples collected after 3 min because the 3 min window to collect baseline samples is a common assumption, so animals often were released without taking a sample if the 3 min window was exceeded.

We included data from a total of 945 individuals from six species. Since many species show seasonal variation in their glucocorticoid concentrations (Romero, 2002), and many of these data were collected to demonstrate that phenomenon, we performed separate analyses for each species at each time of year. The original papers explored this issue in more detail. We were not interested in making comparisons among species or seasons because it is not clear that they would be valid (Romero, 2004), so there are no statistical concerns associated with multiple comparisons. Common redpolls (*Carduelis flammea*; CORE) were trapped both while breeding and while undergoing a prebasic molt (Romero et al., 1998c). We captured Gambel's white-crowned sparrows (*Zonotrichia leucophrys gambelii*; GWCS) while breeding, during winter, and during their fall migration (Romero et al., 1997; Romero and Wingfield, 1998; Romero and Romero, 2002). House sparrows (*Passer domesticus*; HOSP) were trapped at 4 times of the year: while breeding in the spring, while undergoing a postbreeding prebasic molt, in the fall after the end of molt, and in the winter (unpublished data). Lapland longspurs (*Calcarius lapponicus*; LALO) and snow buntings (*Plectrophenax nivalis*; SNBU) also were captured while breeding and during their prebasic molt (Romero et al., 1998a,b). Finally, marine iguanas (*Amblyrhynchus cristatus*) were captured in May and June (nonbreeding) from Santa Fé Island in the Galápagos archipelago (unpublished data and Romero and Wikelski, 2001; Woodley et al., 2003).

In all cases, we observed animals being captured and bled them as quickly as possible. The time after capture until completing sample collection was recorded, often to the nearest 15 or 30 s. Stress-induced samples were then collected after 30 min of handling and restraint in a cloth bag. Samples were stored on ice until centrifuged to collect plasma. Plasma was then frozen, transported to the University of Washington or Tufts University, and analyzed for CORT by radioimmunoassay. See the previous papers for further details on the radioimmunoassay and capture details.



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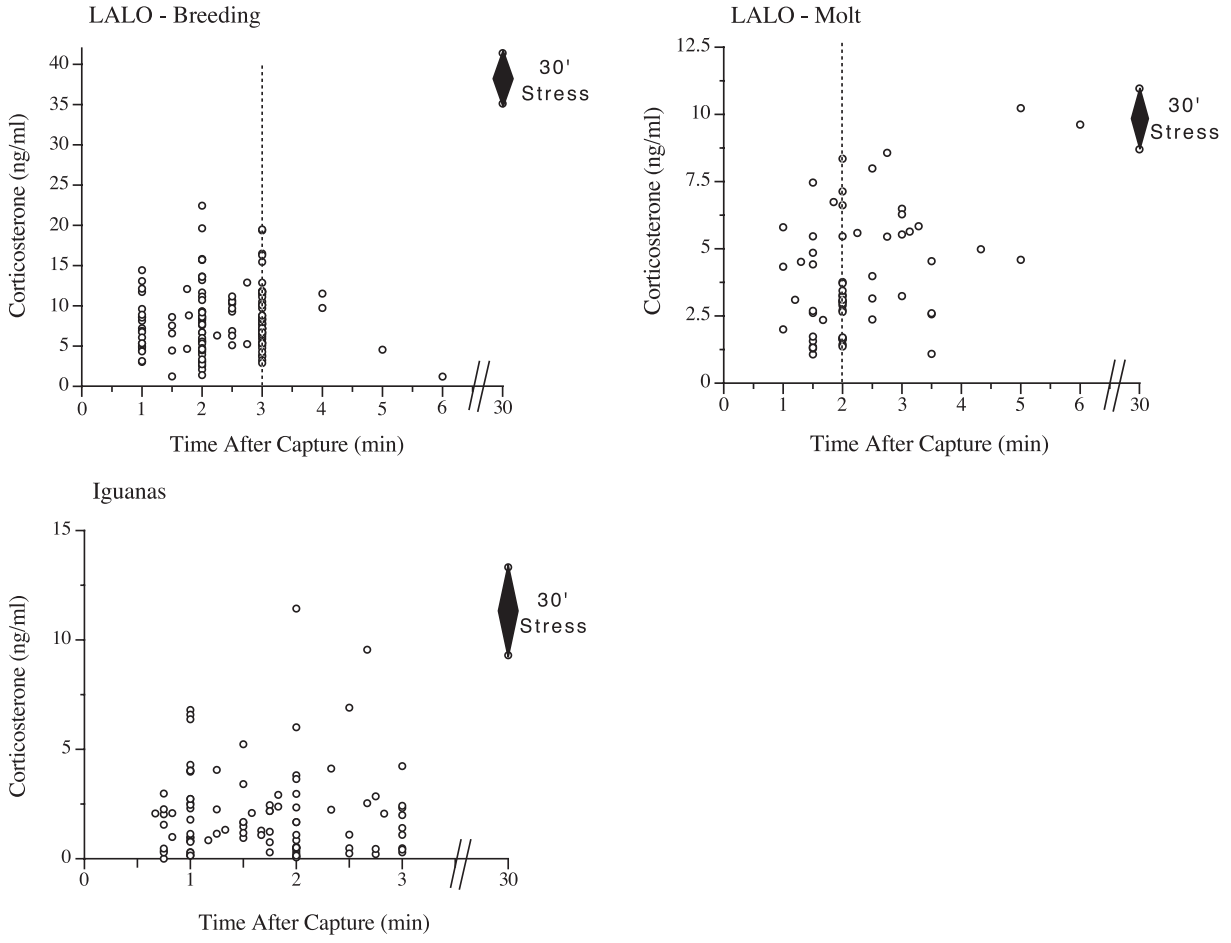
2.2. Statistical methods

Traditionally, researchers have tested for potential increases in the first 3 min, using linear regression. However, given the known delay resulting from the multi-step pathway from detecting a stressor to releasing glucocorticoids, we cannot assume that there is a linear relationship between CORT level and time. Consequently, we used a statistical approach that would be able to detect linear or nonlinear relationships. Our null hypothesis was that there was no significant change in CORT levels as a function of time after capture within the first 3 min. To make this determination, we used a nonparametric change-point test for continuous variables (Siegel and Castellan, 1988). This test determined at what time after capture CORT values from shorter times were most different from CORT values taken from longer sample times, and the significance level of the difference. As a brief description of this technique, the first step in this test is to rank the data from lowest to highest CORT values then order the data from shortest bleeding time to longest. The sum of the cumulative ranks for CORT values is then calculated and subtracted from the predicted cumulative sum. The greatest absolute difference is identified as the change-point, and ranks of CORT values below and above the change-point are compared to a critical value to determine statistical significance. Because there is no accepted way in this test to deal with ties in time (i.e., multiple values of CORT for a given time; ties in CORT values were assigned midrank values), we omitted the sample size for values of CORT at the change-point time when calculating the test statistic. This does not affect the ranking of CORT values or identification of the change-point, just the calculation of the variance and z-score.

When we found a significant change-point at a time under 3 min, we determined whether CORT values above the change-point but ≤3 min were significantly different from stress-induced (30 min) samples and made the evaluation using a t-test (SAS Institute, 1999). This was to determine if these post change-point values were statistically distinct enough from the elevated CORT response as to be functionally near baseline, or if the increase was sufficient to warrant concern over sampling timing.

3. Results

The raw data for each species at each time of year are presented in Fig. 1. For seven of 14 data sets, including five



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species (breeding redpolls, white-crowned sparrows, Lapland longspurs, snow buntings, and iguanas), the nonparametric change-point test found no significant increase in corticosterone titers within 3 min of capture (Table 1). In seven of the 14 cases, the test found a significant change-point at less than 3 min (white-crowned sparrows during migration and winter, all house sparrows except during winter, and molting snow buntings and Lapland longspurs). In all cases where the nonparametric change-point test indicated a significant change-point under 3 min, however, corticosterone titers from the time of increase to 3 min were significantly lower than titers after 30 min of restraint stress.

4. Discussion

Our analyses clearly show that, in most instances, circulating CORT concentrations were not increasing within the first 2 min of capture and often not in the first 3 min. This is consistent with most studies that have analyzed corticosterone in the first few minutes of capture in free-living vertebrates. Three avian studies have indicated that CORT does not change in the first 3 min (Wingfield et al., 1982; Schoech et al., 1999; Sockman and Schwabl, 2001), although the largest sample size for any of these studies was 20 individuals, and most studies included fewer than 10. An additional avian study showed that CORT titers did not change between 3 and 5 min, although no samples were collected earlier than 3 min (Mizrahi et al., 2001). CORT titers also did not change in the first 3 min after capture in a lizard (*Sceloporus cyanogenys*), although, again, the study included only 15 individuals (Manzo et al., 1994). These field data are consistent with laboratory data on birds (Wingfield et al., 1982), lizards (Dauphin-Villemant and Xavier, 1987), and many studies in laboratory rodents (reviewed by Dallman and Bhatnagar, 2001), showing that CORT titers do not increase in the first 3 min after the introduction of a stressor.

In contrast, our data show that CORT titers are beginning to rise after approximately 2 min in seven of the 14 data sets. The rise in CORT earlier than 3 min is consistent with two other studies. CORT titers were reported to begin rising after only 1 min in European starlings (Dawson and Howe, 1983), and a linear regression shows CORT titers increasing in dark-eyed juncos (*Junco hyemalis*) that were given testosterone implants (Schoech et al., 1999). In addition, CORT titers in red-eared slider turtles (*Trachemys scripta elegans*) show a linear increase starting at 5 min, although

Table 1
Nonparametric change-point test results on all data (≤6 min after capture)

Species[a]	Season	*N*	Range of times (min)	Change-point (min)[b]	*P*-value	Postchange vs. 30 min[c]	
						t-value (*df*)	*P*-value
CORE	Breeding	52	1.50–5.00	3.0	<0.0001	–	
	Molt	46	1.00–5.00	(2.0)	0.4940	–	
SNBU	Breeding	72	1.50–6.00	3.0	<0.0001	–	
	Molt	100	0.75–6.00	2.0	0.0132	5.90 (47.7)	<0.0001
GWCS	Breeding	52	0.75–5.00	3.0	<0.0001	–	
	Migrate	44	1.00–5.50	1.5	<0.0001	7.66 (23.1)	<0.0001
	Winter	71	0.58–5.00	2.0	<0.0427	12.58 (18)	<0.0001
HOSP	Breeding	63	0.75–3.33	2.0	<0.0022	9.51 (42.9)	<0.0001
	Fall	57	0.75–3.50	2.0	<0.0035	10.41 (48.9)	<0.0001
	Molt	75	0.50–4.50	2.0	<0.0107	12.04 (41.4)	<0.0001
	Winter	21	1.17–4.00	(2.0)	0.2068	–	
LALO	Breeding	130	1.00–6.00	3.0	<0.0009	–	
	Molt	62	1.00–6.00	2.0	<0.0036	3.52 (23.4)	0.0018
Iguana	Nonbreeding	100	0.67–3.00	(1.0)	0.1492	–	

[a] CORE—common redpoll, SNBU—snow bunting, GWCS—white-crowned sparrow, HOSP—house sparrow, LALO—Lapland longspur, Iguana—marine diving iguana. See legend of Fig. 1 for species names.
[b] Change points that were not statistically significant are indicated in parentheses.
[c] For treatments with a significant change-point in less than 3 min, we asked the question 'are the data collected after the change-point but ≤3 min significantly different from those at 30 min?' Done using a *t*-test; *P*-value and (*df*) presented. When there were unequal variances, it was corrected for, which explains the noninteger *df* values (SAS Institute, 1999).

no samples were taken earlier (Cash et al., 1997). Only the Dawson and Howe (1983) study, however, had a sample size (57) similar to those reported here. Consequently, it does appear that some species, at some times of the year or in certain physiological conditions, can initiate CORT release in less than 3 min. The weight of evidence from our study, with its robust sample sizes, however, indicates that, for most species, at most times of the year, CORT titers from samples collected within 2 min of capture most likely reflect prestressed concentrations.

The change-point tests consistently indicated a change-point near 2 min if a change-point existed in under 3 min, with the exception of migrating white-crowned sparrows where the increase started in 1.5 min. The only published study indicating that CORT titers rise in less than 2 min is by Dawson and Howe (1983) on European starlings. However, this increase is not evident in studies of captive wild starlings, although CORT titers appear to be approximately threefold higher in captive compared to free-living starlings (Romero and Remage-Healey, 2000). Furthermore, even when samples taken between 1.5 and 3 min are not baseline, they are near baseline. We found that, in those cases where CORT titers significantly increased after the change-point, titers were still highly significantly lower than titers after 30 min of stress. For example, although the CORT concentration from a fall house sparrow collected at 3 min is not equivalent to baseline, it is still much closer to baseline than to stress-induced concentrations.

The weight of evidence therefore indicates that samples collected in less than 2 min can be assumed to reflect unstressed concentrations with a high degree of confidence, and samples collected within 3 min most likely reflect unstressed concentrations. Even when samples collected within 3 min do not reflect unstressed concentrations, they will likely be near enough to baseline to be distinguishable from stress-induced concentrations. Samples collected after 3 min, however, will likely not reflect baseline titers since many studies show substantial increases in CORT by this time.

Acknowledgements

We thank D. Palmquist for statistical discussions. We also thank A. Dawson and S. Meddle for providing the impetus for conducting these analyses. This work was funded by NSF grants IBN-9975502 and IBN-0235044 to LMR.

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