General and Comparative Endocrinology 270 (2019) 35–40

Contents lists available at ScienceDirect

General and Comparative Endocrinology

journal homepage: www.elsevier.com/locate/ygcen

Using a keratinase to degrade chicken feathers for improved extraction of glucocorticoids

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ARTICLE INFO

Keywords:
Avian
Keratinase
Feather
Solid-phase extraction
Stress
Corticosterone

ABSTRACT

Stress in animals is a concern in conservation breeding programs and livestock production facilities. The biological stress response is mediated by the release of glucocorticoids, which can suppress reproduction, growth, and immunity if recurrently activated. Feathers can be used to extract and monitor concentrations of corticosterone, a primary glucocorticoid in birds. However, current techniques for extracting feather corticosterone present challenges, including difficulty assessing extraction efficiency or hormone recovery, inconsistent extraction across feather lengths or pieces, and several uncertainties regarding the mechanisms of hormone deposition into feathers. To overcome such challenges and to provide tools useful for facilitating conservation breeding and livestock production, we developed and validated an alternative procedure for extracting feather glucocorticoids. We first developed a protocol to enzymatically digest the protein matrix of feathers using a keratinase, such that non-protein analytes could be isolated by organic extraction. We then developed an extraction protocol and evaluated techniques by measuring extraction efficiency and by testing parallelism and hormone recovery (accuracy) using radioimmunoassay. Our results demonstrated high and consistent extraction efficiency, as well as high accuracy and reliable parallelism to a standard curve upon measurement of corticosterone concentrations from extracts. By dissolving feather material into solution prior to extraction, we were able to replicate hormone deposition into the feather matrix and ensure consistent extraction across feathers. This work provides additional support for the validity and practicality of extracting glucocorticoids from feathers. Our extraction protocol is likely to extend to other applications as well, including the isolation of numerous non-protein analytes from various keratinized tissues.

1. Introduction

Stress in animals under human care is a concern in conservation breeding programs and livestock production facilities (Morgan and Tromborg, 2007). Maintaining healthy, successful, and self-sustaining breeding populations requires stress management. The biological stress response is mediated by the release of glucocorticoids (Romero, 2004; Romero and Butler, 2007), which can suppress reproduction, growth, and immunity if recurrently activated (Charmandari et al., 2005). Long-term stress, or the confounding and persistent effect of multiple environmental stressors, can be monitored using glucocorticoid hormone measurements over time (Sheriff et al., 2011; Washburn et al., 2002). Glucocorticoid concentrations can be modeled in relation to a variety of conditions including sensory stimuli, enclosure limitations or confinement, human presence, diet, and social grouping (Morgan and Tromborg, 2007). Conditions most likely to reduce long-term stress can thereby be identified and implemented.

Corticosterone, a primary glucocorticoid in birds (Holmes and Phillips, 1976), has been indexed from blood (Washburn et al., 2002), feces (Möstl et al., 2005), and feathers (Bortolotti et al., 2008), but current techniques for measuring corticosterone concentrations present challenges. During blood collection, capture and handling methods elicit a measurable stress response unassociated with other factors of interest (Romero and Reed, 2005; Romero and Romero, 2002). Further, blood glucocorticoid analyses provide a short-term measure of corticosterone concentrations, and multiple samples must be collected over time to quantify a long-term response (Sheriff et al., 2011). Feces or feathers can be collected opportunistically, with feather glucocorticoid

Abbreviations: RIA, radioimmunoassay; PBS, phosphate buffered saline; SPE, solid-phase extraction; ANCOVA, analysis of covariance

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https://doi.org/10.1016/j.ygcen.2018.10.002
Received 24 April 2018; Received in revised form 28 September 2018; Accepted 1 October 2018
Available online 03 October 2018
0016-6480/

analyses offering a more long-term assessment of circulating corticosterone concentrations (Bortolotti et al., 2009). The amount of corticosterone in a feather reflects concentrations of circulating corticosterone during its growth period, and many feathers generated at different times can be used to measure concentrations over a period of weeks or months (Bortolotti et al., 2009, 2008). Finally, whereas blood and fecal samples require immediate refrigeration or extraction, feather corticosterone is stable, and samples can be stored at room temperature (Bortolotti et al., 2009). Feathers, therefore, offer substantial advantages for the measurement of glucocorticoids in birds.

Techniques for corticosterone extraction from feathers were first described by Bortolotti et al. (2008) and have proven useful in numerous studies (e.g., Fairhurst et al., 2015, 2013, 2011; Kennedy et al., 2013; Lattin et al., 2011; Lendvai et al., 2013; López-Jiménez et al., 2017, 2016; Perez et al., 2016), but investigation and discussion have revealed several limitations and technical concerns (Berk et al., 2016; Bortolotti, 2010; Romero and Fairhurst, 2016). For example, though studies have reported high extraction efficiencies as the percentage of added radioactivity recovered through filtering after adding radiolabeled corticosterone to chopped feathers in methanol (e.g., 98%, Fairhurst et al., 2013), these methods do not replicate hormone deposition into the feather. Thus, it is not the true extraction efficiency that is reported (Romero and Fairhurst, 2016). Others have pulverized feathers to create a homogenous "dust" to which radiolabeled corticosterone was added, but retention of methanol in this "dust" can decrease the efficiency of extraction (70–80%; Lattin et al., 2011). Both approaches can lead to inconsistent recovery of methanol depending on the mass and surface area of feather pieces, and thus, inconsistent extraction efficiencies (Romero and Fairhurst, 2016). Additionally, some studies have encountered the mass-dilution effect, finding disproportionally high concentrations of corticosterone in lighter sections of single feathers, or in small feather samples compared to larger ones (Berk et al., 2016; Bortolotti, 2010; Romero and Fairhurst, 2016). Finally, there remains uncertainty regarding which glucocorticoid metabolite is being measured in feathers (Romero and Fairhurst, 2016). We sought to identify an alternative feather corticosterone extraction technique to avoid some of the aforementioned limitations and provide additional support for the validity and practicality of extracting glucocorticoids from feathers.

Keratinolytic digestion of feather material offers a promising lead for analysis of non-protein biomolecules, such as corticosterone. Bacteria-derived keratinases can effectively degrade feather keratin (Gurav and Jadhav, 2013; Mazotto et al., 2011; Mukherjee et al., 2011; Poovendran et al., 2011; Tiwary and Gupta, 2012). By increasing the concentration of keratinase, feather material can be liquefied for use in laboratory applications initially intended for serum analysis.

Our main objective was to develop an improved steroid extraction method using keratinolytic digestion of feather material from the domestic chicken (*Gallus gallus domesticus*). Digestion of the feather matrix permitted the use of extraction methods intended for serum, avoiding many of the technical flaws accompanying previous feather extraction methods. Further objectives of this study included the assessment of this approach by (1) testing the efficiency of extraction, (2) checking parallelism of measured corticosterone concentrations from serial dilutions of digested feather material to a corticosterone standard response curve, and (3) evaluating accuracy of corticosterone concentrations measured from extracts using radioimmunoassay (RIA).

2. Materials and methods

To our knowledge, this is the first published account of steroid extraction from feather material digested by a keratinase. Thus, optimization and validation of extraction protocols were essential (Sheriff et al., 2011). We performed all tests described below in triplicate unless otherwise stated.

2.1. Subjects and sample collection

In accordance with approved Animal Care and Use Protocol 7617 through the University of Missouri, we collected feathers from 3 female domestic chickens (carcasses provided by a private farm in Columbia, MO) for protocol development and validation. We clipped feathers at the base of the vane using scissors, leaving calami attached to the skin. Consistent with Bortolotti et al. (2008), we did not include feather calami for steroid extraction.

2.2. Feather digestion

We used the keratinase Cibenza IND900 (Novus International, Inc., St. Charles, MO), which consists of *Bacillus licheniformis* fermentation solubles and sodium sulfate, to create a medium to digest feathers. To determine the amount of keratinase needed to degrade feathers, we evaluated digestibility across a gradient of enzyme concentrations. We prepared a concentrated stock of keratinase solution by adding Cibenza IND900 to alkaline phosphate buffered saline (PBS, pH 9.0) at a rate of 1 g of enzyme per 30 ml of PBS. We selected the pH of the solution and incubation temperature to support maximum enzymatic activity (N. Odetallah, Novus International, Inc., personal communication), while maintaining immunological activity of corticosterone and safe operating conditions for electronic equipment. We diluted the stock with additional alkaline PBS to prepare solutions at the following rates: 1:30000, 1:9000, 1:3000, 1:900, 1:300, 1:60, and 1:30 (original solution). We prepared test vials of each enzyme concentration by adding 1 feather to 3 ml of each of the solutions. We used primary flight feathers for these tests, which are larger and more robust than other feather types, of similar mass (88 ± 8.8 mg per feather). We placed vials in a drying oven (45 °C) and provided agitation using a tube rotator (model 60448; Scientific Equipment Products, Baltimore, MD). We observed the vials every 12 h to monitor the rate of digestion. We terminated incubation after visually determining that no further liquefying occurred between 2 observation periods.

2.3. Sample preparation

After identifying the conditions at which feathers were almost fully digested (see Results), we created a homogeneous pool of digested feathers to use in performing validation steps. For this step, we cut 20 g of contour feathers from the same 3 birds into < 1 cm pieces and added the feathers to a 1000 ml Erlenmeyer flask. We prepared a 1:30 solution of Cibenza IND900 in alkaline PBS by mixing 600 ml of PBS (pH 9.0) with 20 g of Cibenza IND900, and we added the solution to the flask. We added a stir bar and placed the flask on a magnetic stirrer (Corning Hot Plate Stirrer model PC-351; Corning Inc., Corning, NY) inside a drying oven (45 °C). After 5 days, we removed the flask from the oven and centrifuged the solution (15 min at 3750 × *g*) to separate remaining solids. We aliquoted the aqueous component into 50 ml conical tubes and collected the solid component into a separate 50 ml conical tube. Aqueous and solid components were frozen at −15 °C until extracted. We used the sample pool for all subsequent development and validation steps described herein.

To determine whether corticosterone was present in the aqueous or solid component of the digested sample pool, we reconstituted the solid component in the aqueous component at a rate of 1:30 (ratio of solid recovered from the pool, see Results) in 1 ml aliquots and added 50 µl of corticosterone-^{125}I (product 07-120121; MP Biomedicals, LLC, Santa Ana, CA). We vortexed the reconstituted solution vigorously to recombine the solid and aqueous components. We then centrifuged the mixture for 15 min at 3750 × *g*, after which we separated the supernatant from the pellet. We used a calibrated gamma counter to determine the amount of radioactivity present in the supernatant and pellet. Hereinafter, mention of the reconstituted sample pool will reference a reconstitution of the solid component into the aqueous

component in this ratio (1:30).

2.4. Extraction efficiency

To isolate steroids present, we used Strata-XL 100 μm polymeric reversed-phase solid-phase extraction (SPE) cartridges (model 8B-S043-UBJ, 3 ml, 60 mg; Phenomenex, Inc., Torrance, CA) conditioned with 2 ml of methanol followed by 2 ml of deionized water according to the manufacturer's protocol. We used a vacuum manifold to control the flow rate across cartridge sorbents at 2 ml/min. We collected sample pool solutions, washing solvents, and eluates from each cartridge into 1.2 ml polypropylene tubes. To assess the amount of radiolabeled corticosterone recoverable, we loaded cartridges with 1 ml aliquots of the reconstituted sample pool spiked with 50 μl of corticosterone-^{125}I. We then washed sorbents with 1 ml of 20% methanol in water and set the flow rate to maximum for 5 min to dry the cartridges. To optimize analyte recovery and assess extraction efficiency, we evaluated a range of elution solvents including 2% acetic acid in each of the following compounds: acetone, acetonitrile, ethyl acetate, methanol, methyl tertiary butyl ether, methylene chloride, and petroleum ether. We returned the flow rate to 2 ml/min and eluted sorbents with 2 rinses of 0.5 ml of elution solvent. We used a calibrated gamma counter to measure radioactivity in the collected sample solutions, washes, and eluates, and we calculated percent recovery from each. We also calculated the percent of radiolabeled corticosterone recovered from 1 ml aliquots of the aqueous component of the sample pool spiked with 50 μl of corticosterone-^{125}I using the same extraction procedures, except we only evaluated extraction efficiency using 2% acetic acid in acetone as the elution solvent.

2.5. Parallelism

To confirm that a standard corticosterone response curve could be used to determine corticosterone concentrations in dilutions of the reconstituted sample pool of digested feather material, we tested parallelism of the standard curve against respective curves resulting. We prepared 1 ml dilutions of the reconstituted sample pool in alkaline PBS at the following rates: neat, 1:2, 1:4, and 1:10 to assess parallelism of sample extracts to standard corticosterone concentrations. We used corticosterone calibrators (provided at a 1:200 dilution ratio; product 07-120130; MP Biomedicals, LLC) to develop a standard curve with corticosterone concentrations of 6.25 pg/ml, 12.5 pg/ml, 25 pg/ml, 50 pg/ml, 125 pg/ml, 250 pg/ml and 500 pg/ml. To determine if corticosterone concentrations could be determined similarly in the aqueous portion of the sample pool or a blank keratinase solution, we prepared dilutions of the aqueous component of the feather sample pool and a blank solution (i.e., no tissue was added) of the stock feather digestion medium in the same rates and assessed the parallelism of each to standard corticosterone concentrations.

2.6. Recovery

To determine if substances in the sample matrix affected the measured corticosterone concentrations differently than the standard curve (corticosterone calibrators in PBS), we conducted a spiked hormone recovery test. We used corticosterone calibrators 1 through 5 and diluted Standard 1 at 1:1 to spike 0.5 ml aliquots of the reconstituted sample pool with corticosterone concentrations of 6.25 pg/ml, 12.5 pg/ml, 25 pg/ml, 50 pg/ml, 125 pg/ml, and 250 pg/ml. We compared recovered corticosterone concentrations from samples to the concentrations calculated from the standard curve to evaluate the accuracy of corticosterone measurement from digested feather material. To determine how much hormone the SPE cartridges retained after extraction, we calculated the percent recovery of a known concentration of corticosterone in alkaline PBS (i.e., no tissue or keratinase was added). We used calibrators 1 through 5 to prepare 1 ml aliquots with corticosterone concentrations of 12.5 pg/ml, 25 pg/ml, 50 pg/ml, 125 pg/ml, and 250 pg/ml.

2.7. Elution and corticosterone measurement

We isolated steroids from prepared solutions using SPE cartridges as described above. To elute steroids from cartridges, we used 2 rinses of 0.5 ml of 2% acetic acid in acetone (the most effective elution solvent, see Results). We evaporated eluates to dryness in a vacuum centrifuge (Thermo Savant Speed-Vac Plus Concentrator model SC110A and Thermo Savant Universal Vacuum System model UVS400; Thermo Fisher Scientific, Waltham, MA) at 43 °C. We reconstituted residues in 325 μl of neutral PBS (pH 7.24) and assayed samples immediately or refrigerated (6 °C) samples overnight before performing additional steps.

We used a corticosterone RIA kit (product 07-120103; MP Biomedicals, LLC) previously validated for immunoreactive plasma avian corticosterone (e.g., Duffy et al., 2000; Palacios et al., 2007) to measure corticosterone concentrations in reconstituted samples. The assay had minimal cross-reactivity with other steroids (desoxycorticosterone, 0.34%; testosterone, 0.10; cortisol, 0.05%). The sensitivity of the assay was 6.25 pg/ml. We determined the intra- and interassay coefficients of variation using repeated measurements of the second highest (250 pg/mL) and second lowest (12.5 pg/mL) standards across assays. The intra- and interassay coefficients of variation were < 10%.

2.8. Statistical analysis

We used R Version 3.1.1 in RStudio Version 0.98.1028 (R Core Team, 2014) to complete all statistical tests at a significance level of 0.05. Using analysis of covariance (ANCOVA), we assessed the interactions between sample type (i.e., standard or sample/blank) and concentrations of corticosterone to determine if the slope of the standard curve was parallel to each of the following: (1) the slope of the curve formed using the reconstituted sample pool, (2) the slope of the curve formed using the aqueous component of the sample pool, or (3) the slope of the curve formed using the blank solution. We defined binding curves as parallel if the interaction term was non-significant ($P > 0.05$). To evaluate spiked hormone recovery, we calculated percent recovery by dividing the concentration of corticosterone observed (measured concentration of spiked samples minus the background concentration of 0.5 ml of sample) by the expected concentration of corticosterone added to each sample (as determined by the standard curve). We then plotted the expected concentrations versus the measured concentrations and conducted a linear regression analysis.

3. Results

3.1. Feather digestion

We determined that of those tested, undiluted keratinase stock solution (1:30) was most effective at liquefying feathers. Only the undiluted stock solution yielded a liquefied mixture suitable for SPE techniques. We first observed evidence of digestion in the 2 strongest concentrations (1:60 and 1:30) at 12 h. After 24 h, the feathers in vials prepared at a concentration of 1:300 began to degrade. Digestion of feather material in the 3 strongest concentrations continued to progress over the next several observations, with nearly complete digestion in the undiluted stock (a small amount of powder-like material approximately equal to the mass of feather used remained). Digestion activity plateaued on day 5, whereupon we discontinued incubation. None of the feather material incubated in the 4 weakest concentrations (1:30000, 1:9000, 1:3000, 1:900) degraded.

Table 1
Extraction efficiency.

Elution solvent	% radioactivity recovered*
Acetone	97.4% (89–100%)
Acetonitrile	83.2% (80–85%)
Ethyl acetate	60.6% (53–68%)
Methanol	63.8% (63–65%)
Methyl tertiary-butyl ether	19.4% (19–20%)
Methylene chloride	11.7% (9–14%)
Petroleum ether	0% (0%)

* Mean percent of radioactivity recovered across replicates is provided first, followed by the range of efficiency across replicates rounded to the nearest whole percent in parentheses.

3.2. Sample preparation

We recovered 20.017 g of solid white material and 600 ml of liquid. Corticosterone was present in the aqueous and solid components of the pool. After centrifugation, the pellet containing the solid component of the sample pool retained approximately 80% of radiolabeled corticosterone, whereas the other 20% was recovered in the supernatant. We reconstituted the sample pool at a rate of 1:30 in further analyses.

3.3. Extraction efficiency

The amount of radiolabeled corticosterone recovered from the reconstituted sample pool after extraction using SPE cartridges varied with elution solvent used (Table 1). Acetone performed most effectively with 89–100% (n = 5, average = 97.4%, standard deviation = 6.4%) of radiolabeled corticosterone recovered in the eluate. Negligible radioactivity was lost during sample loading or wash steps. Radioactivity recovered in either step was ≤250 counts per minute or ≤2.4% radiolabeled corticosterone. Extraction efficiency from the aqueous component of the sample pool using acetone was similarly high and repeatable (n = 4, range = 94–100%, average = 98.6%, standard deviation = 5.0%).

3.4. Parallelism

The slope of the curve formed using dilutions of the reconstituted sample pool was parallel to the slope of the standard curve (interaction: $F_{1,25} = 2.96$, $P = 0.10$; Fig. 1A). The slope of the curve formed using dilutions of the aqueous component of the sample pool was also parallel to the slope of the standard curve (interaction: $F_{1,25} = 0.042$, $P = 0.84$; Fig. 1B). The slope of the curve formed using the blank keratinase solution was not parallel to the slope of the standard binding curve (interaction: $F_{1,25} = 81.48$, $P < 0.001$; Fig. 1A). However, we detected a small amount of analyte in the blank solution.

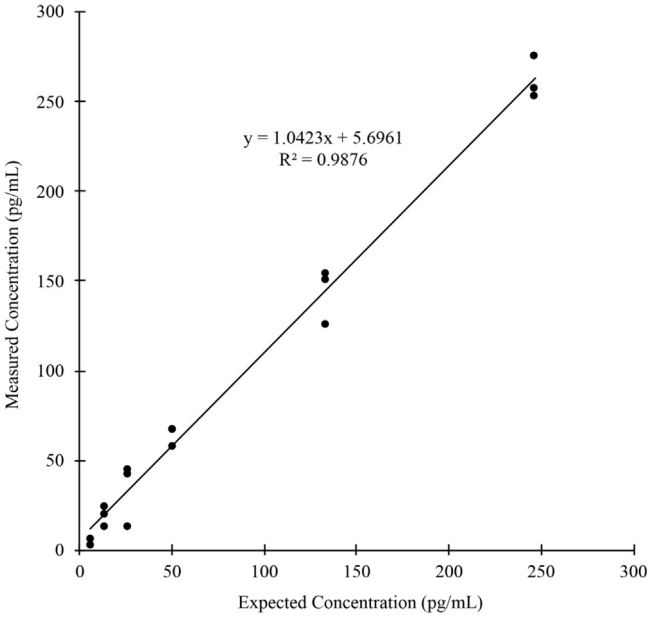

Fig. 2. Spiked hormone recovery test. We conducted a linear regression analysis on the concentrations of feather corticosterone expected versus the concentrations observed in spiked samples (y = 1.0423x + 5.6961, $r^2 = 0.9876$). The slope (1.0423) was > 1, indicating a slight overestimation of corticosterone mass.

3.5. Recovery

Our procedures produced a slight overestimation of corticosterone mass (see Brown et al., 2004) as evidenced by a slope of > 1 when expected concentrations were plotted against measured concentrations in samples spiked with hormone ($r^2 = 0.9876$; Fig. 2). For standard concentrations ≥12.5 pg/ml, > 94% of added corticosterone was recovered. For the standard concentration of 6.25 pg/ml, we could not calculate the percent of hormone recovery due to variation in background measurements. Although the coefficient of variation was < 5%, the difference between the lowest and highest corticosterone concentrations in the background was > 6.25 pg/ml. An average of 98% of standard corticosterone solution (no tissue or keratinase added) was

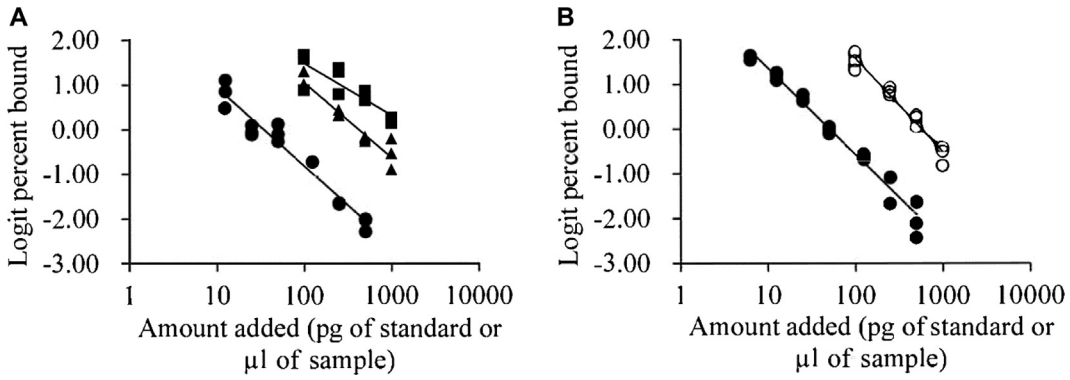

Fig. 1. (A) Parallelism of the reconstituted sample pool and nonparellelism of the blank keratinase solution to the corticosterone standard curve. Standards (closed circles): y = −1.7596x + 2.7025, $r^2 = 0.9731$; Reconstituted Sample Pool (triangles): y = −1.6818x + 4.4192, $r^2 = 0.9629$; Blank (squares): y = −1.1626x + 3.8110, $r^2 = 0.8595$. (B) Parallelism of the aqueous component of the sample pool to the corticosterone standard curve demonstrated in a separate assay. One replicate was removed from the lower end of the standard curve (6.25 pg/ml) prior to analysis due to unreliable measurement by the assay (binding > 95%). Standards (closed circles): y = −1.9209x + 3.2736, $r^2 = 0.9863$; Aqueous Component of Sample Pool (open circles): y = −2.0771x + 5.7357, $r^2 = 0.9790$.

recoverable from SPE columns.

4. Discussion

We present the first evidence of an improved steroid extraction procedure using keratinolytic digestion of chicken feathers. We tested several feather digestion conditions and extraction protocols and selected the methods resulting in the most complete digestion and highest efficiency of extraction. Our results demonstrated high and repeatable extraction efficiency, as well as high accuracy and reliable parallelism to a standard curve upon measurement of corticosterone concentrations from extracts using RIA.

Keratinolytic digestion of feather material for extraction of corticosterone is advantageous for many reasons. To our knowledge, this is the first feather corticosterone study to report results of a spiked hormone recovery test, as we were able to replicate the deposition of hormone into the digested feather matrix prior to extraction procedures. Using previous methods, exogenous steroids were added to the feather pieces during the methanol extraction step (see Romero and Fairhurst, 2016). We also achieved easily interpretable and consistent extraction efficiency using SPE procedures. Previous tests of extraction efficiency either estimated hormone recovery through filtering (not a true measure of extraction efficiency, see Romero and Fairhurst, 2016) or reported inconsistent extraction after adding radiolabeled corticosterone to pulverized feather "dust" (Lattin et al., 2011; Romero and Fairhurst, 2016). While most previous feather corticosterone studies did not report a direct measure of the consistency of extraction efficiency, we improved upon that reported in Berk et al. (2016), in which methanol extraction yielded an average of 86% efficiency (standard deviation = 10%). Another advantage to dissolving the entire feather into solution is that it permitted consistent extraction of corticosterone across all sections of the feather. While this aspect of our digestion and extraction methods limited the ability to investigate the timing of corticosterone deposition into feathers, it simplified the interpretation of corticosterone deposition along feather lengths. For example, if feathers of similar size, morphology, and condition are compared, our protocol can thereby avoid the mass-dilution effect, which erroneously inflates corticosterone concentrations measured from smaller portions of feather (Bortolotti et al., 2009, 2008). It does, however, remain unclear if corticosterone deposition varies across the length of a feather or between different feather types (Bortolotti, 2010; Romero and Fairhurst, 2016). Finally, given the availability and low cost of commercial keratinases, our protocol could extend to crude studies monitoring corticosterone concentrations within populations. Feathers from multiple birds can be dissolved (i.e., homogenized), and small aliquots can be extracted and measured for quick and inexpensive assessments of feather corticosterone concentrations on the population level.

Conversely, several limitations remain unchecked and should not be overlooked. Studies across populations are challenging because variation in molt schedules can require assumptions about molt synchronization when the dates of feather growth are unknown (Romero and Fairhurst, 2016). All studies using feathers to index corticosterone concentrations require an understanding of feather development and molting in target species. Feather corticosterone concentrations reflect circulating hormone levels during the period of feather growth (Bortolotti et al., 2008), and analytes measured from mature feathers thus represent the biological condition of the animal in preceding times. This limitation can be avoided if feathers are pulled prior to molt to initiate redevelopment, and newly grown feathers are monitored and collected to obtain information about the time of feather re-growth (Fairhurst et al., 2011). Additionally, there has been difficulty correlating feather corticosterone concentrations with plasma corticosterone concentrations as expected (Fairhurst et al., 2013). Given our sampling methods, we could not collect whole blood samples from subjects (opportunistically collected broiler carcasses, which were frozen before feather collection) to test for an association between feather and plasma corticosterone concentrations. It remains unclear how short-term elevations of plasma corticosterone are integrated into feathers over time (see Romero and Fairhurst, 2016). Finally, we did not experimentally elevate corticosterone concentrations or infer high or low corticosterone animals across subjects because mature feathers were opportunistically obtained, and thus the condition of animals during feather growth was unknown. A biological validation would provide an informative link between corticosterone extracted from feathers after keratinolytic digestion and other factors associated with stress. Moreover, our study focused solely on the extraction of steroids from the contour feathers of the domestic chicken, and further validations would be necessary to interpret measurement of corticosterone from other feather types (Bortolotti et al., 2008) or feathers from new species (Sheriff et al., 2011).

We found that a reconstitution of the solid and aqueous components of the sample pool of digested feather material provided the most complete record of corticosterone concentrations within the feather. Therefore, we suggest the elimination of the step to separate solids from the sample pool, which was completed here for the purpose of testing and evaluating methodologies. We determined our reconstitution ratio based on the amount of solid recovered from the pool and, thus, intended to replicate conditions in which separation did not occur. However, performing the protocol using the reconstituted sample pool required SPE cartridges with a large pore size and resulted in sluggish flow through the cartridges. We therefore suggest that it is possible to use only the supernatant for further simplification of the procedure. While only a portion of radiolabeled corticosterone was recoverable in the supernatant, the consistency of the ratio of radiolabeled corticosterone recovered, as well as the high extraction efficiency and parallelism to standard corticosterone concentrations using only the aqueous component suggest the usefulness of this liquid portion of the sample pool in measuring relative corticosterone concentrations. We did not perform a spike recovery test using only the aqueous component of the sample pool. Future studies should test this to evaluate the extraction of steroids from the aqueous component only.

This work provides additional support for the validity and practicality of measuring glucocorticoid concentrations from feathers. We provided evidence that another commercial antibody (product 07-120114; MP Biomedicals, LLC) can detect feather corticosterone concentrations. This antibody has been used to measure plasma concentrations of avian corticosterone (e.g., Duffy et al., 2000; Palacios et al., 2007), but to our knowledge had not yet been used in feather corticosterone studies. Although the assay had minimal cross-reactivity with other steroids, we detected small amounts of analyte in the blank keratinase solution. This could suggest additional cross-reactivity with analytes not tested by the manufacturer, as it is unlikely the keratinase itself contained corticosterone. In future studies using this method, the blank keratinase solution should be measured and final sample concentrations adjusted accordingly. More broadly, this protocol may extend to glucocorticoid monitoring in a variety of other species. While our specific motivation was stress management in avian husbandry, keratinases like Cibenza IND900 are likely to digest other protein matrices with similar efficacy. For example, it may be possible to extract corticosterone from digested hair, skin, scales, nails, hooves, horns or other keratinized tissues from various taxa. We further speculate that isolation and measurement of other non-protein biomolecules in keratinized tissues may be possible.

We encourage others to adopt this approach in studies aimed at stress management in facilities housing bird populations. As long-term exposure to corticosterone can have immunosuppressive and anti-reproductive effects (Charmandari et al., 2005), the mitigation of stressful conditions may improve health and breeding. We further encourage the standardization of feather corticosterone extraction across labs and argue that this method provides a repeatable and effective alternative to previous methods.

Acknowledgments

We thank M. Smith, E. Benavides, and A. Abbasian for assistance with laboratory techniques; B. Lamberson and K. Pohler for help with statistical analyses; and D. Lyons and D. Ledoux for helping with sample collection. Keratinase for feather digestion was donated by Novus International, Inc., St. Charles, MO. This work is published contribution number 594 from The Institute for Bird Populations, and it was funded by the University of Missouri.

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