

Fecal steroid monitoring for assessing gonadal and adrenal activity in the golden eagle and peregrine falcon

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Abstract We examined the efficacy of noninvasive monitoring of endocrine function via fecal steroid immunoassays in the golden eagle and peregrine falcon. High-pressure liquid chromatography analyses of fecal glucocorticoid metabolites (fGCM) revealed that minor percentages of immunoreactive fGCM co-eluted with [³H]corticosterone in both sexes of the eagle (2.5–2.7%) and falcon (7.5–11.9%). In contrast, most fecal estrogen metabolites in eagle and falcon females co-eluted with radiolabeled estradiol-17β ([³H]; 57.6, 64.6%, respectively) or estrone ([³H]; 26.9, 4.1%, respectively). Most fecal progestin metabolite immunoreactivity in the female eagle (24.8%) and falcon (21.7%) co-eluted with progesterone ([¹⁴C]). Most fecal androgen metabolite immunoreactivity in eagle (55.8%) and falcon (63.7%) males co-eluted with testosterone ([¹⁴C]). Exoge-

nous adrenocorticotropin hormone induced increased fGCM excretion above pre-treatment in both species, but only significantly ($P < 0.05$) in the eagle. Both species showed increased fGCM after saline administration, suggesting the detection of ‘handling stress.’ Both species exhibited enterohepatic and renal recirculation of administered steroids as demonstrated by biphasic and triphasic excretion patterns. Thus, noninvasive fecal hormone monitoring is a valid and promising tool for assessing gonadal and adrenal status in rare and threatened birds-of-prey.

Keywords Fecal metabolites · ACTH · HPLC · Avian

Abbreviations

ACN	Acetonitrile
ACTH	Adrenocorticotropin hormone
CERI, JCCP	Centro de Estudios de Rapaces Ibericas
CORT	Corticosterone
CRC	Conservation and Research Center
c.p.m.	Counts per minute
dH ₂ O	Distilled water
E	Estrogen
E ₁	Estrone
E ₂	Estradiol-17β
EtOH	Ethanol
fAM	Fecal androgen metabolites
fEM	Fecal estrogen metabolites
fGCM	Fecal glucocorticoid metabolites
fPM	Fecal progestin metabolites
HPA	Hypothalamic-pituitary axis
HPLC	High-pressure liquid chromatography
MeOH	Methanol
P	Progesterone
P ₄	Progesterone
T	Testosterone

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Introduction

Understanding reproductive function in raptors is important because more than 30 species are threatened by extinction (del Hoyo et al. 1994). There is a paucity of information regarding behavioral–hormonal correlates for raptors, which is unfortunate given that these species help balance ecosystems through predator–prey interactions while serving as important ‘bioindicators’ of environmental change. Knowledge about endocrine patterns can help in determining an individual’s reproductive status and physical fitness and can even suggest management steps for developing safe natural or assisted reproduction techniques.

Noninvasive fecal steroid monitoring offers a potential alternative method for studying long-term gonadal and adrenal rhythms in stress-susceptible birds-of-prey that often fail to reproduce *ex situ*. The main advantage is avoiding repeated blood sampling and animal handling, both of which can activate the hypothalamic–pituitary–adrenal (HPA) axis [including corticosterone (CORT) secretion] (Hiebert et al. 2000; Sockman and Schwabl 2001) and compromise reproduction (Pottinger 1999). Despite being a well-accepted means of assessing endocrine status in wild mammals, fecal steroid monitoring has rarely been used in raptors and specifically in only three species of owls (Wasser et al. 1997; Wasser and Hunt 2005).

Positive correlations between fecal and plasma hormones in matched samples [testosterone (T)/fecal androgen metabolites (fAM) and progesterone (P₄)/fecal progestin metabolites (fPM), Bishop and Hall 1991; T/fAM and estradiol-17β (E₂)/fecal estrogen metabolites (fEM), Cockrem and Rounce 1994; CORT/fecal glucocorticoid metabolites (fGCM), Dehnhrad et al. 2003; CORT/fGCM, Wasser et al. 1997] support the validity of fecal steroid monitoring as a potentially valuable tool for avian physiological studies. Fecal steroid analysis has been used in birds to study sex determination (Tell and Lasley 1991), stress (Wasser et al. 1997), seasonality (Hirschenhauser et al. 1999), gonadal activity (Albaugh 1999; Bishop and Hall 1991; Cockrem and Rounce 1994), breeding behaviors (Albaugh 1999; Bishop and Hall 1991), morphological development (Frigerio et al. 2001), and the influence of steroids on incubation onset (Sockman and Schwabl 1999).

Fecal and urinary endocrine techniques must be validated for each species of interest (Goymann 2005; Touma and Palme 2005). This is largely due to inherent species-specific differences in steroid metabolism, gut microflora, and in the quantity and variety of excreted metabolites that may not contain the parent hormone (Goymann 2005; Wasser et al. 2000; Whitten et al. 1998). Other ‘nonspecific’ substances also may be present in the excreta that interfere with assay antibodies (Whitten et al. 1998). Additionally,

assays specific for a particular hormone in serum or plasma may be inappropriate for quantifying the corresponding excreted metabolite in urine or feces (Goymann 2005; Whitten et al. 1998).

Our long-term aim is to develop and implement noninvasive endocrine monitoring in birds-of-prey as a means of creating fundamental knowledge that has application in enhancing management and conservation of rare species *ex situ* and *in situ*. In this first of multiple studies, we focused on developing assays and testing their efficacy in monitoring gonadal and adrenal function. To begin to understand the influence of species variations on results, we compared data between the golden eagle (*Aquila chrysaetos*) and peregrine falcon (*Falco peregrinus*). The specific objectives were to: (1) conduct laboratory validations of fecal processing and immunoassay procedures for quantifying reproductive and adrenal steroid metabolites, without radiolabeled steroid infusion; and (2) physiologically and biologically validate fecal steroid monitoring for assessing gonadal and adrenal status. We also sought to validate the inexpensive, rapid, one-step ethanol (EtOH) procedure described by Wasser et al. (1994, 2000) that extracts conjugated and unconjugated steroids alike. By injecting birds with a bolus of unlabeled steroid, it was possible to validate steroidal immunoassays without the special storage and disposal required using radioactive approaches. To measure adrenal function, we relied upon an adrenocorticotropin hormone (ACTH) challenge, a common method for assessing stress responsiveness in birds (Ludders et al. 1998, 2001; Romero and Wingfield 1999; Wasser et al. 1996, 2000). Documenting a temporal cause-and-effect relationship between a pharmacological hormonal challenge or exogenous stressor (e.g., an ACTH injection or a stressful event such as animal handling) and a subsequent increase in steroid metabolite in feces is a common means of documenting the physiological or biological validity of such assays (Wasser et al. 2000; Touma and Palme 2005). Finally, by examining endocrine profiles over time after ACTH and steroid hormone injections, we sought to generate new information on lag-time (the interval from hormone secretion to excretion) as well as the significance of enterohepatic circulation that is known to occur in other avian species (Holmes and Phillips 1976).

Materials and methods

Study area, animal subjects, and housing

Birds were located at the Centro de Estudios de Rapaces Ibericas (CERI, JCCM) near Sevilleja de la Jara, Spain. All animals were nonreleasable (due to past injury) or captive bred and were considered healthy at study onset. Birds

were housed as singletons or in pairs in $\sim 6 \times 6 \times 10 \text{ m}^3$ outdoor enclosures and exposed to natural photoperiod. For the ACTH and steroid infusion studies (see below), six adult golden eagles and six adult peregrine falcons (three males : three females/species) were randomly selected. The pens used for the ACTH and steroid infusions were $\sim 3 \times 3 \times 3 \text{ m}^3$ indoor enclosures, each with a large window. Raptors are a reverse-sexually dimorphic genre (females being larger than males) and were sexed accordingly after attaining adult plumage. Diets for both species consisted of rat, quail, rabbit, and hare fed 6 day/week with fresh water provided ad libitum.

Fecal steroid extraction

All endocrine evaluations were conducted at the Conservation and Research Center (CRC) of the Smithsonian's National Zoological Park near Front Royal, VA, USA. The fecal steroid extraction method of Wasser et al. (1994) was used with minor modifications. Fecal samples were dried in a SpeedVac® Rotary Evaporator (Thermo Savant, Holbrook, NY, USA), crushed, and stored at -20°C in labeled, plastic tubes. For extraction, feces (0.05–0.06 g) were suspended in 6 ml of 100% EtOH, vortexed, boiled in a water bath (95°C , 20 min), centrifuged ($500 \times g$, 20 min), and the supernatant decanted into clean tubes. After evaporation under an air stream, extractant was re-suspended in 4 ml EtOH, vortexed, sonicated in ultrasonic glass cleaner (Mettler Electronics, Anaheim, CA, USA), and evaporated. This step was repeated with 2 ml EtOH to maximize the amount of extractant in the bottom of the tube. The final extract was re-suspended in 1 ml methanol (MeOH), vortexed and sonicated, and then diluted (1:4–1:16 for fGCM; 1:50–1:500 for fEM; 1:40–1:250 for fPM; 1:8–1:64 for fAM) in assay buffer (for fA, fGC, and fE metabolite extracts: steroid diluent [ICN Pharmaceuticals Inc., Costa Mesa, CA, USA]; for fPM extracts: phosphate buffer [0.01 M PO_4 , 0.14 M NaCl, 0.5% BSA, 0.01% sodium azide]) and stored at -20°C before analysis. Mean fecal extraction efficiency (88.7%, $n = 196$ samples) was determined by estimating the recovery of radiolabeled (^3H) CORT ($\sim 13,500$ counts per minute [c.p.m.]) added before extraction.

Fecal radioimmunoassays (RIAs)

Assays were validated for each species by demonstrating: (1) efficacy and specificity of antibodies by showing parallel binding between the standard hormones and serial dilutions of pooled fecal extracts in a dose-dependant fashion; and (2) accuracy of recovered exogenous hormone added to low-mass pools of fecal extracts. Fecal GC, E, and A metabolite excretions were measured in duplicate using commercial, double-antibody ^{125}I RIA kits (ICN Pharma-

ceuticals Inc.), halving the manufacturer's reagents. Fecal PM excretion was measured in duplicate using an in-house P_4 assay (Brown et al. 1994). Data were expressed as mass units of hormone excreted per gram of dry feces. Samples were randomly distributed among assays so that each analysis involved multiple birds.

For the CORT assay, samples were incubated (25°C) with standards (50 μl), anti-CORT (100 μl), and [^{125}I]CORT antigen (100 μl) for 2 h before precipitating the antibody-bound antigen using 250 μl of a precipitating solution. For the estrogen (E) assay, standards and samples (250 μl), anti-total estrogens (50 μl), and [^{125}I]E₂ (50 μl) were incubated for 1.5 h at room temperature (25°C). Second antibody (500 μl) was added, and the assay incubated for 1 h to precipitate the antibody–antigen complexes. For the P_4 assay, standards and samples (100 μl) were incubated (4°C) with first antibody (provided by Dr. Jan Roser, University of California, Davis, CA, USA; 100 μl , 1:150,000), assay buffer (200 μl), and [^{125}I] P_4 (100 μl) (ICN Pharmaceuticals Inc.) for 3 h; then for 1 h with sheep anti-mouse gamma globulin second antibody [1:1,000, 1 ml in buffer containing 5% polyethylene glycol, 8000M, (Sigma-Aldrich Corp., St. Louis, MO, USA)] to precipitate antibody–bound complexes. For the T assay, standards and samples (25 μl) were incubated (2 h, 37°C) with anti-T (250 μl), SGBI Solution (50 μl), and [^{125}I]T (250 μl). Second antibody (50 μl) was added, and the assay incubated for 1 h at 37°C to precipitate the antibody–antigen complexes.

HPLC

To determine the polarity, relative quantity, distribution, and immunoreactivity of fecal steroidal metabolites, reverse-phase High-pressure liquid chromatography (HPLC) columns were used (Varian Microsorb C-18/mv 100 Å, Varian, Analytical Instruments Inc., Walnut Creek, CA, USA) with minor modifications from Monfort et al. (1991) and Wasser et al. (1993). Fecal extracts were pre-processed with reverse-phase C-18 matrix cartridges (Spice™, Analtech, Newark, DE, USA) and eluted in 5 ml MeOH to remove contaminants, combined with [^3H]CORT, [^3H]E₂, [^3H]estrone (E₁), [^{14}C]T, or [^{14}C] P_4 ($\sim 9,000$ c.p.m.), evaporated, and suspended in 300 μl MeOH. About 55 μl of fecal extract were injected for metabolite separation. The solvent gradients for HPLC were: (1) fAM, 45% isocratic acetonitrile (ACN) gradient over 80 min (0.33 ml/min flow rate, 1 fraction/min); (2) fGCM, 20–100% MeOH: distilled water (dH_2O) over 80 min (1.0 ml/min flow rate, 1 fraction/min); (3) fEM, 32–45% ACN: dH_2O for 20 min, increasing to 60% over 40 min and then to 100% over 20 min (1.0 ml/min flow rate, 1 fraction/min); (4) fPM, 20–32% ACN: dH_2O over 15 min, increasing to 50% over 45 min, and then

increasing to 100% over 60 min (1.0 ml/min flow rate, 1 fraction/min). A 100 µl aliquot of each fraction was combined with scintillation cocktail and counted directly for radioactivity (LS5801, Beckman Instruments Inc., Nuclear Systems Operation, Fullerton, CA, USA) and the resulting fraction evaporated (SpeedVac Rotary Evaporator, Thermo Savant). The remaining fractions were suspended in steroid diluent for fA, fGC, and fE metabolites and phosphate buffer for fPM, then analyzed, and the results compared to the radiolabeled portion of the fraction. Retention times of labeled hormone fraction(s) served as reference for identifying steroid metabolites. Immunoreactive assessments helped identify the quantity and relative distribution of fecal steroid metabolites.

Experimental design

Three males and three females of each species were studied over a 4 week interval (August–September). Some were captive bred (eagles, *n* = 2; falcons: *n* = 4) and others were wild-born but nonreleasable (eagles, *n* = 4; falcons: *n* = 2). Each bird was treated with ACTH, nonradiolabeled steroids, and saline in a split-plot, repeated measures design with a 4 day washout period (Table 1). The afternoon before treatment, each was moved from its outdoor home pen to an indoor treatment pen to allow short acclimation. At 0800 hour on the following day, all fecal samples were collected followed by treatment administration at 0900 hour. Blood was collected (1 ml from each eagle, 0.5 ml from each falcon) immediately before treatment administration to monitor general health (data not shown). Catching, restraining, blood collection, and treatment administration required <2 min. Attempts were made to collect only feces (without urates), but this was not always possible, especially in falcons. Fecal samples were collected from each individual every 4 h from 0 to 24 h, every

6 h from 24 to 48 h, and every 8 h from 48 to 72 h post-injection. This protocol was chosen because: (1) previous studies revealed that peak steroid excretion occurred ≤16 h of treatment administration (Wasser et al. 1997, 2000; Lee et al. 1999; Ludders et al. 2001); and (2) by expanding the interval on Days 2 (from 24 to 48 h) and 3 (from 48 to 72 h), stress caused by human presence might be minimized. Each fecal sample was placed in a labeled, plastic tube (16 × 100 mm), stored (−20°C), and then shipped frozen to CRC for analysis.

ACTH challenges, steroid infusions, and saline controls

To minimize handling, body weights (as a measure of animal well-being and to calculate ACTH dose) were obtained before the first and after the second treatments. Porcine ACTH (~25 IU/kg, Sigma-Aldrich Corp.) was administered i.m. into the pectoral muscle of each bird using a 1-ml tuberculin syringe and a 26 gauge needle. The ACTH dosage was based on previous challenges found in the literature (Astheimer et al. 1994; Deviche et al. 1980; Wasser et al. 1996). The ACTH solution for eagles (0.705 mg/100 µl or 56.4 IU/100 µl) was prepared by dissolving 14.1 mg ACTH in 200 µl 100% EtOH, followed by adding 2.0 ml of sterile physiological saline (0.9% NaCl). The ACTH solution for falcons (0.151 mg/100 µl or 12.1 IU/100 µl) was prepared by dissolving 3.0 mg ACTH in 200 µl 100% EtOH, followed by adding 2.0 ml of sterile saline. Injections were prepared using the stock solutions and the most current body weight at treatment onset; saline was added to ensure that the total injection volume for all treatments was 500 µl.

For the steroid infusions, a minimum of 1.0 µg/500 µl was administered to eagles and 0.5 µg/500 µl to falcons by i.m. injection, as described above. The steroid infusion doses were extrapolated from similar experiments conducted previously in mammalian species (Morrow and

Table 1 Experimental design for adrenocorticotropin hormone (ACTH) challenge, steroid infusions [estradiol-17β (*E*₂), progesterone (*P*₄), testosterone (*T*)], and collection (C)

Species	Bird	Treatment number 1			Treatment number 2			Treatment number 3		
		Day 1	2	3	Day 1	2	3	Day 1	2	3
Peregrine falcon	1	E ₂ , P ₄ , C	C	C	Saline, C	C	C	ACTH, C	C	C
	2	T, C	C	C	Saline, C	C	C	ACTH, C	C	C
	3	ACTH, C	C	C	E ₂ , P ₄ , C	C	C	Saline ^a , C ^a	C ^a	C ^a
	4	ACTH, C	C	C	T, C	C	C	Saline, C	C	C
	5	Saline, C	C	C	ACTH, C	C	C	E ₂ , P ₄ , C	C	C
	6	Saline, C	C	C	ACTH, C	C	C	T, C	C	C
Golden eagle	7	E ₂ , P ₄ , C	C	C	Saline, C	C	C	ACTH, C	C	C
	8	Saline, C	C	C	ACTH, C	C	C	T, C	C	C
	9	Saline, C	C	C	ACTH, C	C	C	E ₂ , P ₄ , C	C	C
	10	ACTH, C	C	C	T, C	C	C	Saline, C	C	C
	11	ACTH, C	C	C	E ₂ , P ₄ , C	C	C	Saline, C	C	C
	12	T, C	C	C	Saline, C	C	C	ACTH, C	C	C

^a Bird not available for treatment

Monfort 1998; Thompson et al. 1998). Previous studies suggest that the dosages used here greatly exceeded normal circulating hormone concentrations in the blood (Rehder et al. 1986; 1988) of raptors. Hormone treatments were prepared by dissolving the steroid (eagles, $E_2 = 0.0128$ mg, $P_4 = 0.0120$ mg, $T = 0.0125$ mg; falcons, $E_2 = 0.0061$ mg, $P_4 = 0.0070$ mg, $T = 0.0067$ mg) in 200 μ l EtOH, followed by dilution in 6 ml (eagles) or 3 ml (falcons) saline. The final concentrations were as follows: eagle males ($T = 0.208$ μ g/100 μ l), eagle females ($E_2 = 0.213$ μ g/100 μ l and $P_4 = 0.200$ μ g/100 μ l), falcon males ($T = 0.223$ μ g/100 μ l), and falcon females ($E_2 = 0.203$ μ g/100 μ l and $P_4 = 0.233$ μ g/100 μ l). Each injection was adjusted to a total volume of 500 μ l. For the saline controls, a volume of 500 μ l sterile saline (0.9% NaCl) was injected i.m. as described above as a control for the ACTH challenges and steroid infusions.

Statistical analysis

A split plot design with repeated measures for the ACTH challenges, steroid infusions and saline controls was used. The wholeplots were a completely randomized design with bird nested within sex. The subplots were the treatments (ACTH, steroids, saline) with repeated measures on time. Fecal metabolite values are presented as log-transformed means (\pm SEM, where applicable). Data were log-transformed to achieve a normal distribution and rank-transformed if the data were still abnormal, skewed, or had outliers. The assumptions for normality were met after transformations in most cases; however, the pooled ACTH and saline hourly data for the falcons were not normal according to the Shapiro-Wilk normality test ($P < 0.05$). However, after removing one outlier from the male falcon pool, the data returned to normal. Variances were determined to be equal after analyzing the residuals by predicted plots. To determine if there was a species effect in response to treatment, we included species in the model; if significant, this effect was removed. Daily means of both ACTH and saline treatments were analyzed simultaneously to determine if sex and overall treatment effects existed (Tables 2, 3). If there was a significant time, but not treatment by time effect, ACTH and/or steroid treatments were analyzed separately from the saline treatments to determine differences between pre-treatment and post-treatment samples (Tables 4, 5). We conducted and analyzed a priori Tukey's adjusted pair-wise comparisons to determine specific differences. Additionally, we analyzed all fecal samples collected (pre- to 72-h post-injection) in the same fashion as described for daily means to characterize the excretion patterns over time (Tables 6, 7, 8). This statistical model allowed for missing data points, and all analyses were performed using SAS (Version 8.01, 1999–2000).

Table 2 Results of mixed factorial ANOVA on log- and rank-transformed daily means data for ACTH challenges and saline controls in the golden eagle

Source	df	F	P
Sex	1	0.25	0.646
Error [bird (sex)]	4		
Treatment	1	47.96	0.002
Treatment \times sex	1	0.55	0.500
Error [treatment \times bird (sex)]	4		
Time	3	7.49	0.001 ^a
Time \times sex	3	1.22	0.325
Time \times treatment	3	2.28	0.105
Time \times sex \times treatment	3	0.54	0.658
Error [time \times treatment \times bird (sex)]	24		
Total	47		

Sex was the whole plot factor, treatment was the subplot factor, and time was the repeated measures factor

^a Treatments were separated due to a significant time effect (see Table 4)

Table 3 Results of mixed factorial ANOVA on log- and rank-transformed daily means data for ACTH challenges and saline controls in the peregrine falcon

Source	df	F	P
Sex	1	0.85	0.424
Error [bird (sex)]	3		
Treatment	1	1.22	0.350
Treatment \times sex	1	1.33	0.332
Error [treatment \times bird (sex)]	3		
Time	3	3.15	0.048 ^a
Time \times sex	3	0.55	0.651
Time \times treatment	3	1.64	0.211
Time \times sex \times treatment	3	0.68	0.575
Error [time \times treatment \times bird (sex)]	20		
Total	41		

Sex was the whole plot factor, treatment was the subplot factor, and time was the repeated measures factor

^a Treatments were separated due to a significant time effect (see Table 5)

Results

Animal subjects

Each peregrine falcon lost body mass during the course of the study (range = 4.9–23.9%; mean \pm SEM = 11.4 ± 2.8), suggesting that experimental procedures were stressful. In contrast, each golden eagle gained weight (0.5 – 15.5% ; 6.8 ± 2.2). Peregrine falcon female 3 experienced significant body mass loss after two treatments and later died as a

Table 4 Results of individual mixed factorial ANOVA on log- and rank-transformed daily means data for ACTH challenges and saline controls in the golden eagle

Source	ACTH challenges			Saline controls		
	df	F	P	df	F	P
Sex	1	1.86	0.244	1	0.00	0.912
Error [bird (sex)]	4			4		
Time	3	22.58	<0.001	3	1.27	0.330
Time × sex	3	3.36	0.055	3	0.14	0.933
Error [time × bird (sex)]	12			12		
Total	23			23		

Sex was the whole plot factor and time was the repeated measures factor

Table 5 Results of individual mixed factorial ANOVA on log- and rank-transformed daily means data for ACTH challenges and saline controls in the peregrine falcon

Source	ACTH challenges			Saline controls		
	df	F	P	df	F	P
Sex	1	0.03	0.874	1	2.58	0.206
Error [bird (sex)]	4					
Time	3	3.76	0.044 ^a	3	0.63	0.615
Time × sex	3	0.93	0.458	3	0.04	0.988
Error [time × bird (sex)]	11			9		
Total	22			19		

Sex was the whole plot factor and time was the repeated measures factor

^a After Tukey’s adjustment, there was no significant time effect ($P = 0.058$)

Table 6 Results of mixed factorial ANOVA on log- and rank-transformed hourly data for ACTH challenges and saline controls in the golden eagle

Source	df	F	P
Sex	1	0.87	0.404
Error [bird (sex)]	4		
Treatment	1	153.09	<0.001
Treatment × sex	1	2.13	0.212
Error [treatment × bird (sex)]	4		
Time	13	6.65	<0.001 ^a
Time × sex	13	1.22	0.277
Time × treatment	13	1.48	0.140
Time × sex × treatment	13	0.89	0.567
Error [time × treatment × bird (sex)]	97		
Total	160		

Sex was the whole plot factor, treatment was the subplot factor, and time was the repeated measures factor

^a Treatments were separated due to a significant time effect (see Table 7)

Table 7 Results of individual mixed factorial ANOVA on log- and rank-transformed hourly data for ACTH challenges and saline controls in the golden eagle

Source	ACTH challenges			Saline controls		
	df	F	P	df	F	P
Sex	1	3.40	0.139	1	0.07	0.804
Error [bird (sex)]	4			4		
Time	13	6.45	<0.001	13	2.77	0.005
Time × sex	13	1.31	0.244	13	0.63	0.816
Error [time × bird (sex)]	47			50		
Total	78			81		

Sex was the whole plot factor and time was the repeated measures factor

Table 8 Results of mixed factorial ANOVA on log-transformed (removed one outlier to meet assumptions of normality and equal variances) hourly data for ACTH challenges and saline controls in the peregrine falcon

Source	df	F	P
Sex	1	0.40	0.571
Error [bird (sex)]	3		
Treatment	1	7.27	0.074
Treatment × sex	1	2.95	0.184
Error [treatment × bird (sex)]	3		
Time	13	1.48	0.146
Time × sex	13	1.26	0.256
Time × treatment	13	0.71	0.749
Time × sex × treatment	13	1.40	0.180
Error [time × treatment × bird (sex)]	69		
Total	130		

Sex was the whole plot factor, treatment was the subplot factor, and time was the repeated measures factor

result of a Newcastle’s viral infection. Because her endocrine data were comparable to counterparts, these results were included in the statistical analysis.

Fecal RIAs: parallelism and recovery

For the CORT assay, serial dilutions (1:1–1:2,048) of pooled golden eagle or peregrine falcon fecal extracts yielded displacement curves parallel to those of standard preparations. Recovery of known concentrations of unlabeled CORT (range, 6.3–500 ng/ml) in male and female eagles were $117.2 \pm 10.7\%$ ($y = 1.11x - 0.38$, $R^2 = 1.00$) and $119.4 \pm 5.3\%$ ($y = 1.04x + 4.11$, $R^2 = 1.00$), respectively. For male and female falcon extracts, recovery of added CORT was $100.7 \pm 4.1\%$ ($y = 1.07x - 1.00$, $R^2 = 1.00$) and $118.4 \pm 4.6\%$ ($y = 1.12x + 10.16$, $R^2 = 0.99$), respectively. Inter-assay coefficients of variation for two separate internal controls were 10.1% and 9.7% ($n = 24$)

assays). Intra-assay coefficients of variation were <10%, and assay sensitivity was 12.5 ng/ml at 87% maximum binding.

For the E assay, dilutions (1:1–1:2,048) of pooled eagle or falcon fecal extract yielded displacement curves parallel to standards. Recovery of unlabeled E (range, 1.25–50 pg/tube) from female eagle and falcon extracts were $103.3 \pm 6.5\%$ ($y = 0.97x - 0.08$, $R^2 = 1.00$) and $97.3 \pm 9.8\%$ ($y = 1.03x - 0.63$, $R^2 = 0.98$), respectively. Inter-assay coefficients of variation for three controls were 10.0, 9.8, and 9.1% ($n = 8$ assays). Intra-assay coefficients of variation were <10%, and assay sensitivity was 0.31 pg/tube at 90% maximum binding.

For the P assay, dilutions (1:1–1:2,048) of pooled eagle or falcon fecal extract yielded displacement curves parallel to standards. Recovery of unlabeled P (range, 0.63–50 pg/tube) from female eagle and falcon extracts were $105.7 \pm 9.4\%$ ($y = 0.88x + 3.88$, $R^2 = 1.00$) and $113.4 \pm 6.7\%$ ($y = 1.06x + 0.21$, $R^2 = 1.00$), respectively. Inter-assay coefficients of variation for two controls were 12.8 and 17.8% ($n = 11$ assays). Intra-assay coefficients of variation were <10%, and assay sensitivity was 1.88 pg/ml at 93% maximum binding.

For the T assay, dilutions (1:1–1:2,048) of pooled eagle or falcon fecal extract yielded displacement curves parallel to standards. Recovery of unlabeled T (range, 0.03–5 ng/ml) from male eagle and falcon extracts were $100.1 \pm 3.4\%$ ($y = 1.04x - 0.00$, $R^2 = 1.00$) and $112.8 \pm 6.4\%$ ($y = 0.92x + 0.08$, $R^2 = 0.99$), respectively. Inter-assay coefficients of variation for two controls were 6.0 and 14.2% ($n = 15$ assays). Intra-assay coefficients of variation were <10% and assay sensitivity was 0.03 ng/ml at 90% maximum binding.

HPLC

For the golden eagle, HPLC analyses of fGCM revealed one polar immunoreactive peak in both sexes (fractions 15–19 of 80 total fractions) and three less polar counterparts in males (fractions 43, 46–53) and females (fractions 44–51), of which 2.7% (fractions 46–49) and 2.5% (fractions 46–48), respectively, co-eluted with [³H]CORT reference radiolabeled steroid. The first polar peak comprised most of the immunoreactive fGCM (males, 79.3%; females, 88.4%). Chromatographic profiles of fEM in females revealed three immunoreactive peaks (two major and one minor). Most immunoreactivity (57.6%, fractions 21–24 of 80 total) co-eluted with [³H]E₂ and 26.9% (fractions 27–29) aligned with [³H]E₁. HPLC separation of fPM in female feces revealed five polar peaks (fractions 2–39 of 120 total) and seven less polar peaks (fractions 65–118), of which 24.8% (fractions 70–72) co-eluted with [¹⁴C]P₄. Separation of fAM in males revealed six peaks (four minor, fractions 11–28, 44–49 and two major, fractions 29–32, 33–38 of 80

total), of which 55.8% (fractions 33–38) co-eluted with [¹⁴C]T.

For the peregrine falcon, HPLC separation of fGCM yielded one polar immunoreactive peak (males, fractions 27–35; females, fractions 15–22 of 80 total) and two less polar peaks (fractions 45–52). In males (fractions 45–48) and females (fractions 45–49), 7.5 and 11.9% of total immunoreactivity, respectively, co-eluted with [³H]CORT. Separation of fEM in females revealed three peaks; 64.6% (fractions 18–23 of 80 total) and 4.1% (fractions 24–27) of the immunoreactivity co-eluted with [³H]E₂ and [³H]E₁, respectively. Separation of fPM revealed at least 13 peaks (fractions 2–69, one relatively major peak, fraction 57 of 120 total) having higher polarity than [¹⁴C]P₄, which co-eluted with 21.7% of the total immunoreactivity (fractions 70–74). There also were two major (fractions 77–79, 88–90) and four minor (fractions 81–84, 96, 98, 114–118) peaks less polar than the reference radiolabeled steroid. In males, HPLC separation of fAM revealed two major (fractions 30–40) and two minor (fractions 11–12, 46–49 of 80 total) peaks with 63.7% (fractions 35–40) co-eluting with [¹⁴C]T.

ACTH challenges and saline controls

The golden eagle excreted more fGCM (2.65 ± 0.07 ng/g; $P < 0.001$) after ACTH or saline administration (pooled data) than the peregrine falcon (1.31 ± 0.07 ng/g); thus, there was a species effect [$F_{(1,7)} = 187.32$, $P < 0.0001$]. Because there were no sex differences in fGCM excretion within species (eagle, $P = 0.645$; falcon, $P = 0.424$), data were pooled across genders within species. In both species and over time, there was no impact ($P > 0.05$) of saline on daily fGCM excretion (Fig. 1). For the eagle, significant treatment and time effects were found ($P < 0.05$, Table 2); ACTH increased fGCM output from 2.47 ± 0.13 ng/g (pre-treatment) to 3.50 ± 0.13 ng/g on Day 1, which remained elevated on Days 2 and 3 ($P < 0.05$, Table 4; Fig. 1a). For the falcon, there was only a time effect ($P < 0.05$, Table 3), which revealed a rise in excreted fGCM from 1.23 ± 0.13 ng/g pre-treatment to 1.68 ± 0.11 ng/g on Day 1 ($P < 0.05$, Table 5; Fig. 1b). After Tukey's adjustment, this difference was marginally significant ($P = 0.058$), and all values were similar ($P > 0.05$) on Days 2 and 3.

Analysis of hourly data for the eagle again revealed a treatment (ACTH versus saline) and time effect ($P < 0.05$, Table 6; Fig. 2a). Fecal GCM excretion was elevated ($P < 0.05$, Table 7) above pre-ACTH concentrations (2.47 ± 0.16 ng/g) at 4 (3.14 ± 0.16 ng/g), 8 (3.66 ± 0.16 ng/g), 12 (3.25 ± 0.19 ng/g), and 30 (3.22 ± 0.16 ng/g) h after ACTH injection. In contrast, the falcon showed marginal significance in treatment effect ($P = 0.074$), and there were no significant time or treatment by time effects ($P > 0.05$, Table 8; Fig. 2b).

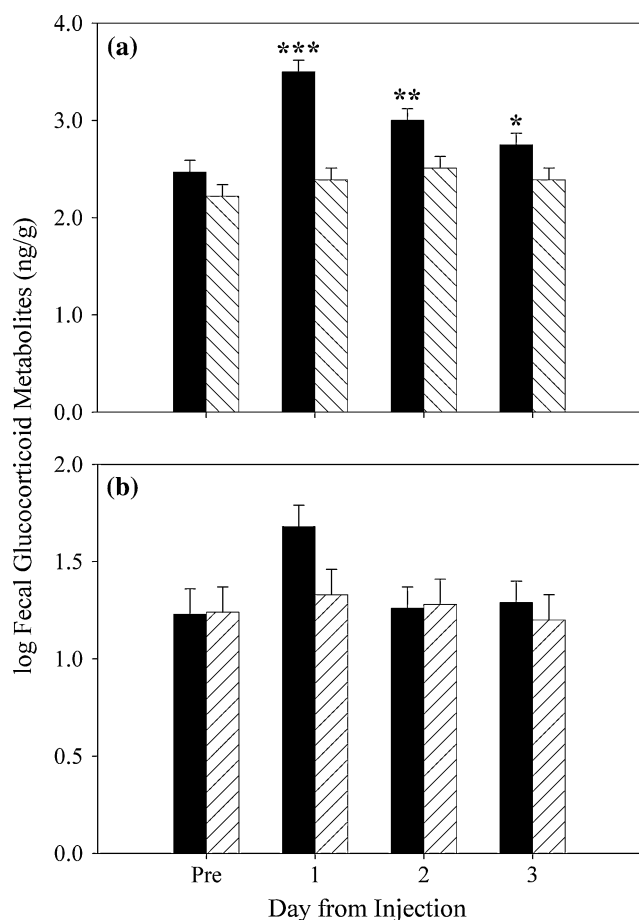


Fig. 1 Fecal glucocorticoid metabolite concentrations in the golden eagle (a, $n = 6$) and peregrine falcon (b, $n = 6$) after ACTH (black bars) and saline (striped bars) treatments for daily means (log-transformed mean \pm SEM). Data were aligned to day of treatment injection (Day 1). Asterisks indicate a difference (* $P < 0.05$, ** $P < 0.001$, *** $P < 0.0001$) among pre- (Pre) and post- (Day 1, 2, 3) fecal glucocorticoid metabolite concentrations over time within treatment (ACTH or saline)

As the saline treatment also served as an index of ‘handling stress,’ we evaluated the hourly data for this particular treatment in more detail. For the eagle, there was a time effect ($P < 0.05$, Table 7), with significant peaks occurring at 8 (2.46 ± 0.16 ng/g) and 56 (2.54 ± 0.16 ng/g) h after treatment onset (pre-treatment value, 2.22 ± 0.12 ng/g; Fig. 2a). In contrast, hourly data in falcon was more random and not different ($P > 0.05$, Table 8) over time (Fig. 2b).

Steroid infusions and saline controls

Overall, the peregrine falcon excreted more fA [$F_{(1,4)} = 46.79$, $P = 0.002$; 1.08 ± 0.06 ng/g] and fE [$F_{(1,3)} = 11.83$, $P = 0.041$; 1.18 ± 0.09 ng/g] metabolites after steroid or saline administration (pooled data) than the golden eagle (0.51 ± 0.06 ng/g and 0.78 ± 0.08 ng/g, respectively).

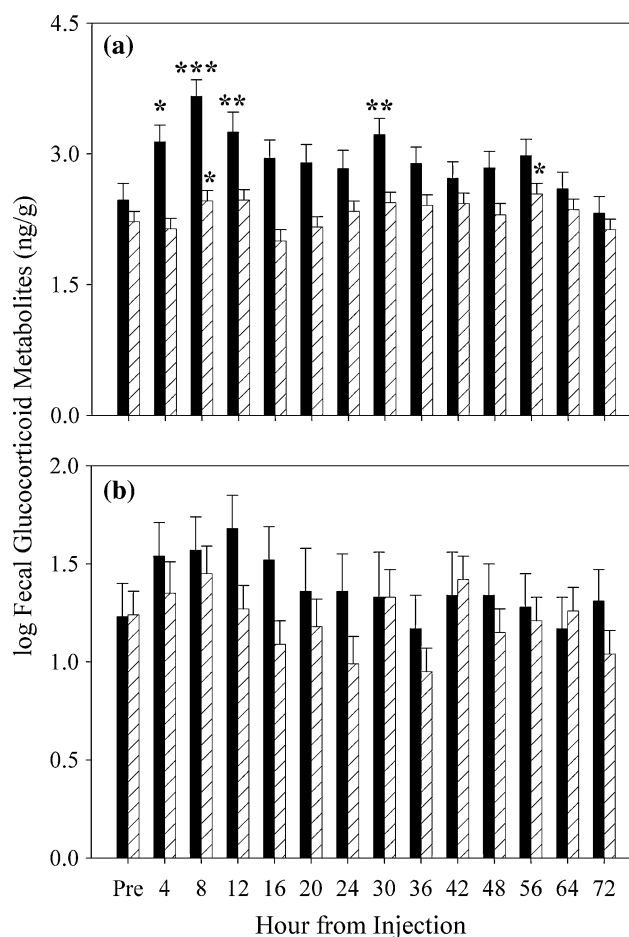


Fig. 2 Fecal glucocorticoid metabolite concentrations in the golden eagle (a, $n = 6$) and peregrine falcon (b, $n = 6$) after ACTH (black bars) and saline (striped bars) treatments for each collection interval (log-transformed mean \pm SEM). Data were aligned to hour of treatment injection (0 h). Asterisks indicate a difference (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$) among pre- (Pre) and post- (4–72 h) fecal glucocorticoid metabolite concentrations over time within treatment (ACTH or saline)

There were no treatment effects ($P > 0.05$) after any of the steroid injections for the eagle or falcon (Table 9).

Results for individual steroid infusions were variable in both species. However, of the 18 occasions during which treatments were given, six produced profiles revealing clear trends in excretion patterns. For a male eagle (Fig. 3a), there was nearly a three-fold increase in fAM excretion from 8 to 20 h post-T administration compared to the pre-treatment values. The profile in the falcon showed peak fAM excretion 12 h after T infusion (Fig. 3b). For eagle female 7 (Fig. 4a), fEM excretion doubled above the pre-treatment concentration from 12 through 44 h post-E₂ infusion. In contrast, eagle 9 (Fig. 4b) expressed a biphasic fEM excretion pattern with peaks at 8 and 30 h. The fEM excretion profile in falcon female 3 also was biphasic (Fig. 5a) with an initial peak (10 h) that was followed by sustained elevated excretion (30–56 h). Falcon female 1 produced an

Table 9 Daily logged means (\pm SEM) of fecal hormone concentrations (ng/g) from steroid [estradiol-17 β (E_2), progesterone (P_4), testosterone (T)] and saline infusions in the golden eagle and peregrine falcon

Species	Sex	<i>n</i>	Treatment	Day				<i>P</i> -value ^a
				Pre	1	2	3	
Eagles	M	3	T	0.62 \pm 0.21 ^b	0.61 \pm 0.21	0.39 \pm 0.21	0.54 \pm 0.21	0.692
		3	Saline	0.29 \pm 0.21 ^b	0.54 \pm 0.21	0.56 \pm 0.21	0.50 \pm 0.21	
	F	3	E_2	0.89 \pm 0.12 ^c	1.05 \pm 0.12	1.06 \pm 0.12	0.67 \pm 0.12	0.089
		3	Saline	0.39 \pm 0.12 ^c	0.76 \pm 0.12	0.74 \pm 0.12	0.67 \pm 0.12	
		3	P_4	2.58 \pm 0.13 ^d	2.36 \pm 0.13	2.47 \pm 0.13	2.24 \pm 0.13	
3	Saline	2.26 \pm 0.13 ^d	2.12 \pm 0.13	2.35 \pm 0.13	2.27 \pm 0.13			
Falcons	M	3	T	1.28 \pm 0.12	1.14 \pm 0.12	1.00 \pm 0.12	1.09 \pm 0.12	0.471
		3	Saline	1.07 \pm 0.12	0.96 \pm 0.12	1.06 \pm 0.12	1.06 \pm 0.12	
	F	3	E_2	1.38 \pm 0.19	1.12 \pm 0.19	1.30 \pm 0.19	1.18 \pm 0.19	0.513
		2	Saline	1.17 \pm 0.22	1.00 \pm 0.22	1.11 \pm 0.22	1.17 \pm 0.22	
		3	P_4	2.07 \pm 0.16	2.16 \pm 0.16	2.35 \pm 0.16	2.52 \pm 0.16	
2	Saline	1.80 \pm 0.19	2.05 \pm 0.19	2.00 \pm 0.19	2.21 \pm 0.19			

Saline treatments correspond to, and were assayed for, preceding hormone treatment

^a *P*-value indicates results from ANOVA comparisons between steroid treatment and corresponding saline control

^b Fecal androgen metabolite concentration

^c Fecal estrogen metabolite concentration

^d Fecal progestin metabolite concentration

initial peak at 8 h (Fig. 5b) that was followed by sustained fPM excretion from 25 to 64 h after P_4 administration.

Steroid metabolism

Time of peak excretion after ACTH administration (excretion lag-time) was 8 and 12 h for the golden eagle ($P < 0.05$) and peregrine falcon, respectively (Fig. 2). Excretion lag-times for the steroidal infusions ranged from 4 to 20 h for both species (Figs. 3, 4, 5). Data indicated enterohepatic recirculation was occurring in both species and sexes, largely from observations of bi- and triphasic patterns of steroid excretion after treatment injections. For example, the eagle expressed a significant ($P < 0.05$) biphasic response in fGCM post-ACTH treatment with a trend toward a triphasic response (Fig. 2a). The falcon exhibited a nonsignificant ($P > 0.05$), but nonetheless trend toward a biphasic response (Fig. 2b). Recycling patterns also were observed for fGCM after saline administration ($P < 0.05$ for the eagle; Fig. 2) and for fE, fP, and fA metabolites post-steroid administration (Figs. 3, 4, 5).

Discussion

Our findings clearly demonstrate the potential utility of noninvasive fecal hormone monitoring for assessing endocrine function in raptors. It is important to characterize reproductive and adrenal physiology in birds-of-prey, many

of which are ecologically threatened and most of which fail to reproduce in captivity. Nonetheless, one of our most important findings was the variation in resilience to experimental use between species. On the basis of weight alone, the golden eagle withstood the rigors of study (gained body mass), whereas the peregrine falcon was highly sensitive to any manipulations (consistently lost mass). Thus, our results suggested that, for some species (in this case the peregrine falcon), even occasional human presence to recover feces appeared stressful, sufficiently so to compromise well-being. This means that preemptive characterization and validation studies (like the ones described here) will be essential for identifying those raptor species most appropriate for application of noninvasive endocrine monitoring to investigate physiological phenomena.

Extraction efficiency, parallelism, and recovery results demonstrated that the hydrolytic steps used in earlier studies for other bird species (Tell and Lasley 1991; Cockrem and Rounce 1994) were unnecessary to identify specific fecal steroidal metabolites in these raptors. Wasser et al. (1994, 2000) have demonstrated that boiling feces in 80 to 100% EtOH does not alter steroidal immunoreactivity or cleave conjugates from the parent hormone. Ethanol extracts both free and conjugated fecal metabolites because of its relative polarity (being less polar than water, but more so than most organic solvents). Furthermore, EtOH extraction is a simple and effective method for extracting steroidal metabolites from raptor feces; we achieved good recovery using 100%EtOH. It is worth noting that Wasser and Hunt

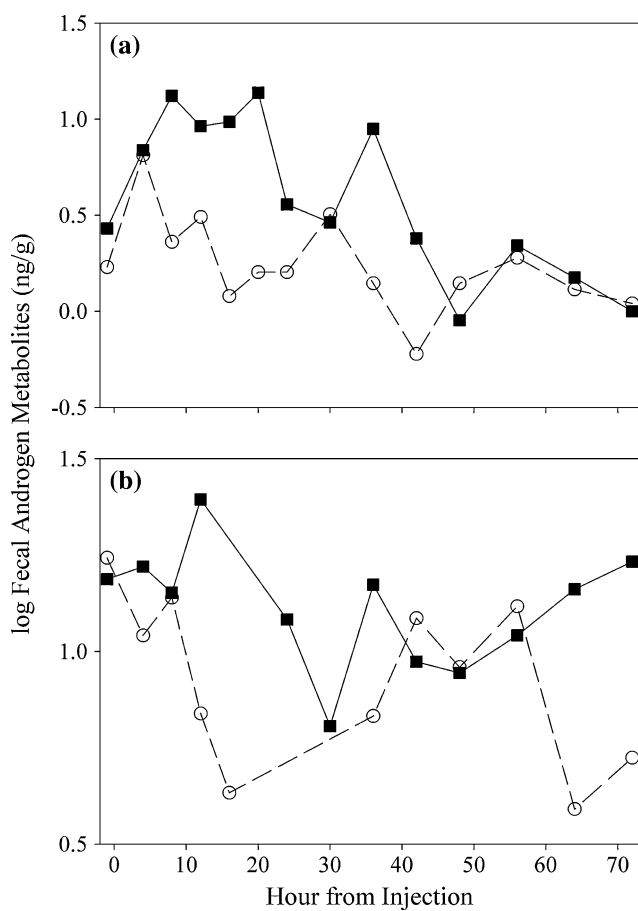


Fig. 3 Log-transformed fecal androgen metabolite concentrations in golden eagle male 8 (a), and in peregrine falcon male 4 (b) after testosterone (*squares*) or saline (*open circles*) treatment. Data were aligned to hour of treatment injection (0 h)

(2005) recently reported that using 80% EtOH will yield higher recoveries in owl feces than a 100% solution.

Separation of fGCM via HPLC in both species and sexes revealed that most of these compounds were conjugated to a highly polar side-chain moiety (e.g., glucuronide or sulfate) or had become structurally modified before excretion. These polar metabolites had identical retention times in both eagle genders, which suggested that males and females were excreting the same metabolite. In contrast, differential retention times of some polar metabolites in the falcon suggested the existence of a sex-dependent fGC metabolism mechanism, which also has been observed in other avian species (Goymann et al. 2002; Goymann 2005; Touma and Palme 2005). Wasser and Hunt (2005) found a similar polar peak in the barred owl (*Strix varia*) after radiolabeled infusion that was attributed to a solvent front of radioactivity that separated from the radiolabeled hormone. However, the polar peaks observed in the eagle and falcon occurred later in the elution profile, which may indicate that it is an fGCM. Helton and Homes (1973) found no glucuronidated fGCM in the duck (*Anas platyrhynchos*), although some

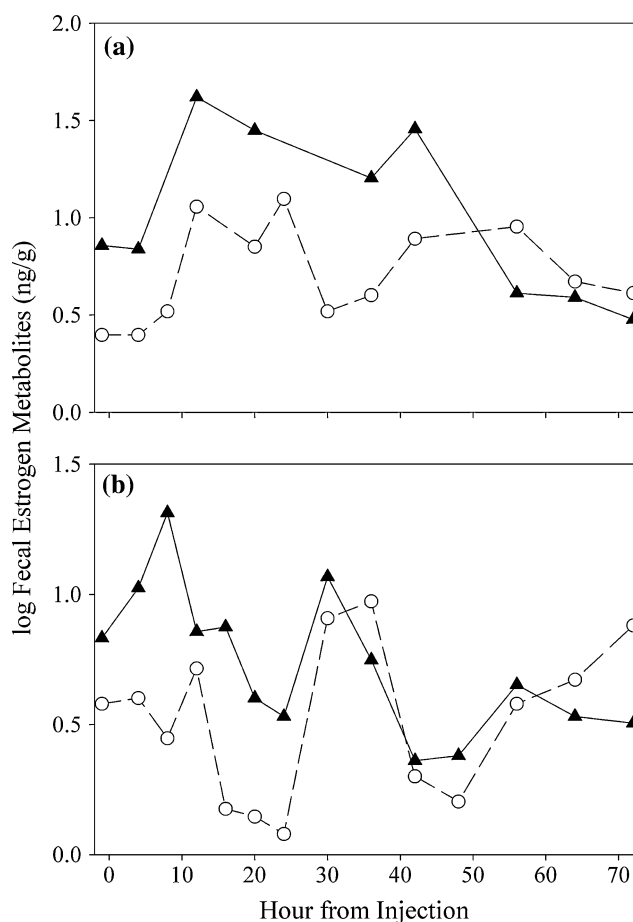


Fig. 4 Log-transformed fecal estrogen metabolite concentrations in golden eagle females 7 (a) and 9 (b) after estradiol-17β (*triangles*) or saline (*open circles*) treatment. Data were aligned to hour of treatment injection (0 h)

were sulfated and excreted unconjugated. In the hen, CORT is metabolized to 20-dihydrocorticosterone, and 11-dehydrocorticosterone is converted to 11-dehydro-20-dihydrocorticosterone (Vylitová et al. 1998), neither of which are conjugated fGCM. Wasser et al. (2000) found that the ICN corticosterone RIA antibody (used in the present study) appears sensitive to changes at the C-11 and C-21 positions of the steroid structural framework, which makes this assay particularly well-suited for birds.

The fEM identified via HPLC elution was similar in the eagle and falcon. Estradiol and E₁ represented most of the fEM, suggesting that the unconjugated metabolite predominated in feces. Our estrogen profile was similar to the HPLC elution profile of the barred owl after radiolabeled-E₂ infusion (Wasser and Hunt 2005). However, only the falcon had a small polar peak that may also have been a solvent front (Wasser and Hunt 2005). Furthermore, Albaugh (1999), using a similar extraction technique and HPLC, has found that the Bali mynah (*Leucopsar rothschildi*) primarily excretes fEM co-eluting with E₂. In contrast, HPLC

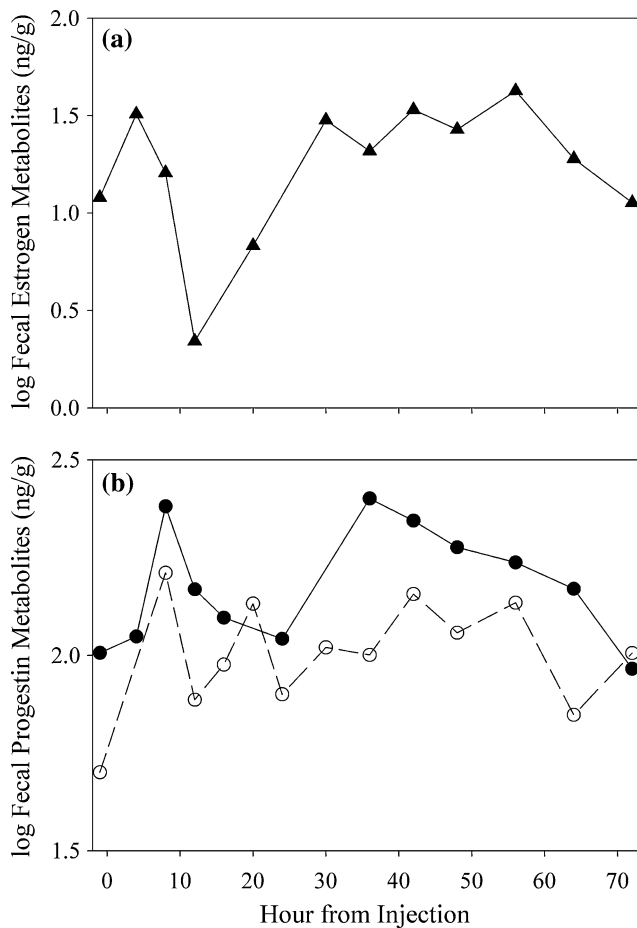


Fig. 5 Log-transformed fecal estrogen metabolite concentrations in peregrine falcon female 3 (a) and fecal progestin metabolite concentrations in peregrine falcon female 1 (b) after estradiol-17 β (triangles), progesterone (solid circles), or saline (open circles) treatment. Data were aligned to hour of treatment injection (0 h)

after radiolabeled E₂ infusion has revealed that the cockatiel (*Nymphicus hollandicus*), orange-winged Amazon parrot (*Amazona amazonica*), budgerigar (*Melopsittacus undulatus*), and Japanese quail (*Coturnix coturnix japonica*) excrete mostly conjugated fEM (Bishop and Hall 1991; Lee et al. 1999; Tell 1997). This may be due, in part, to the aqueous buffer extraction system used that preferentially extracts conjugated (polar) metabolites. Like the cockatiel, Amazon parrot, and Japanese quail, the eagle and falcon excreted more E₂ than E₁. In contrast, the budgerigar excretes more E₁ than E₂ (Lee et al. 1999; Tell 1997). Our findings demonstrated that our methods for analyzing fecal steroids in raptors yielded the primary forms of E (E₂ and E₁) known to be most closely associated with reproductive events in female birds (Rehder et al. 1986; Wingfield and Farner 1978).

Chromatographic evaluation of fPM in the eagle and falcon revealed a combination of polar and nonpolar metabolites, probably derived from circulating P₄, common

pregnanolone or pregnanediol metabolites, or other hormones metabolized to P₄ (e.g., corticosteroids) (McKerns 1969). Resulting profiles in the eagle and falcon were quite similar to the eclectic HPLC patterns elicited in the Bali mynah (Albaugh 1999) and barred owl (Wasser and Hunt 2005). Our data suggested that P₄ was a major fPM in raptor feces, which was important because this hormone has been associated with specific reproductive events, such as egg laying in raptors (Rehder et al. 1986).

Both the male eagle and falcon excreted polar and nonpolar fAM, and it was apparent that unconjugated T constituted the majority of immunoreactive androgens. This is in contrast to other species where radiolabel T infusion studies have indicated that excreta contain nonhydrolyzable fAM conjugates (Lee et al. 1999; Tell 1997). The Amazon parrot produces mostly hydrolyzable conjugates of T, and a majority co-elutes with radiolabeled-T after HPLC separation (Lee et al. 1999). In contrast, there is a single immunoreactive peak that co-elutes with radiolabeled-T in the Bali mynah (Albaugh 1999). Using similar techniques to that used for the mynah, we determined that eagle and falcon fAM primarily contained unconjugated T. Thus, our procedure was useful for tracking T, the primary steroid associated with spermatogenesis and breeding behavior in birds (Meijer and Schwabl 1989; Rehder et al. 1988; Wingfield and Farner 1978).

Exogenous ACTH consistently induced adrenal activation that was characterized by a rapid rise in fGCM excretion, followed by a return to baseline in both species. The parallel qualitative fGCM excretion patterns in both species studied here were similar to those found in serum/plasma (Ludders et al. 1998; Wilson and Holberton 2001) or feces (Ludders et al. 2001; Wasser et al. 1997, 2000) of other ACTH-challenged birds. There were no sex-related differences in ACTH responsiveness within the eagles or falcons, which also was consistent with other ACTH or handling studies (Astheimer et al. 1994; Romero and Ramage-Healey 2000).

Although the ACTH challenge fGCM excretion pattern in the falcon was analogous to the pattern observed in the eagle, it was not statistically significant in the falcon. This was likely due to the hypersensitivity of the falcon to handling (or simply our presence in the enclosure to collect feces). Ludders et al. (2001) observed increased plasma CORT without a concomitant elevation in fGCM in a Florida sandhill crane (*Grus canadensis pratensis*) given ACTH. This was partially explained by aggressive and nervous behavior, which may have activated the HPA axis before treatment. Thus, anxious individuals may have prematurely and maximally activated adrenal function, and one result would be variable and confounded profiles post-ACTH. Alternatively, adrenal refractoriness can occur in individuals under chronic stress, making the HPA axis unresponsive

to ACTH (Gray et al. 1990; Heath and Dufty 1998). Other studies have documented the significance of handling stress in birds (Zenoble et al. 1985; Hiebert et al. 2000; Sockman and Schwabl 2001), and our findings emphasized that this can be a major perturbation in the peregrine falcon. Additionally, these evaluations were conducted during the time of natural molt, which may influence basal B concentrations and adrenal responsiveness to handling stress (Romero and Ramage-Healey 2000). The highly variable and modest adrenal responsiveness observed in our falcons also may be explained by the low-sample size and individual variation. It also was possible that porcine ACTH was not as effective as other hormone preparations in this species (Spelman et al. 1995). Additionally, the CORT RIA antibodies used may not have been the most effective at detecting fGCM in the peregrine falcon. However, based on the HPLC profile, ACTH challenge and saline infusion data, we are confident that the antibodies in this assay detect biological and physiological changes in fGCM in the falcon.

Finding a cause and effect response after steroid injections in only one-third of the infusion cases suggested that either that the assays were unable to detect some relevant metabolites or, more likely, the mass of hormone administered generally was too low and probably should be increased in future studies of this type. Nonetheless, the positive responses measured in one-third of the birds were typical of expectations and, in conjunction with HPLC data, further confirmed the validity of the various assays. Handling-induced stress also may have influenced the secretion of hormones other than CORT, which could explain some of the similarities between the profiles after steroid versus saline injection. Welsh and Johnson (1981) found a positive correlation between serum P_4 and corticosteroids (which may exist because P_4 is an obligate precursor of corticosteroids), and both steroids also increase when bulls are electroejaculated. Domestic male fowl also experience increased CORT secretion from the adrenals, but also T from the testes in response to restraint (Heiblum et al. 2000). Additionally, the effects of negative feedback may be difficult to predict or interpret. In the present study, handling (in the presence of saline only) induced increases in fAM and fPM on five occasions. Such observations emphasize the need for more detailed investigations that examine the impact of species and handling on adrenal activity in raptors. Such studies are important for understanding the physiological mechanisms that allow species and individuals to be sustained or adapt *ex situ* or *in situ*, and to successfully reproduce and thrive. At the same time, such investigations are highly complex in design and logistics and must address many factors. For example, these type studies also are influenced by receptor and binding protein concentrations (e.g., corticosterone-binding globulin) that,

in turn, are affected by season (Breuner and Orchnik 2001). The relative proportions of bound-to-unbound hormone can impact hepatic and renal clearance rates for steroids, effectively reducing biological activity and/or altering metabolism and excretion (Wingfield 1980; Wingfield et al. 1984).

Lag-time from secretion to excretion is another important variable. The ACTH challenges revealed intervals in the golden eagle and peregrine falcon that were similar to what has been reported for the spotted owl (Wasser et al. 1997, 2000). In contrast, fGCM concentration peaks within 3 h of ACTH administration in the Florida sandhill crane (Ludders et al. 2001). Similarly, European stonechats (*Saxicola torquata rubicola*) excrete peak fAM within 3 h of [3 H]T administration (Goymann et al. 2002). Direct between-species comparisons in CORT metabolism are complicated by differences in sample handling, sex, age, reproductive status, circadian or circannual rhythms, diet, and captive management (Goymann 2005; Touma and Palme 2005).

It was important to document enterohepatic recirculation of both adrenal and gonadal hormones in these two species, a phenomenon whereby endogenous steroids are removed from plasma via hepatic and renal circulation, and reabsorbed from the filtrate or excreted into the cloaca. This mechanism has been identified previously in the duck (Helton and Holmes 1973), great-horned owl (*Bubo virginianus*), and barred owl, but interestingly not in the Northern spotted owl (*Strix occidentalis caurina*) (Wasser et al. 2000; Wasser and Hunt 2005). We also recorded for the first time what appeared to be triphasic recycling. Such observations are important for ensuring adequate design of future studies. For example, it is critical for investigators to allow sufficient time for steroid excretion in metabolism studies. Likewise, it is essential that sample collection frequency be adequate to capture temporal rise and falls in excreted metabolites to avoid confounding data interpretation.

In summary, the present work has demonstrated the potential applicability of noninvasive fecal hormone monitoring for assessing gonadal and adrenal activity in raptors. We have identified some of the requisite endocrine traits through HPLC analysis, ACTH challenges, and steroid infusions. Additionally, there were marked species differences, which reinforce the need for conducting more investigations in the diverse species within this taxon. However, it also suggests that, although noninvasive monitoring was less informative for the peregrine falcon than the golden eagle, this technology may well be ideal under a different experimental design. The point is that data recovered from excreted feces were physiologically and biologically valid and reflective of endogenous endocrine function. As a result, there are fascinating possibilities for applying these techniques to understand the physiological complexities of

birds-of-prey. Among the many candidate topics are studying the impact of: (1) habitat degradation and fragmentation, (2) captive management techniques (i.e., enrichment to reduce aggression or crowding), (3) social status, (4) reproductive status on fitness and well-being, and (5) developing assisted breeding to enhance reproduction in ex situ breeding programs.

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