Synthesis of Measuring Steroid Metabolites in Goose Feces

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ABSTRACT: **The reliability of noninvasively measuring steroid hormones from feces in greylag geese (*Anser anser*) and domestic geese (*A. domesticus*), both qualitatively and quantitatively, was tested experimentally. Geese are mainly herbivorous birds with a short gut-passage time (2–3 h). Groups of eight outdoor-housed male domestic geese were subjected to two different experiments, injection of either GnRH or ACTH, which were replicated in three different seasons (spring, summer, and fall). GnRH stimulation resulted in significant increases of response fecal testosterone metabolites (TM; 17β-OH-androgens) in spring and fall, but not during the summer photorefractoriness. Testosterone response patterns obtained from plasma samples paralleled those from feces; however, no direct correlation between individual immunoreactive plasma and feces contents was observed. To improve the sample handling during extraction and the assay sensitivity, we promote the use of a group-specific antibody against 17-oxoandrogens that does not require deconjugation prior to the analysis. ACTH robustly increased fecal corticosterone in all seasons. The polar nature of glucocorticoids, however, seems to make a distinction between conjugated and nonconjugated types difficult, and the available avian literature on this topic is discussed.**

KEYWORDS: ***Anser anser; Anser domesticus;* fecal hormones; steroid excretion; testosterone; corticosterone; progesterone; estradiol; enzyme immunoassay; fecal sample storage; GnRH; ACTH; seasonal patterns; diurnal patterns**

INTRODUCTION

Hormones are produced by endocrine glands and are transported via the bloodstream. They are rapidly metabolized and excreted. Measuring immunoreactive steroid metabolites from excrements carries several advantages over more traditional blood sampling. Collection of fecal samples enables continuous monitoring from the same individual, even over long periods, with minimal disturbance of its activities and social environment. Handling of the animal is not required, which is especially valuable in studies with a focus on adrenal stress hormones (i.e., glucocorticoids).[1]

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However, gonadal steroid secretion may also be affected by adrenal stress responses (e.g., due to the sampling procedure), probably by an intrahypothalamic neuroendocrine circuit through which stress may inhibit the release of GnRH.[2] This factor particularly delays egg laying in female birds.[3,4]

Fecal measures of steroids are cumulative levels over the gut-passage time, and therefore reflect environmental effects over time.[5] In contrast, steroid measures from blood represent single-condition snapshots in time. The drawbacks of fecal hormone measurements are that care must be taken to prevent misidentification of samples when animals group closely together or when the vegetation level is high. Optimal storage of samples in the field may be problematic.[6–8] The separation of urinary and fecal compounds may be unfeasible,[9] further confounding the results. In birds, as in mammals, renal excretion via urine is faster than excretion via feces. However, fecal and urinary glucocorticoid metabolite concentrations were highly correlated in Wilson's storm petrels (*Oceanites oceanicus*[10]) and chicken (*Gallus domesticus*[11]). In the case of geese, however, the uric acid compound is placed as a cap at one end of the dropping (8% of the dropping in barnacle geese *Branta leucopsis*[12]), and so at least collection of the white urea may easily be avoided.

Increasing evidence indicates that there are large differences between species and that, so far, no single method for noninvasive assessment of steroid hormones can be used for cross-species application, which necessitates customized approaches.[5–6,13–15] The optimization of the mode of extraction and validation of the assay system are time-consuming and expensive. Validation and the specificity of the assay should always be stringent requests when fecal steroid data are presented.[16–19]

The free-ranging nonmigratory population of monogamous and biparental greylag geese (*Anser anser*) in Grünau is an exceptionally suitable subject for the use of fecal steroid measurements. All birds are marked with colored leg bands and habituated to the close presence of human observers; the individual life histories have been recorded since 1973.[20–21] As a starting point for hormonal studies in the greylag geese, the diurnal[22] and ontogenetic[23] patterns have been examined for integration into sampling paradigms. From individual fecal samples of adult male and female geese, we monitored the seasonal patterns of immunoreactive corticosterone,[24] testosterone,[25,26] estradiol, and progesterone[27] metabolites. We focused on the behavioral modulation of steroid hormones by comparing the seasonal patterns of different social categories, such as paired or unpaired, parental or nonparental. To do this, a large set of fecal samples was collected throughout a complete annual cycle. Fecal steroid data were grouped by means of seasonal phase; the phases were individually arranged by the date a female laid her first egg. Several issues had to be resolved beforehand, such as the collection and storage of the samples and the qualitative and quantitative clarifications of what the assay basically detected in the goose excrement. The fundamental indication for the method's applicability was the biological relevance of a match between hypotheses and first results. For example, the annual cycles of fecal sex steroids in greylag geese[25,27] generally matched those of plasma in barheaded geese (*A. indicus*),[28] and the seasonal patterns of fecal corticosterone were associated with social status and behavioral interactions.[24,26] Also, androgen metabolite levels in males were affected by the winning of interactions and pair-bond challenges during the breeding season,[26] as predicted by the "challenge hypothesis."[29] And finally, implants of exogenous testosterone resulted in higher levels of androgen metabolites in the feces of adult males.[30] Meanwhile, we have

done more detailed analyses of short-term hormonal responses to behavior and changes in the social environment.[31–34] Here, we aim to present a synthesis of the technical aspects of measuring steroids (particularly androgens and glucocorticoids) in goose feces.

GUT-PASSAGE TIME AND REFLECTANCE OF CIRCULATING LEVELS

The lag between a plasma hormone peak and its appearance in the feces depends on gut-passage time.[13] The time course of excretion varies widely between species and is faster via urine than via feces,[5,10,11] in which it may be influenced by dietary fiber content.[16,35] Steroid hormones reach the gut after passing through the liver and the bile.[36,37] Peak excretion of both androgens and glucocorticoids is in the range of days in large mammals,[36,38–41] and in the range of minutes to hours in birds.[13,40,42–48]

To determine the basic gut-passage time of geese, we attempted two different methods. For greylag geese, as a marker we used a silver mica powder nucleus set inside a piece of bread (W. Haberl, personal communication), which after continuous observation was excreted after precisely 2 h.[49] In the second method we used charcoal dust–marked food, which revealed a gut-passage time of 2–5 h in domestic geese (*A. domesticus*[13]). Both approaches corresponded with the average throughput time reported for domestic geese (between 44 and 137 min[50]). Therefore, we assume that steroid levels obtained from goose feces reflect cumulative secretion over the 2 h preceding defecation. However, defecation rates of geese may vary between seasons,[12] even if provided with standardized food,[13] which indicates a potential overlap of the periods reflected in a dropping.

The central assumption that fecal levels of hormone metabolites reflect circulating levels of biologically active compounds is inevitable. However, the relationship between fecal and plasma steroid concentrations is not always a direct one. High plasma–feces correlations for testosterone and estradiol were reported in domestic fowl using the droppings during the 3 h before the plasma was sampled[43] or, alternatively, using the fecal samples at a lag of 4 h after the plasma sampling.[48] Correlations of corticosterone were reported in the Northern spotted owl (*Strix occidentalis caurina*), with blood samples taken on three mornings just after a fresh fecal sample was obtained.[45] However, the pulsatile and episodic pattern of glucocorticoid secretion, for example, may complicate this validation approach. In geese, we observed no short-term correlations between individual plasma and fecal testosterone measures, although plasma and feces resulted in parallel androgen response patterns to GnRH challenges[13] (FIG. 1, and see below). It seems that the amount of "noise" in fecal steroid data[51] suggests that they cannot be used to distinguish between animals that have only small differences in plasma steroid concentrations. Rather, fecal steroids are useful to distinguish between animals with large plasma differences (J. Cockrem, personal communication). In fact, for most questions the use of integrative cumulative hormone measurements from feces may even provide a better picture of hormone patterns than data derived from blood sampling.[5]

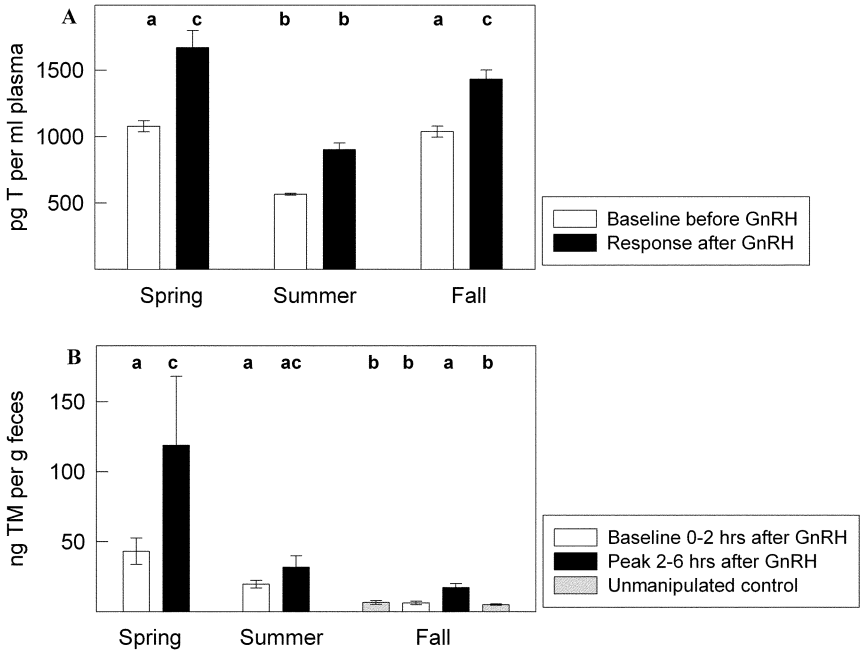

FIGURE 1. Seasonal comparison (mean ± SEM) of baseline and androgen response in domestic ganders. (**A**) Baseline testosterone levels (T) in plasma were sampled before GnRH stimulation and responses 90 min after injection. (**B**) In feces, baseline testosterone metabolites (TM) were sampled within 0–2 h after stimulation and peak response from samples within 2–6 h after stimulation. An unmanipulated control group was available for feces sampling only in fall. Two groups of eight ganders were available per season. Sample sizes vary because individuals were included only when both baseline and response values were available (depending mainly on irregular defecation). Significant differences between seasons, as well as between baseline and response levels, are indicated by different letters, a–c. In the (**A**) plasma, both baseline and response T differed significantly between seasons (one-way ANOVA: baseline, $F = 24.32$, $df = 2$, $P < .0001$; response, $F = 9.14$, $df = 2$, $P = .0012$); spring and fall levels significantly exceeded summer levels (Bonferroni *post hoc* test). Within seasons, response T significantly exceeded baseline T in spring and fall (Wilcoxon: spring, $Z = -2.80$, $n = 10$, $P = .005$; fall, $Z = -3.06$, $n = 12$, $P = .002$) but non-significantly so in summer ($Z = -1.83$, $n = 4$, $P = .068$). In the (**B**) feces, baseline TM significantly varied between seasons (one-way ANOVA: $F = 10.46$, $df = 2$, $P = .0006$); spring baseline was significantly higher than fall baseline (Bonferroni *post hoc* test). Seasonal differences of peak TM responses were only marginally significant ($F = 3.13$, $df = 2$, $P = .063$). Peak TM levels significantly exceeded baseline levels in spring (Wilcoxon: $Z = -2.40$, $n = 10$, $P = .017$) and in fall ($Z = -3.06$, $n = 12$, $P = .002$), but not in summer ($Z = -0.73$, $n = 4$, $P = .465$). (Figure modified from Hirschenhauser *et al.*,[13] with permission from Elsevier.)

SAMPLE HANDLING

The stability of the immunoreactive content in fecal samples due to handling and storage is an essential topic, particularly for field work, and may potentially be one source of noise in fecal steroid data.[8] The time between defecation and freezing the droppings is critical, because oxidation and bacterial metabolism can alter the steroid contents in the feces within hours after deposition.[6,52] In unpreserved baboon (*Papio cynocephalus*) fecal samples kept at room temperature, Wasser *et al*.[53] showed that changes in fecal estrogen and progesterone levels occurred within 6 h. In chicken droppings, no effect of room temperature on testosterone metabolites (TMs) or estrogen concentrations was observed for up to 48 h.[43] This may be another species-specific issue. It is probably essential to collect fresh droppings immediately after deposition and to preserve them as quickly as possible, optimally by freezing at −20°C. If freezing is not available, it is possible to store aliquots of the sample in alcohol.[6,54] However, caution is suggested because long-term preservation of elephant (*Loxodonta africana*) feces in ethanol resulted in elevated immunoreactive glucocorticoid concentrations of up to 300% of those in lyophilized frozen control samples.[55] Furthermore, repeated sample thawing and the exposition of the droppings to rainfall increased fecal glucocorticoid metabolite measurements.[7] A study on fecal glucocorticoid and estrogen metabolite content of baboon feces stored in 95% ethanol at room temperature or at −20°C, suggested keeping the samples no longer than a month at either temperature and if possible to extract and deconjugate the fecal sample as soon as possible.[6]

Stability of Testosterone in Fecal Samples: From Collection to Storage

We stored the fecal samples from geese by freezing unpreserved dry matter at −20°C within 2 h of collecting fresh feces. Care was also taken to keep them frozen, or at least cool, during transport. In a small data set, we aimed to define a sampling–freezing field protocol for fecal androgen measurements from goose feces taken during the summer season. We were also interested in whether the collection of "older" feces—from incubating females at their nests, for example—would be feasible.[49] To determine the latest possible time before freezing the fecal samples from geese, we divided large droppings into four portions, one of which was stored at −20°C immediately after deposit, while the remaining three portions were kept at room temperature (20°C) and then frozen after 1 h, 2 h, and 3 h (n = 3 females and 4 males). The samples were then extracted and analyzed for levels of testosterone (TM),[25] progesterone (PM), and estradiol (EM)[27] metabolites. Sample sizes were too small to statistically test the resulting patterns; however, variation within individuals was especially large with regard to TM in males (>200% of the initial TM content; FIG. 2B). In all cases, freezing of the fecal samples as soon as possible after collection is highly recommended.

To compare the storage of fecal samples as untreated dry matter with that stored in alcohol, we divided the available three to four feces portions of one female and one male into halves. One half was frozen at −20°C with no preservatives, and we stored 0.5 g of the other half in a 6-mL aqueous methanol dilution, and then stored it at −20°C immediately, after 1 h, 2 h, and 3 h at room temperature. The immuno-

reactive contents of TM, PM, and EM in the male samples were hovering around 100% of the initial value (FIG. 2B, 2D, 2F), and the same result was observed for the EM contents in female goose feces stored in alcohol (FIG. 2E). However, PM levels in female fecal samples frozen in alcohol were considerably higher than the initial values of dry frozen matter (FIG. 2C), whereas in the samples from males this pattern was less pronounced and, if any, reversed (FIG. 2D). TM in the female fecal samples preserved in alcohol started with an identical 100% of the initial value in the unpreserved frozen sample, but then increased to more than 200% (FIG. 2A).

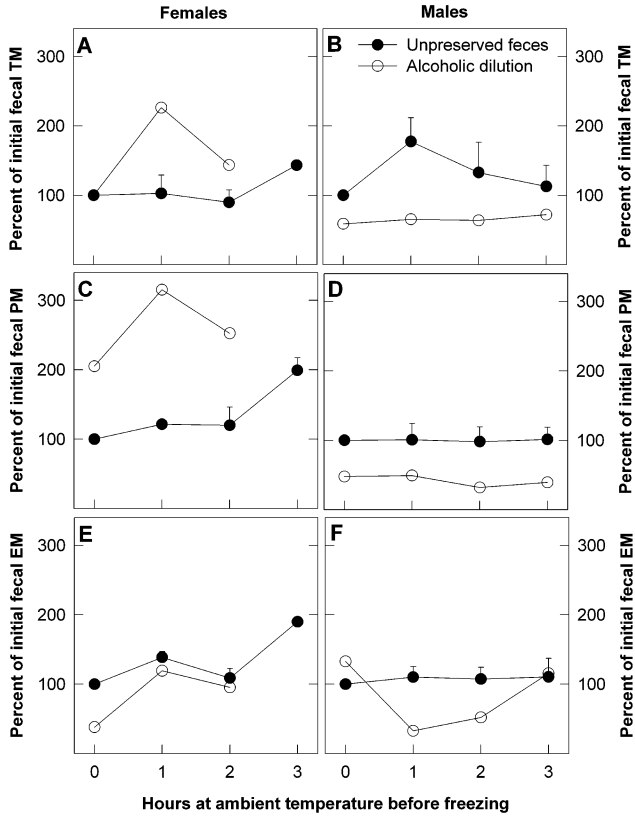

FIGURE 2. Test series of the immunoreactive steroid contents [(**A**, **B**) TM: fecal 17β-OH-androgen metabolites; (**C, D**) PM: fecal progesterone metabolites; (**E, F**) EM: fecal estrogen metabolites] in greylag goose feces frozen as dry matter or in alcohol at different times at ambient temperature. Up to four portions of the same fecal samples were frozen as unpreserved fecal matter (*filled symbols*) immediately after collection (0 h), and after 1 h, 2 h, and 3 h at room temperature (20°C). For comparison, 0.5-g aliquots of the fecal sample portions of one female and one male were preserved in aqueous alcoholic dilution before freezing (*open circles*). Immunoreactive steroid contents are expressed as percent of the initial value (frozen unpreserved immediately after deposition). Left-side panels show the fecal steroid patterns from females ($N = 3$), right-side panels from males ($N = 4$).

QUANTITATIVE TESTS OF STEROID EXCRETION IN GEESE

Testosterone

To test the basic assumption that TM levels in feces are biologically meaningful and proportionally reflect plasma levels, we compared basal and GnRH-stimulated (10 g Ovurelin per individual; Reana, Budapest, Hungary) plasma T concentrations with fecal TM in two groups of eight domestic ganders.[13] The experiment was repeated during three seasons: the spring peak of reproductive activity, the summer photorefractoriness, and the fall sexual reactivation. Plasma was sampled 90 min following the treatment; feces were collected continuously from all individuals throughout the day of the experiment.

Plasma T-level increases in response to GnRH were followed by significantly elevated fecal TM 2–6 h after the GnRH treatment in spring and fall (FIG. 1). During the summer refractoriness, however, T responses to GnRH remained nonsignificant in both plasma and feces. The high correspondence between general androgen response patterns measured from plasma and feces emphasizes the reliability of using fecal androgen measures for pattern comparisons.[13] On the other hand, direct correlations between individual plasma T and fecal TM levels remained nonsignificant ($P > .05$), which indicates the lack of a short-term relationship between plasma and excreted androgen metabolites. Episodic fluctuations seem to be dampened rather than reflected by the fecal metabolite concentration.[48]

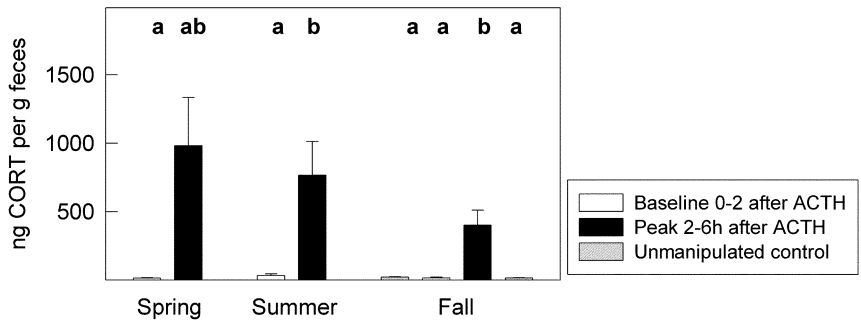

FIGURE 3. Seasonal comparison (mean ± SEM) of fecal baseline corticosterone metabolites (CORT) sampled 0–2 h after ACTH treatment and peak responses within 2–6 h after ACTH injection in domestic geese. An unmanipulated control group was available only in fall. Two groups of eight ganders were available per season. Different letters (a, b) indicate significant differences between baseline and response levels, as well as between seasons. Neither baseline CORT levels nor peak response levels differed significantly between seasons (one-way ANOVA: baselines $F = 0.68$, $df = 2$, $P = .523$; peaks $F = 1.09$, $df = 2$, $P = .363$). However, in all seasons, peak CORT levels were significantly elevated in response to the ACTH treatment (Wilcoxon baseline vs. peak response: spring $Z = -1.825$, $n = 4$, $P = .068$; summer $Z = -2.52$, $n = 8$, $P = .012$; fall $Z = -2.02$, $n = 5$, $P = .043$). Sample sizes vary because individuals were included only when both baseline and response values were available (mainly depending on defecation, which varied between seasons). (From Kotrschal *et al.*[46] Reprinted with permission from Springer.)

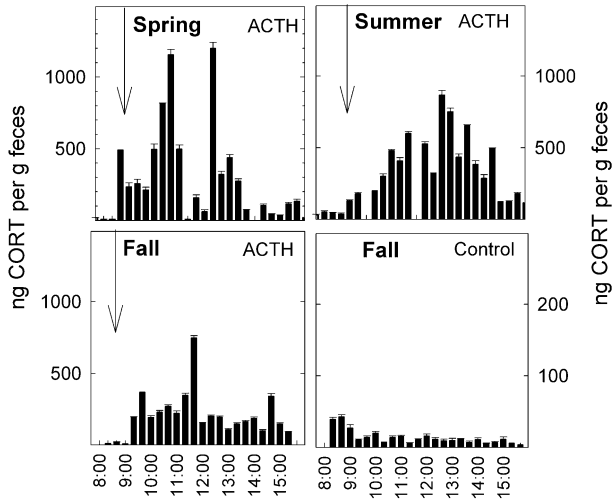

FIGURE 4. Diurnal excretion patterns of mean fecal corticosterone metabolites (CORT) of eight domestic ganders ± SEM within 20-min sampling periods following ACTH injection (indicated by the *arrows*) in spring (**Upper-Left Panel**), summer (**Upper-Right Panel**), fall (**Lower-Left Panel**), and an unmanipulated control group in fall (**Lower-Right Panel**). Because individual defecation rates were irregular, the number of samples integrated per bar varies between one and eight. In bars lacking SEM, $n = 1$. (From Kotrschal *et al.*[46] Reprinted with permission from Springer.)

Corticosterone

Two groups of eight domestic ganders were also treated with ACTH in spring, summer, and fall to stimulate corticosterone (CORT) responses. Individual fecal samples were collected throughout the treatment day.[46] ACTH clearly increased peak fecal CORT levels in all seasons (FIG. 3), with two to three peaks over the day (one after approximately 2 h, a second one after 4 h, and possibly a third one after 6 h; FIG. 4). In the unmanipulated fall control group we observed the typical small CORT peaks in the morning,[22,56] followed by consistently low levels throughout the day. We therefore consider the oscillatory excretion pattern of CORT throughout the day as specific responses to the treatment rather than an effect of an endogenous circadian excretion regimen.

QUALITATIVE TESTS OF STEROID CONTENT IN GOOSE EXCREMENT

Androgens

To determine the excretory testosterone metabolites in domestic geese, we injected intravenously two ganders with 370 kBq of ^{14}C-testosterone (NEC-101; New England Nuclear, Vienna). All droppings were collected, and 0.5 g of each sample was

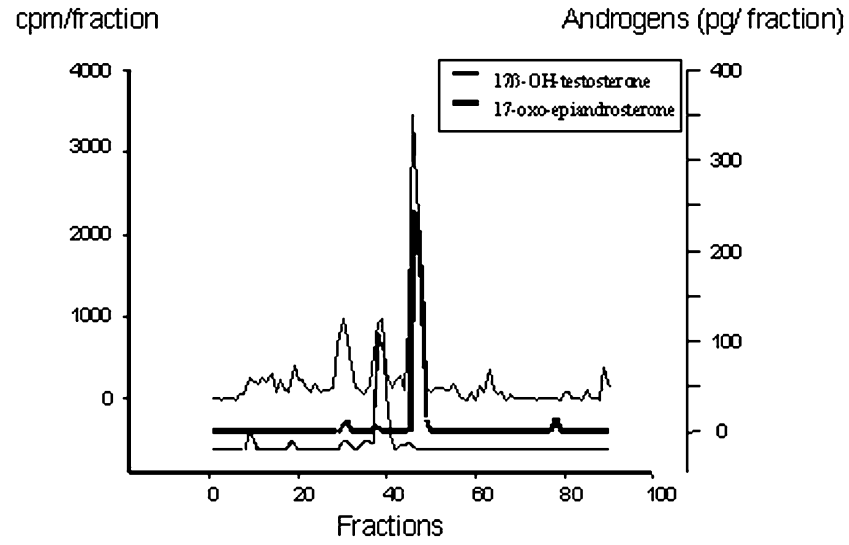

FIGURE 5. HPLC immunogram after intravenous application of ^{14}C-testosterone into a domestic gander. The fecal sample with the peak radioactivity was separated via a SepPac C_{18} column, followed by reverse-phase HPLC. The 17β-OH-androgen assay bound to a few minor metabolites, whereas the 17-oxoandrogen assay detected the major metabolite peak. (Modified from Hirschenhauser *et al.,*[13] with permission from Elsvier.)

extracted with methanol, centrifuged, and the supernatant measured by liquid scintillation counting. The samples with highest radioactivity were then assayed with and without prior deconjugation (enzymatic hydrolysis with β-glucuronidase/arylsufatase; Merck 4114[25,27]) using enzyme immunoassays. We compared two group-specific antibodies,[52,57]one against 17β-OH-androgens (4-androstene-17β-ol-3-on-carboxymethyloxine-albumine-CMO from rabbits), which we had used hitherto,[13,25–27,46] with an epiandrosterone antibody against 17-oxoandrogens. The 17-oxoandrogen assay detected more immunoreactive metabolites, and the biological sensitivity of the assay therefore increased. Using the 17β-OH-androgen assay, immunoreactive metabolites were measured mostly after deconjugation. When using the assay for the 17-oxoandrogens, however, hydrolysis diminished the immunoreactivity.[13] An HPLC immunogram (high-pressure liquid chromatography separation) indicated that all three major radioactive peaks showed immunoreactivity (FIG. 5). Both assays reacted with testosterone metabolites in the feces, but the dominating radioactive peak was detected by the 17-oxoandrogen assay without prior hydrolysis. Thus, the 17-oxoandrogen antibody allows a direct assay without the necessity of deconjugation. Because this antibody binds to another position of the androgen molecules, one can measure both unconjugated and conjugated fractions in one procedure, which is probably a solution comparable to the steroid–conjugate assays proposed by Bishop and Hall.[42]

To test the robustness of our previous results, we reanalyzed selected samples from domestic ganders (three individuals in spring, summer, and fall) with high TM

TABLE 1. Summary of the literature on measuring immunoreactive steroids from avian feces with or without deconjugation prior to the analysis

Order	Species	Focal immunoreactive steroid	Hydrolysis	Reference
Galliformes	Japanese quail (*Coturnix coturnix japonica*)	Testosterone	Yes	42
Galliformes	Japanese quail	17-oxoandrogens	No[a]	Own unpublished data
Galliformes	Chinese painted quail (*Excalfactoria chinensis*)	17-oxoandrogens	No[a]	Own unpublished data
Galliformes	Chicken (*Gallus domesticus*)	Testosterone	No[b]	43
Anseriformes	Greylag goose (*Anser anser*)	17β-OH-androgens	Yes	13, 25, 27
	Domestic goose (*A. domesticus*)	17β-OH-androgens	Yes	13
Anseriformes	Domestic goose	17-oxoandrogens	No[a]	13
Piciformes	Downy woodpecker (*Picoides pubescens*)	Testosterone	No[b]	64
Psittaciformes	Cockatiel (*Nymphicus hollandicus*)	Testosterone	Yes	65
Passeriformes	Brown dipper (*Cinclus pallasii*)	Testosterone	No[b]	66
Passeriformes	European stonechat (*Saxicola torquata rubicola*)	Testosterone	Yes[b]	47
Galliformes	Chicken	Corticosterone	No	48
Galliformes	Chicken	Cortisone	No	11
Anseriformes	Greylag goose	Corticosterone (11β,21-diol-20-one)	Yes	24
	Domestic goose			46, 60
Anseriformes	Greylag goose	Corticosterone (11β-Hydroxyetiocholanolone)	Yes	62
Sphenisciformes	Adelie penguin (*Pygoscelis adeliae*)	Corticosterone (Tetrahydrocorticosterone)	No	63
Procellariformes	Wilson's stormpetrel (*Oceanites oceanicus*)	Corticosterone (Tetrahydrocorticosterone)	No	10
Apodiformes	Rufous hummingbird (*Selasphorus rufus*)	Corticosterone	No	67
Falconiformes	Goshawk (*Accipiter gentilis*)	Corticosterone	No[b]	48

TABLE 1. (*continued*) Summary of the literature on measuring immunoreactive steroids from avian feces with or without deconjugation prior to the analysis

Order	Species	Focal immunoreactive steroid	Hydrolysis	Reference
Strigiformes	Northern spotted owl (*Strix occidentalis caurina*)	Corticosterone	No	45
	Barred owl (*Strix varia*)			40
	Great horned owl (*Bubo virginianus*)			40
Passeriformes	European stonechat	Corticosterone	Yes	61
Passeriformes	Great tit (*Parus major*)	Corticosterone (11β-Hydroxyetiochol-anolone)	Yes	56
Galliformes	Japanese quail	Estradiol	Yes[c]	42
Galliformes	Japanese quail	Pregnanediol	Yes[c]	42
Galliformes	Chicken	Estradiol	No[b]	43
Psittaciformes	Cockatiel	Estrone/Estradiol	Yes	65
Passeriformes	Brown dipper	Estradiol	No[b]	66

[a]Antibody also detects conjugated androgen molecules.
[b]The original article provided no information on whether testing had proved the necessity of using hydrolysis.
[c]Antisera used against steroid conjugates (i.e., glucuronides).

concentrations after GnRH stimulation[13] by using the 17-oxoandrogen assay. Results obtained with this assay were parallel to the results obtained by the 17β-OH-androgen assay ($r_s = 0.98$, $n = 9$, $P < .0001$[13]), which confirmed the results obtained by the earlier assay. All of these tests were restricted to male geese, and we are currently testing for potential sex-specific differences of immunoreactive androgen metabolites[16,58,59] in goose feces.

Early studies revealed a large proportion of unconjugated steroid metabolites in Japanese quail (*Coturnix coturnix japonica*) feces for both testosterone (31% of TM compared with only 6.5% in the starling's feces) and estradiol (15% of EM). However, in quail feces the dominant metabolites were also present as glucuronide conjugates.[42] Recent 17-oxoandrogen assays of Japanese and Chinese painted quail (*Excalfactoria chinensis*) feces resulted in biologically meaningful androgen patterns without deconjugation of the extracted fecal samples (unpublished data).

The fact that unconjugated metabolites were detectable in chicken androgens,[11,43,48] whereas in the goose model we observed a large proportion of conjugated metabolites,[60] suggests a systematic pattern of the degree of conjugation or polarity. However, a summary of the existing literature on this topic does not confirm this proposition, and a systematic pattern of the degree of excreted steroid metabolites does not seem obvious (TABLE 1).

Glucocorticoids

Until recently, fecal glucocorticoid metabolites in goose feces were assayed by enzyme immunoassay using an antibody against corticosterone-3-CMO:BSA and corticosterone-3-CMO-dioxaoctane-biotin as label.[24,46] We measured the glucocorticoid metabolites after infusions of radioactive corticosterone metabolites in domestic ganders. Most glucocorticoid metabolites were not diethylether extractable, but enzymatic hydrolysis of the methanol-extracted fecal samples with β-glucuronidase/arylsufatase (Merck 4114) increased the recovery of excreted radioactive corticosterone metabolites to 23% compared with almost 0% without prior hydrolysis.[46,60] This finding indicated that in geese, glucocorticoid metabolites were either excreted as predominantly conjugated or as polar unconjugated metabolites. Similar results were meanwhile obtained with great tits (*Parus major*[56]), European Stonechats (*Saxicola torquata rubicola*[61]), and quails (*C. c. japonica* and *E. chinensis*; unpublished data).

More recently, an assay for measuring a group of fecal glucocorticoid metabolites (3,11β-dihydroxyandrogens) has been developed and applied to goose feces[62] (hydroxyetiocholanolone was used as the standard and as label). This assay resulted in considerably higher glucocorticoid metabolite concentrations than did measuring the same samples with the earlier assay and, thus, in more fine-tuned patterns. Preparation and extraction of the fecal samples, however, had to be continued as before.[24,46]

The necessity to deconjugate prior to the assay is not always clear. Glucocorticoids may be excreted as tetra- and pentahydroxylated metabolites. These are highly polar molecules, which are not ether extractable. Therefore, a clear classification of glucocorticoids into conjugated or nonconjugated is problematic.

To find a systematic pattern in the degree of conjugation of excreted steroid metabolites, we compiled a summary of the existing literature (TABLE 1). This summary shows that specifically in the (purely carnivorous) birds of prey[40,45,48] and Adelie penguins (*Pygoscelis adeliae*[63]), deconjugation prior to the analysis curiously did not improve the measured content of immunoreactive CORT in the feces, although the metabolites were highly polar. Deconjugation was also not used to measure TM in woodpeckers (*Picoides pubescens*[64]), which feed exclusively on insects, and for TM[43] and CORT[11,48] in chicken which, if permitted, prefer a high-protein diet. The binding of steroid molecules to glucuronides and sulfates is regulated by enterobacteria in the gut and ceca. A high-protein diet changes the microflora in the ceca, whereas the fiber content of the diet decreases steroid resorption.[9] Whether the location of steroid conjugates may vary with diet or with excretion pathway (i.e., proportion of feces or urine excreted), or whether those are just highly polar substances, remains an open question. A further confounding variable is the use of different antibodies that detect different metabolites.

In summary, over the last decade, several studies in the wild greylag geese, as well as in domestic geese, have demonstrated that fecal steroid measurements from goose feces result in biologically meaningful patterns. This method makes a wide array of questions accessible for research. Nevertheless, researchers in this area of study should be aware of the drawbacks of the method, such as sufficient number of samples to overcome variation within individuals, as well as the potential pitfall of sex-specific differences in excreted metabolites.

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