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Validation of a fecal glucocorticoid metabolite assay to assess stress in the budgerigar (*Melopsittacus undulatus*)

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Abstract

The budgerigar (*Melopsittacus undulatus*) is a small parrot native to Australia that is commonly held in zoos, laboratories, and private homes. Assessment of budgerigar stress levels would aid welfare monitoring and improve our understanding of their biology. Analyzing fecal glucocorticoid metabolites provides a non-invasive method to measure stress levels in birds. For this method to be reliable, the antibody to be used in an immunoassay must be carefully selected for each species, and validation must be performed. A common limitation in many existing assays is the inability to accurately detect variable fecal glucocorticoid metabolites levels in minute quantities of feces, requiring small samples to be combined. We have developed a double antibody radioimmunoassay protocol based on a commercially available ¹²⁵I-corticosterone radioimmunoassay kit for use in detecting fecal glucocorticoid metabolites in small quantities (< 20 mg) of budgerigar droppings. The assay was validated pharmacologically with an adrenocorticotrophic hormone challenge and with oral administration of corticosterone. Our validation has demonstrated our assay is both sensitive and a reliable approach to non-invasive monitoring of stress in budgerigars.

Keywords

radioimmunoassay; ACTH challenge; fecal glucocorticoid metabolite; stress; avian; budgerigar

STATEMENT OF THE PROBLEM

The budgerigar (*Melopsittacus undulatus*) is a small parrot (40 grams) native to Australia that is commonly maintained in captivity in zoos, laboratories, and private homes. Budgerigars have been the focus of a growing number of walk through aviaries in zoos and in laboratory experiments. Non-invasive stress analysis would expand the understanding of the species' physiology and welfare.

The predominant avian glucocorticoid (GC) is corticosterone, a hormone released in response to a potential threat to homeostasis which is used to regulate behavior and suppress bodily processes that are non-essential to survival [Wingfield, 1994]. Levels of circulating GC are considered a reliable indicator of stress levels in birds [Dehnhard et al., 2003; Hartup et al., 2004]. Traditional measuring of GC concentrations in plasma requires handling and blood collection, which may be undesirable as restraint itself induces stress [Goymann, 2005; Mostl et al., 2005; Touma and Palme, 2005] and can cause a substantial rise in GC

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concentrations in the blood after two or three minutes [Hiebert et al., 2000; Romero and Reed, 2005]. Plasma analysis may not be feasible for small birds because size imposes a constraint on the volume and frequency of blood that can be drawn [Goymann, 2005; Hiebert et al., 2000].

Analyses of hormone metabolite levels in feces offer an attractive alternative. Feces can be collected non-invasively, so GC concentrations are not affected by sampling itself [Mostl et al., 2005; Touma and Palme, 2005]. Steroid metabolites in feces are cumulative over a range of time, so they provide an integrated measure of GC concentrations [Goymann, 2005; Hirschenhauser et al., 2005; Ninnis et al., 2010]. However, steroids such as GC are metabolized in the liver and excreted into the gut through bile [Mostl et al., 2005; Palme, 2005; Touma and Palme, 2005]. Therefore, GC are not actually present in the feces, but rather their metabolites [Carere et al., 2003].

A large assumption in analyzing fecal glucocorticoid metabolites (FGM) is that the concentration of hormone metabolites detected in the feces is indicative of the concentration of circulating hormones integrated over time [Goymann, 2005; Hirschenhauser et al., 2005]. FGM assays have been validated for a multitude of species [see Chinnadurai et al., 2009; Touma and Palme, 2005; Wasser et al., 2000], although fewer studies have focused on parrots [Cockrem et al., 2004; Popp et al., 2008], or small (< 200 g body mass) avian species [Hiebert et al., 2000; Washburn et al., 2003]. A common limitation is the inability to accurately detect variable FGM levels in minute quantities of feces. A negative relationship between fecal sample mass and FGM concentration has been found previously [Millspaugh and Washburn, 2004, but see Goymann, 2005], leading to the suggestion of only analyzing larger fecal samples (> 20 mg dried mass). This limit precludes analyzing individual samples from many species of smaller birds which typically produce a fecal sample that is less than 20 mg. Researchers working with small amounts of feces have previously used a charcoal assay [Wasser et al., 1997], which is labor and time intensive, or combined multiple fecal samples [Cyr and Romero, 2008; Harper and Austad, 2001; Millspaugh and Washburn, 2004; Pereira et al., 2010]. A radioimmunoassay (RIA) for smaller samples of avian feces has only been recently reported for spotted owls [Hayward et al., 2010] and stonechats [Goymann et al., 2006a]. Our objective was to validate a double antibody RIA capable of detecting FGM in small amounts of budgerigar droppings based on a commercially available ^{125}I -corticosterone RIA kit (MP Biomedicals, Orangeburg, NY).

DESCRIPTION OF THE PROCESS

All research was approved by the NMSU Institutional Animal Care and Use Committee (# 2007-011).

Collection and Preparation of Droppings

We housed adult, captive bred budgerigars in same sex groups of four in wire-mesh cages (50 × 60 × 90 cm). They were given water and a commercial seed diet (Brooks Brand Seeds, Lawton, OK) *ad libitum* and housed on a 12/12 light and dark cycle. We caught budgerigars by hand and placed them individually into smaller wire-mesh cages (11 × 20 × 15.75 cm) lined with paper. After budgerigars had been isolated for 20 to 30 min, individual samples comprising the entire fecal and urate mass were collected and stored frozen at -20°C.

Before assay, droppings were thawed at room temperature, placed in 12 × 75 mm borosilicate glass tubes and weighed wet. We dried samples in a forced-air oven at 90°C for 1 h to destroy bacterial enzymes that can further metabolize feces [Mostl et al., 2005; Palme, 2005]. As it was not always possible to separate the fecal and urate component of the sample, both components were ground, homogenized, and weighed. FGM were extracted by

adding 2 mL of methanol to each tube (dry sample 3 to 30 mg each) followed by vigorous vortexing for 5 min, centrifugation ($2,300 \times g$, 15 min, 4°C), and freezing (-85°C for 15 min). This process resulted in the fecal/urate pellet being frozen to the bottom of the tube, so we could then easily decant the methanol solvent supernatant to another 12×75 mm glass tube and evaporate it to dryness under a stream of air at 90°C . We added Phosphate Buffered Saline (PBS, 0.01M) with 0.1% gelatin (Knox Gelatine, Kraft Foods, Tarrytown, NY; PBS + Gel, pH 7.0, 1 mL) to each tube, vortexed them vigorously for 5 min, and stored them at -20°C until the assay was performed.

Radioimmunoassay of Fecal Glucocorticoid Metabolites

Sample preparation and RIA of FGM were similar to the method described by Washburn et al. (2003) and Hayward et al. (2010). The same rabbit anticorticoesterone and ^{125}I -corticoesterone from MP Biomedicals (Solon, OH) were used but several procedural modifications were employed. The primary antibody (MP 07-120116) was diluted 1:10,000 in PBS + EDTA (ethylenediaminetetraacetic acid, 0.05M) + 1:400 normal rabbit serum (pH 7.0). The ^{125}I -corticoesterone ($10 \mu\text{Ci}$, MP 07-120126) was suspended in PBS + Gel such that 0.1 mL contained approximately 15,000 cpm. A second antibody was prepared by immunizing castrated male sheep against rabbit gamma globulins (Sigma, St. Louis, MO). The second antibody (sheep anti-rabbit gamma globulin) was diluted 1:10 in PBS + EDTA before use in the RIA. Two stock standard solutions were prepared for use in the working standards of each assay by suspending corticoesterone (Sigma) at 1 ng/mL and 10 ng/mL in PBS + Gel.

On the day of assay, we thawed glass tubes containing the dried methanol extracts in 1.0 mL PBS + Gel and centrifuged them for 30 min at $2,300 \times g$ and 4°C . We divided the 1.0 mL of PBS + Gel for each sample such that 0.4 mL was transferred to each of two 12×75 mm borosilicate glass assay tubes. We prepared duplicate standards by pipetting appropriate volumes of the 1 ng corticoesterone/mL stock to 12×75 mm glass tubes that contained the dried extract from 2 mL of methanol to yield 0, 20, 40, 80, 160 (using the 1 ng/mL stock), 320, and 640 (using the 10 ng/mL stock) pg of corticoesterone per tube. We then normalized all tubes containing standards to 0.4 mL using PBS + Gel. Each assay and standard tube subsequently received 0.2 mL of the primary antibody (1:10,000 dilution) followed by 0.1 mL of the ^{125}I -corticoesterone. We vortexed tubes briefly and incubated them at room temperature for 4 hours. Following incubation, we added 0.2 mL of second antibody, vortexed tubes, then incubated them overnight at 4°C , after which we centrifuged tubes at $2,300 \times g$ for 15 min at 4°C . We discarded the supernatant and counted the bound fraction in the precipitate for 1 min in a Packard Cobra II gamma counter. We calculated concentrations of corticoesterone using the four-parameter logistic method to fit the sigmoid nature of the standard curve.

DEMONSTRATION OF EFFICACY

Although antibody specificity was not determined in this experiment, the manufacturer reported a 100% cross reactivity with corticoesterone and less than 0.5% cross reaction with 17 other steroids including desoxycorticoesterone, cortisol, 11-desoxycortisol, and aldosterone.

Assay performance was evaluated using a series of recovery experiments. Sensitivity of the assay was determined by conducting a close interval spike experiment. When the fecal pool was spiked with 2, 4, 8, and 12 pg/mg of corticoesterone, 94, 109, 115, and 113%, respectively, were recovered (all coefficients of variation were 14% or less). Based on these results, we determined the assay sensitivity to be 2 pg/mg of dried feces. To verify assay accuracy and precision, a pool of dried budgerigar feces was spiked with 12 (low), 25

(medium), and 50 (high) pg corticosterone/mg. When these samples were assayed, 89.3, 87.8, and 93.9% of the added corticosterone was recovered, respectively. To assess assay precision, budgerigar droppings containing the low, medium, and high amounts of FGM metabolites were measured in 10 assays. Between assay coefficients of variation for the three respective samples were 19, 18, and 15%. Within assay coefficients of variation over the 10 assays averaged 12, 11, and 12% for the low, medium, and high pools, respectively. Assay of 10, 20, 40, and 60 mg of the pool resulted in displacement parallel to the standard curve. Specific binding determined over 10 assays was $55.3 \pm 2.0\%$ (mean $\pm$ SE) and addition of 20 pg of corticosterone resulted in 14% of the ^{125}I -competitor being displaced from the antibody.

ACTH Challenge

To determine whether our RIA could detect changes in FGM corresponding to pharmacologically induced changes in circulating GC, we isolated 16 adult male budgerigars in small cages and injected either saline ($n = 8$; 0.9% sodium chloride solution) or an ACTH saline solution ($n = 8$; 10 μg ACTH hormone fragment 1-24 human, Sigma-Aldrich Co., St. Louis, MO, in 10 μL of saline) into the brachial vein with a 25-gauge needle fitted on a 1-mL syringe. Droppings were then collected for the next 8 hr during which time all 16 birds were kept in the smaller cages. We replaced budgerigars into their larger group cages overnight; at 25 hr after the challenge, we again hand caught and isolated them until they defecated. If an individual defecated more than once during the sample collection period, these samples were combined while wet.

A significant interaction between treatment and sampling time was observed in a repeated measures ANOVA ($F_{(7,91)} = 2.61$, $P = 0.017$; Fig. 1), necessitating examination of treatment effects within sampling time. FGM concentrations in the ACTH-treated group were greater than those in the controls for the first ($F_{(1,13)} = 4.77$, $P = 0.048$) and second ($F_{(1,14)} = 5.46$, $P = 0.035$) time periods after treatment, 77 ± 14 and 134 ± 14 min (mean $\pm$ SD) after injection. These responses demonstrate that the assay was able to detect a pharmacologically induced elevation in FGM approximately an hour after injection of ACTH.

Both treatment groups experienced an increase in FGM after being confined for several hours. Classic restraint stress induction in birds involves immobilizing a bird in the hand for minutes or in a bag for up to an hour. The small cages we used were familiar to the budgerigars and did allow for minimal movement, but they previously had not spent more than 20 minutes at a time in them. Thus, we consider the rise in FGM concentrations over time in the control group to be indicative of an increased stress response due to restraint. An increase in corticosterone production over restraint time has also been demonstrated in male rats [Livezey et al., 1985].

Oral Administration of Corticosterone

In addition to an ACTH challenge, we validated our assay by feeding budgerigars exogenous corticosterone suspended in peanut oil following Spencer *et al.* [2003] and Spencer and Verhulst [2007]. We isolated 16 adult male budgerigars in cages ($50 \times 60 \times 45\text{cm}$) and allowed for visual contact through a plexiglass barrier with one other male. Droppings were collected in the morning, midday, and afternoon. Birds were stratified by weight and randomly assigned to a treatment group of either a) control (0.0 mg), b) low corticosterone (0.05 mg), c) medium corticosterone (0.1 mg) or d) high corticosterone (0.2 mg). Corticosterone was suspended in three solutions of peanut oil (0.25 mg/mL, 0.50 mg/mL, and 1.00 mg/mL), and administered orally to budgerigars in the morning via a .2-mL dose every day for four consecutive days.

FGM results were not normally distributed, so we log transformed the data prior to statistical analysis. We observed a significant interaction between treatment and time in a repeated measures ANOVA ($F_{(66, 264)} = 4.47$, $P < 0.001$). A Tukey HSD detected significant differences between treatment groups, with means increasing in a dose dependent manner (Fig 2). The low dose of corticosterone was sufficient to statistically detect elevated FGM levels above control levels.

Conclusions

Our data demonstrate the accuracy, precision, parallelism, and validity of using an altered commercial double antibody RIA kit to detect FGM concentrations in small amounts of budgerigar droppings. The assay detected a pharmacologically induced elevation in FGM approximately an hour after an injection of ACTH, and a dose dependent increase in FGM after oral administration of corticosterone. Due to the difficulty of separating fecal and urates in budgerigar droppings, it is not clear whether any one component contributes more to the FGM concentrations. The assay should be generally useful for the non-invasive monitoring of stress using small amounts of droppings in captive budgerigars. Caution should be exercised if applying this method to wild birds or those living in a variety of settings as diet and temperature can affect FGM concentrations [Goymann, 2005; Goymann et al., 2006b; Klasing, 2005].

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REFERENCES

- Carere C, Groothuis TGG, Möstl E, Daan S, Koolhaas JM. Fecal corticosteroids in a territorial bird selected for different personalities: Daily rhythm and the response to social stress. *Horm and Beh.* 2003; 43(5):540–548.
- Chinnadurai SK, Millspaugh JJ, Matthews WS, Canter K, Slotow R, Washburn BE, Woods RJ. Validation of fecal glucocorticoid metabolite assays for south african herbivores. *J Wildl Manage.* 2009; 73(6):1014–1020.
- Cockrem, JF.; Adams, DC.; Bennett, EJ.; Candy, EJ.; Henare, SJ.; Hawke, EJ.; Potter, MA.; Gordon, MS.; Bartol, SM. Endocrinology and the conservation of New Zealand birds.. In: Gordon, MS.; Bartol, SM., editors. *Experimental Approaches to Conservation Biology*. University of California Press; Los Angeles: 2004. p. 101-121.
- Cyr NE, Romero LM. Fecal glucocorticoid metabolites of experimentally stressed captive and free-living starlings: Implications for conservation research. *Gen Comp Endocrinol.* 2008; 158(1):20–28. [PubMed: 18554600]
- Dehnhard M, Schreer A, Krone O, Jewgenow K, Krause M, Grossmann R. Measurement of plasma corticosterone and fecal glucocorticoid metabolites in the chicken (*Gallus domesticus*), the great cormorant (*Phalacrocorax carbo*), and the goshawk (*Accipiter gentilis*). *Gen Comp Endocrinol.* 2003; 131(3):345–352. [PubMed: 12714017]
- Goymann W. Noninvasive monitoring of hormones in bird droppings: Physiological validation, sampling, extraction, sex differences, and the influence of diet on hormone metabolite levels. *Annals NY Acad Sci.* 2005; 1046(1):35–53.
- Goymann W, Geue D, Schwabl I, Flück H, Schmidl D, Schwabl H, Gwinner E. Testosterone and corticosterone during the breeding cycle of equatorial and European stonechats (*Saxicola torquata axillaris* and *S. t. rubicola*). *Horm and Beh.* 2006a; 50(5):779–785.
- Goymann W, Trappschuh M, Jensen W, Schwabl I. Low ambient temperature increases food intake and dropping production, leading to incorrect estimates of hormone metabolite concentrations in European stonechats. *Horm and Beh.* 2006b; 49(5):644–653.

- Harper JM, Austad SN. Effect of capture and season on fecal glucocorticoid levels in deer mice (*Peromyscus maniculatus*) and red-backed voles (*Clethrionomys gapperi*). *Gen Comp Endocrinol*. 2001; 123(3):337–344. [PubMed: 11589634]
- Hartup BK, Olsen GH, Czekala NM, Paul-Murphy J, Langenberg JA. Levels of fecal corticosterone in sandhill cranes during a human-led migration. *J Wildl Dis*. 2004; 40(2):267–272. [PubMed: 15362826]
- Hayward LS, Booth RK, Wasser SK. Eliminating the artificial effect of sample mass on avian fecal hormone metabolite concentration. *Gen Comp Endocrinol*. 2010; 169:117–122. [PubMed: 20709063]
- Hiebert SM, Ramenofsky M, Salvante K, Wingfield JC, Gass CL. Noninvasive methods for measuring and manipulating corticosterone in hummingbirds. *Gen Comp Endocrinol*. 2000; 120(2):235–247. [PubMed: 11078634]
- Hirschenhauser K, Kotschal K, Mostl E. Synthesis of measuring steroid metabolites in goose feces. *Annals NY Acad Sci*. 2005; 1046(1):138–153.
- Klasing, KC. Potential impact of nutritional strategy on noninvasive measurements of hormones in birds.. In: Bauchinger, U.; Goymann, W.; JenniEiermann, S., editors. *Bird hormones and bird migrations: Analyzing hormones in droppings and egg yolks and assessing adaptations in long-distance migration*. 2005. p. 5-16.
- Livezey GT, Miller JM, Vogel WH. Plasma norepinephrine, epinephrine and corticosterone stress responses to restraint in individual male and female rats, and their correlations. *Neurosci letters*. 1985; 62(1):51–56.
- Millspaugh JJ, Washburn BE. Use of fecal glucocorticoid metabolite measures in conservation biology research: Considerations for application and interpretation. *Gen Comp Endocrinol*. 2004; 138(3): 189–199. [PubMed: 15364201]
- Mostl E, Rettenbacher S, Palme R. Measurement of corticosterone metabolites in birds' droppings: An analytical approach. *Annals NY Acad Sci*. 2005; 1046(1):17–34.
- Ninnes CE, Waas JR, Ling N, Nakagawa S, Banks JC, Bell DG, Bright A, Carey PW, Chandler J, Hudson QJ. Comparing plasma and faecal measures of steroid hormones in Adelie penguins *Pygoscelis adeliae*. *J Comp Phys B*. 2010; 180(1):83–94.
- Palme R. Measuring fecal steroids: Guidelines for practical application. *Annals NY Acad Sci*. 2005; 1046(1):75–80.
- Pereira RJG, Granzinolli MAM, Duarte JMB. Annual profile of fecal androgen and glucocorticoid levels in free-living male American kestrels from southern mid-latitude areas. *Gen Comp Endocrinol*. 2010; 166(1):94–103. [PubMed: 20026334]
- Popp LG, Serafini PP, Reghelin ALS, Sperscoski KM, Roper JJ, Morais RN. Annual pattern of fecal corticoid excretion in captive red-tailed parrots (*Amazona brasiliensis*). *J Comp Phys B*. 2008; 178(4):487–493.
- Romero LM, Reed JM. Collecting baseline corticosterone samples in the field: Is under 3 min good enough? *Comp Biochem and Physi A*. 2005; 140(1):73–79.
- Spencer KA, Buchanan KL, Goldsmith AR, Catchpole CK. Song as an honest signal of developmental stress in the zebra finch (*Taeniopygia guttata*). *Horm and Beh*. 2003; 44(2):132–139.
- Spencer KA, Verhulst S. Delayed behavioral effects of postnatal exposure to corticosterone in the zebra finch (*Taeniopygia guttata*). *Horm and Beh*. 2007; 51(2):273–280.
- Touma C, Palme R. Measuring fecal glucocorticoid metabolites in mammals and birds: The importance of validation. *Annals NY Acad Sci*. 2005; 1046(1):54–74.
- Washburn BE, Millspaugh JJ, Schulz JH, Jones SB, Mong T. Using fecal glucocorticoids for stress assessment in mourning doves. *The Condor*. 2003; 105(4):696–706.
- Wasser SK, Bevis K, King G, Hanson E. Noninvasive physiological measures of disturbance in the northern spotted owl. *Cons Biol*. 1997; 11(4):1019–1022.
- Wasser SK, Hunt KE, Brown JL, Cooper K, Crockett CM, Bechert U, Millspaugh JJ, Larson S, Monfort SL. A generalized fecal glucocorticoid assay for use in a diverse array of nondomestic mammalian and avian species. *Gen Comp Endocrinol*. 2000; 120(3):260–275. [PubMed: 11121291]

Wingfield JC. Modulation of the adrenocortical response to stress in birds. *Perspec Comp Endocrinol.* 1994;520–528.

Fig. 1.

ACTH challenge. Fecal glucocorticoid metabolite (FGM) concentrations for budgerigars challenged in response to ACTH. Treatments (10 μ L of saline for controls and 10 μ g of ACTH in 10 μ L of saline for challenged birds) were administered intravenously immediately after the baseline sample was collected. Droppings were collected to minimize time left exposed and maximize sample number, approximately every hour or two after injection, and again the next day. Each point represents the mean $\pm$ SE for control (open grey circle, $n = 8$ droppings, one sample per individual) or ACTH-challenged (filled black circle, $n = 8$ droppings, one sample per individual) birds. There are two control periods and three experimental periods where one or more individuals did not produce a sample, so the mean is based on $n = 6$ or $n = 7$. Asterisks indicate that treatments are significantly different ($P < 0.05$).

Fig. 2.

Log of fecal glucocorticoid metabolite (FGM) concentrations for budgerigars challenged in response to oral administration of corticosterone. Droppings were sampled three times a day (AM: prior to administration of corticosterone, MID: ~1.5 hours after administration, PM: ~5.5 hours after administration) starting the day prior to the first administration, and continuing for a week after the last administration. Four doses of peanut oil with different concentration of corticosterone (control, low, medium, and high) were fed to budgerigars ($n = 4$ per dose) in morning after AM fecals were sampled for Dose Day 1 – Dose Day 4. Each point represents the log transformed FGM mean $\pm$ SE.