Tools and Technology Article

Stability of Avian Fecal Corticosterone Metabolite Levels in Frozen Avian Feces

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ABSTRACT Fecal corticosterone metabolites are commonly used in avian ecology as a measure of response to stress. Recent research on mammals suggested that the manner in which samples are stored could be critical to alleviating any storage handling bias. Cross-reacting metabolites can increase glucocorticoid metabolites even after samples are frozen and, thus, result in an overestimation of hormone levels as the time increases between when samples were collected and when levels are measured. We examined effects of sample storage time on fecal corticosterone metabolites for 2 avian species across 165 days. We observed no change in fecal corticosterone metabolites across the sampling periods in either fulvous whistling-ducks (*Dendrocygna bicolor*) or white ibis (*Eudocimus albus*). Results suggest that avian fecal corticosterone metabolite levels do not change when samples are frozen for long periods of time and that there were no differences in the response between the 2 species we compared. This study demonstrated that avian fecal corticosterone samples are accurate even after freezing and, thus, studies that seek to address conservation questions may rely on these data. Studies of additional bird species are needed to generalize our findings to other avian taxa. (JOURNAL OF WILDLIFE MANAGEMENT 73(6):1010–1013; 2009)

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The physiological condition of avian species is often influenced by changes in their environment and can be measured by endocrinological parameters. Endocrinological techniques can allow for an assessment of the physiological condition of species simply through collection of blood or fecal material. One of the most widespread approaches to studying the physiological response of birds to an environmental stimulus or stressor is through measurement of corticosterone. When the cortex of the brain detects a stressor (e.g., food shortage, large temp change), a neural signal is sent to the hypothalamus (Levine et al. 1989). The hypothalamus then sends a hormone signal to the pituitary gland, resulting in the pituitary sending a signal to the adrenal or inter-renal gland to release corticosterone (Levine et al. 1989). After the stressor has been alleviated a negative feedback loop shuts off the hypothalamus–pituitary–adrenal pathway leading to the corticosterone release (Levine et al. 1989). If the stressor persists and corticosterone remains elevated, the negative feedback loop will cease to function and destructive chronic effects may begin as allostatic overload occurs (Romero 2004).

At lower levels in birds, corticosterone can stimulate foraging activity and lipogenesis (Astheimer et al. 1992, Wingfield 1994). Under conditions of food scarcity, corticosterone stimulates gluconeogenesis, shifting catabolism away from fats towards proteins (Astheimer et al. 1992, Wingfield 1994). Chronic elevated levels of corticosterone can reduce fitness of individuals via immunosuppression and result in reproductive failure (Verme and Doepker 1988, Wingfield et al. 1992). Fecal corticosterone metabolite levels reflect residue levels of plasma corticosterone; however, rapid and extensive metabolization before excretion results in lower overall levels than circulating levels (Wasser et al. 2000).

Increased corticosterone levels in birds have been related to impaired embryonic development and lower quality offspring (Eriksen et al. 2003, Hayward and Wingfield 2004, Saino et al. 2005), changes in habitat use (Thiel et al. 2008), lower fat reserves in chicks (Kitaysky et al. 1999), lower habitat quality (Marra and Holberton 1998), increased response to poor weather conditions (Lobato et al. 2008), and others (see Millspaugh and Washburn 2004). Measurement of corticosterone metabolites in fecal samples has become a common approach to measure stress in birds because of its noninvasive nature (see Wasser et al. 1997, Ludders et al. 2001, Washburn et al. 2003, Millspaugh and Washburn 2004, Palme 2005). Additionally, the elevation of corticosterone metabolite levels in feces does not occur for 2–12 hours after capture (Wasser et al. 1997, Ludders et al. 2001, Washburn et al. 2003) so fecal corticosterone metabolites are not biased from short-term capture and handling stress, as in the case with plasma corticosterone from blood samples collected >3 minutes after a bird is captured (Romero and Reed 2005).

Despite the increased use of fecal corticosterone metabolites to measure stress in birds, factors associated with sample storage that could bias results remain poorly understood (Goymann 2005, Möstl et al. 2005, Palme 2005). Khan et al. (2002) examined potential issues with storage of mammalian fecal samples from baboons (*Papio cynocephalus*) stored in ethanol or frozen and demonstrated that cross-reacting metabolites could result in a consistent increase of nearly 25% in fecal corticosterone metabolite levels after freezing (Khan et al. 2002). These results suggested that if samples were not measured within the first 2 months after collection, fecal corticosterone metabolite levels might be biased high (Khan et al. 2002). To our

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knowledge no study has examined effects of freezing storage time on fecal corticosterone metabolite levels in any avian species.

Our objective was to experimentally test effects of sample storage time on corticosterone metabolite levels from frozen fecal samples over a period of >5 months (165 days) in 2 species of birds. We used this time duration for storage because it was similar to that in the study by Khan et al. (2002) and because changes in fecal glucocorticoid metabolite levels occurred in this period in their study. It is also a reasonable interval between field work and laboratory work for seasonal studies. We compared 2 bird species to determine if there might be species-specific responses associated with sample storage.

METHODS

Fecal Sample Collection and Measurement

We collected fecal samples from 2 sources and 2 species, the fulvous whistling-duck (*Dendrocygna bicolor*) and the white ibis (*Eudocimus albus*). We used these 2 species because of their captive availability for sampling, phylogenic differences (Hackett et al. 2008), and differences in diet (see Kushlan and Bildstein 1992, Hohman and Lee 2001, Dorn et al. 2008) that might result in different gut passage time, gut microflora, and corticosterone metabolization. We collected samples from white ibis on 19 June 2007 from a captive research flock at the University of Florida, Gainesville, Florida, USA. We collected samples from fulvous whistling-ducks on 8 August 2007 at the Palm Beach Zoo, West Palm Beach, Florida. Both species were maintained in enclosures with no access to other species. Floors in both aviaries were cleaned prior to sampling to avoid contamination. On sampling days, we observed birds after feeding during the morning until they defecated on either concrete or a plastic covered floor. We collected sufficient samples (approx. 200 g) within 15 minutes of deposition, stored them in vacuutainers, and froze them immediately at -20° C before shipping them on ice overnight or transporting them to the laboratory at Florida Atlantic University the following day.

In the lab, we thoroughly mixed fecal samples by hand for 1-minute intervals, alternating with 0.5-minute vortexing, 5 times. We then divided each species' sample into 6 equal portions (approx. 33 g); we placed 5 in individual vacuutainers and froze them at -20° C for later analysis. We then proceeded to analyze our 1-day-old sample from each species on that day as below. We considered 1-day-old samples to be fresh for purposes of analysis. Approximately every 30 days for 165 days, we thawed and analyzed 1 frozen sample for each species.

We homogenized fecal samples, divided them into 6 2.0-g wet portions, which we dried using a Labconco CentriVap Concentro (Labconco, Kansas City, MO). We then mixed dried samples (0.5 g) with 5 mL of 95% ethanol and vortexed them for 30 minutes. After centrifugation (15 min, 2,500 $\times$ *g*) we transferred the supernatant to a new vial, which we then evaporated under a stream of nitrogen gas. We then resuspended corticosterone metabolites in diluted extraction buffer and measured using the Correlate-EIA™ Corticosterone Enzyme Immunoassay Kit (ELISA; Assay Design, Inc., Ann Arbor, MI; Rothschild et al. 2008) following the manufacturer's instructions. The Correlate-EIA™ Corticosterone ELISA used a donkey anti-sheep IgG microtiter plate, and a corticosterone enzyme immunoassay antibody as a standard. Cross-reactivity was generally low and unlikely to result in misinterpretation of results and was as follows: deoxycorticosterone (21.3%), desoxycorticosterone (21.0%), progesterone (0.46%), testosterone (0.31%), tetrahydrocorticosterone (0.28%), aldosterone (0.18%), cortisol (0.04%), pregnenolone (0.03%). β-estradiol (0.03%), cortisone (0.03%), 11 = dehydrocorticosterone acetate (0.03%). Minimum detectable level of corticosterone metabolites was 0.05 ng/g. We conducted a standard assay validation for each species, which included an assessment of parallelism, recovery of exogenous corticosterone, and intra- and inter-assay precision to confirm that the ELISAs accurately measured corticosterone metabolites in both fulvous whistling-duck and white ibis fecal samples (Astalis et al. 2004, Goymann 2005, Rothschild et al. 2008).

Statistical Analysis

To validate ELISA test kits for fecal corticosterone metabolites we tested for equality of 2 slopes (parallelism) using a *t*-test (Zar 1996) to determine if curves of serially diluted fecal corticosterone metabolites from both fulvous whistling-ducks and white ibises were parallel to standard curves for corticosterone. Similar slopes between serially diluted samples and standard curves would demonstrate ELISA kits were, in fact, measuring the parameter as purported by the manufacturer.

We log-transformed corticosterone data to meet assumptions of homoscedascity (Levene's test $P = 0.09$ and $P = 0.07$ for fulvous whistling-duck and white ibis, respectively; Sall et al. 2001) and normality after examining residuals. We then ran separate repeated-measures analysis of variance (Sall et al. 2001) tests to determine if there were differences among the 6 fecal-sample storage time periods for each species.

RESULTS

Serial dilutions of fulvous whistling-duck and white ibis fecal corticosterone metabolite extracts yielded displacement parallel to the standard corticosterone curves ($t_{10} = -2.22$, $P = 0.11$, $t_9 = 1.02$, $P = 0.33$, respectively; Fig. 1A–B). Mean recovery of the added exogenous corticosterone spike was 86.3% $\pm$ 7.7 (SE). Inter- and intra-assay coefficients of variation for corticosterone internal standards for fulvous whistling-ducks and white ibises were 8% and 9% and 7% and 11%, respectively. Fecal corticosterone metabolite levels did not differ between storage sample times for either fulvous whistling-duck or white ibis ($F_{1,5} = 0.78$, $P = 0.56$, $F_{1,5} = 0.2$, $P = 0.96$, respectively; Fig. 2).

DISCUSSION

Neither the fulvous whistling-duck nor white ibis showed significant change in fecal corticosterone metabolite levels across time, which suggests that cross-reacting metabolites

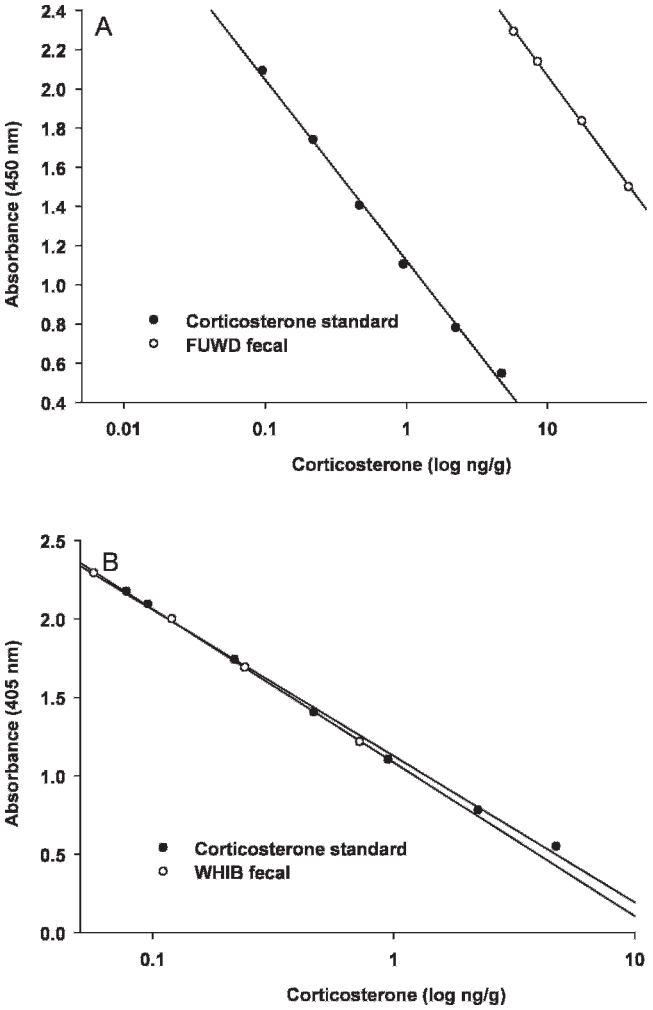

Figure 1. Parallelism between corticosterone enzyme linked immunoassay (ELISA) kit standard curve and serially diluted fecal extracts from (A) fulvous whistling-duck (FUWD; 8 Aug 2007) and (B) white ibis (WHIB; 19 Jun 2007) collected at the Palm Beach Zoo, West Palm Beach and the University of Florida, Gainesville, Florida, USA.

may not bias measures of bird fecal corticosterone metabolite levels as has been reported in mammalian species (Khan et al. 2002). Variation in sample means in our study and standard errors between treatment periods may stem from a failure to completely homogenize samples, resulting in sample-to-sample variance (Millspaugh and Washburn 2003, 2004).

Khan et al. (2002) additionally tested for differences between frozen and non-frozen sample storage, observing that frozen samples were not as biased; that is, cross-reacting metabolites did not change levels of fecal glucocorticoids as greatly as non-frozen stored samples, which increased nearly 100% in the same time period. Although we did not test multiple storage methods, our results do support the contention by Khan et al. (2002) that freezing may be the best option for avian fecal samples.

Although our results suggested frozen sample storage time of up to 165 days may not bias fecal corticosterone metabolite levels, we caution against widespread application of these results to other bird species because our results are the first test for avian fecal samples. Additional tests of other

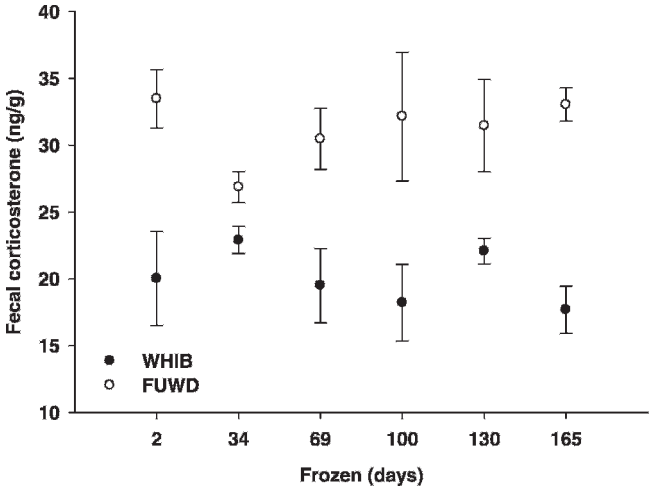

Figure 2. Change in mean (±SE) fecal corticosterone metabolite levels over time after storage at −20° C measured in samples collected from captive fulvous whistling-ducks (FUWD; 8 Aug 2007) at the Palm Beach Zoo, West Palm Beach and white ibises (WHIB; 19 Jun 2007) at the University of Florida, Gainesville, Florida, USA (n = 5–6 for each time period sampled).

bird species are warranted to determine if interspecific differences in cross-reacting metabolites exist, which could result in sample storage issues and biased corticosterone metabolite estimates. Additional studies could also evaluate whether different levels of corticosterone metabolites could produce a bias in response to different storage methods.

MANAGEMENT IMPLICATIONS

An important first step in the use of fecal corticosterone metabolites in avian conservation is to determine if sample storage methods are in fact stable, resulting in accurate measurement of the condition of a bird. For 2 avian species, the first evaluation of freezing as a storage method for fecal corticosterone indicates it is unbiased for up to 165 days.

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