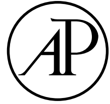
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Non-invasive methods to measure androgen metabolites in excrements of European stonechats, *Saxicola torquata rubicola*

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Abstract

Traditionally androgen concentrations are measured invasively in blood plasma. However, non-invasive methods to detect androgens are desirable, as this reduces interference with the natural behavior of the study species and multiple samples can be obtained relatively easy. The aim of this study was to validate a method to measure androgens non-invasively in excrements of male European stonechats (*Saxicola torquata rubicola*). Extracts of excrements of a male stonechat injected with [^{3}H]testosterone ([^{3}H]T) were chromatographically separated using high performance liquid chromatography (HPLC). The resulting HPLC fractions were then analyzed with a radioimmunoassay against testosterone (T-RIA). The results showed that the assay picked up major metabolites of [^{3}H]T. The physiological relevance of excreted androgen metabolites was further validated by showing that injection of exogenous GnRH to seven males led to a significant increase in excreted androgen metabolites. In contrast, androgen metabolite levels of six saline-injected control males did not increase. Furthermore, excrements from nine males were collected from January until April to see whether the typical seasonal increase in testosterone levels can also be traced when measuring excreted androgen metabolites. As expected, there was a significant seasonal increase in androgen metabolite concentrations. Thus, the T-RIA measures androgen metabolites in droppings of male European stonechats and to our knowledge this study represents the first validation of a non-invasive androgen assay in a passerine bird.
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1. Introduction

During the reproductive season several morphological and behavioral traits of male birds, such as plumage coloration, aggression, song, courtship, and territorial behavior are dependent on androgens (see e.g., in Balthazart, 1983; Ketterson and Nolan, 1992; Silver and Ball, 1989; Wingfield and Ramenofsky, 1985; Wingfield et al., 1990, 1994, 1997a,b). Traditionally, these androgens have been determined from blood samples that provide accurate measures of plasma androgen concentrations. However, this method has obvious drawbacks, as birds must be caught and handled, which is not always feasible or desired. Particularly in small birds, the practicability of hormone studies is often limited by the frequency and volume of blood samples that can be obtained. Also, handling may cause stress (Wingfield et al., 1992) and therefore may influence the measurement of androgens. Thus, non-invasive methods, such as measuring androgen metabolites in excrements of birds (=cloacal mixture of feces and urine) are desirable because sampling does interfere little with behavior and even multiple samples can be obtained from individuals. Furthermore, androgen metabolites measured in excrements represent pooled fractions of plasma androgens, providing an integrated measure of androgen levels. Due to these benefits and due to methodological progress (e.g., Möstl et al., 1987; Palme and Möstl, 1993), the non-invasive approach has gained increasing importance in recent years. Non-invasive measurement of gonadal steroids and corticosteroids in mammalian feces or bird excrements facilitated field-endocrinological studies (e.g., Cavigelli, 1999; Cavigelli and Pereira, 2000; Creel et al., 1997; Goymann et al., 2001; Hiebert et al., 2000b; Hirschenhauser et al., 1999a,b, 2000b;

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Kirkpatrick, 1990; Kotrschal et al., 1998; Wallner et al., 1999; Wasser et al., 1997; White et al., 1995). Furthermore, this method has proven to be a useful tool to monitor reproductive status and stress in captive animals (e.g., Bercovitz et al., 1982; Goymann et al., 1999; Graham and Brown, 1996, 1997; Hirschenhauser et al., 2000a; Hultén et al., 1995; Jurke et al., 1997; Kirkpatrick et al., 1992; Lee et al., 1995; Lucas et al., 1991; Miller et al., 1991; Monfort et al., 1998; Möstl et al., 1984; Palme and Möstl, 1997; Schwarzenberger et al., 1992; Sousa and Ziegler, 1998; Wasser et al., 2000; Whitten et al., 1998; Ziegler et al., 1988).

The validity of non-invasive hormone measurement relies on the assumption that steroid hormone concentrations in feces or excrements proportionally reflect circulating levels of these hormones. A method to detect steroid metabolites in one species does not necessarily work in other species (Goymann et al., 1999). Although androgen metabolite levels have been reported in passerine birds (Lee et al., 1995; Schwabl, 1996), to our knowledge this method has not yet been validated for any passerine bird species.

In this study, we validated the non-invasive measurement of androgens in droppings of European stonechats (*Saxicola torquata rubicola*). The European stonechat is a migratory passerine that breeds from April to August (Cramp, 1988) in northern and central Europe and winters in the Mediterranean area. This species establishes territories and forms pairs not only during the breeding season but also during the non-breeding season (Canoine and Gwinner, 2002; Gwinner et al., 1994; Rödl, 1995). Stonechats frequently use prominent perches (bushes, grasses) to display their territoriality. While perching they often conspicuously defecate. Their excrements can then be collected from leaves or grasses underneath the perch. Thus, this species is not only interesting because of its territorial behavior in the breeding and non-breeding grounds, but collection of excrements is also feasible in the field (Rödl and Flinks, 1996; Scheuerlein, 2000).

The objectives of this study were (1) to validate a radioimmunoassay (RIA) to quantify androgen metabolites in excrements of European stonechats and (2) to assess the potential of this technique to monitor seasonal changes in androgen metabolite excretion, that would allow us the future use of this technique in ongoing studies of captive and free-ranging stonechats (e.g., Canoine and Gwinner, 2002; Gwinner et al., 1994; Rödl, 1995, 1999; Scheuerlein and Nitsche, 1994; Scheuerlein et al., 2001) and possibly also in other passerines. Direct comparisons between circulating androgen levels and excreted metabolites of these androgens are problematic because of two reasons. Firstly, taking a blood sample may cause changes in post-sampling androgen release and thus alter the levels of excreted androgen metabolites. Secondly, plasma and excreted androgen levels represent two entirely different types of measures. While plasma samples give concentrations of androgens at a certain moment of time (point measure), androgen levels in feces or excrements represent a measure over a period of time (integrated measure). We hence used indirect comparisons to validate the non-invasive method to measure androgen metabolites in stonechats. We measured changes in androgen metabolite levels induced by a GnRH injection and by comparing androgen metabolite levels during different seasons.

2. Methods

2.1. Animals

Experimental birds were kept under natural light conditions and housed in individual cages, where they could hear, but not see each other. Radiolabeled testosterone and GnRH injections were carried out in April and May, when the gonads of the stonechats were fully developed and when our captive population typically started to breed (end of April–July; Gwinner, unpublished data). All birds were supplied ad libitum with our standard diet (Gwinner et al., 1995) and water.

2.2. Radiolabeled testosterone metabolism

Tritiated testosterone ([1,2,6,7,16,17-^{3}H-T]; New England Nuclear, Dupont: NET-553) was used to determine how a male stonechat metabolizes and excretes this steroid. The male was injected i.p. with 50 μl [^{3}H]T (400,000 dpm) in isotonic saline using a Hamilton syringe. [^{3}H]T solution (3×50 μl) was pipetted with the same Hamilton syringe into three scintillation vials and counted with a Beckman LS6000 counter with 4 ml scintillation fluid to determine total radioactivity. After isotope administration syringes were rinsed with ethanol and the residual radioactivity counted and subtracted from the pre-injection total. Excrements were collected from non-absorbant plastic sheets that covered the cage floors and walls. Samples were collected after 0, 2.8, 6.3, 9.1, 23.8, 47.8, and 70.8 h after [^{3}H]T administration. After extraction (see below) radioactivity of each sample was determined by counting a fraction of 100 μl in duplicates with 4 ml scintillation liquid to an accuracy of 2–3% in a Beckman LS6000 β-counter. The sample with the highest amount of radioactivity was then further analyzed with HPLC (see below).

2.3. Experiment 1: GnRH-challenge of androgen production

Injections of GnRH have been used as a standard technique to increase plasma androgen levels (e.g., Schoech et al., 1996; Wingfield et al., 1991). To test

whether an increase in androgen production and release induced by GnRH can be traced quantitatively in excrements of European stonechats, seven males were injected i.p. with 1 μg chicken GnRH (Sigma). This amount of GnRH is about 20 times higher than the concentration that elicits a maximum response of gonaldal testosterone production in songbirds (Wingfield and Farner, 1993). Six of these experimental males also served as controls and were injected with 50 μl saline 1 week before the treatment with GnRH. All injections were done at 09:00 h in the morning. Excrements were collected on stainless steel plates underneath the perches of each cage during 3 h preceding the GnRH administration, and 1.3, 2.7, 4.0, 5.3, 6.6, and 9.2 h after GnRH administration.

2.4. Experiment 2: seasonal changes in androgen metabolite excretion

To further validate the non-invasive measurement of androgen metabolites, excrements from nine males kept under natural light conditions were collected in intervals of 3–4 weeks between January and April to investigate whether the typical seasonal increase in plasma T levels can also be traced when measuring androgen metabolites in excrements. Excrements were collected as described for Experiment 1 during a 2-h period between 08:00 and 11:00 h on January 3rd and 24th, on February 16th, on March 3rd, and on April 5th. Temperate bird species, including the stonechat, typically have regressed gonads and very low levels during the non-breeding season (non-breeding baseline). Androgen levels typically increase as the gonads grow and reach breeding baseline levels shortly before the birds establish territories and start breeding (e.g., Wingfield and Farner, 1980; Wingfield et al., 1990). We expected an increase of androgen metabolite levels between January (non-breeding condition) and April (breeding).

2.5. Processing of excrements

Excrements were removed from the stainless steel plates with a spatula, collected in small plastic vials, and kept frozen (−70 °C) until further processing. Differences in water content of the samples did not affect the ability to collect the entire sample. Samples were freeze–dried with a Christ Alpha I-5 lyophilizator, pulverized, weighed to the nearest milligrams with a Sartorius Research R 160 P balance and extracted with 750 μl methanol and 250 μl double-distilled water (ddH_2O). After vortexing for 30 min, the samples were centrifuged (2500*g*, 10 min) and the supernatant transferred to a new tube. A fraction of 200 μl was transferred to a new vial and dried under a stream of nitrogen. Then 200 μl sodium acetate buffer (0.2 M, pH 4.8) were added, containing β-glucuronidase/arylsulfatase (1:100), to cleave conjugated steroids. Then the vials were sealed and incubated for 16 h at 39 °C. After incubation the samples were stored at −40°C until further analysis.

2.6. Radioimmunoassay of androgen metabolites

Androgen metabolites were measured with a testosterone radioimmunoassay (T-RIA) using a commercial antiserum against testosterone (T3-125, Endocrine Sciences, Tarzana, USA). Cross reactivities of this antiserum are testosterone (100%), 5α-dihydrotestosterone (44%), δ-1-testosterone (41%), δ-1-dihydrotestosterone (18%), 5α-androstan-3β, 17β-diol (3%), 4-androsten-3β, 17β-diol (2.5%), δ-4-androstenedione (2%), 5β-androstan-3β, 17β-diol (1.5%), estradiol (0.5%), and less than 0.2% with 23 other steroids tested. Standard testosterone was purchased from Sigma (St. Louis, USA), and labeled testosterone from New England Nuclear, Dupont (NET-553). Standard curves were set up by serial dilution of stock standard solutions with a concentration range of 0.391–200 pg standard hormone in duplicates. The testosterone antiserum (100 μl) was added to the standard curve, the controls, and to duplicates of the respective sample fractions (2×100 μl). After 30 min, 13,000 dpm of the [^{3}H]T was added to each sample and incubated for 20 h at 4 °C. Bound and free fractions were separated at 4 °C by adding 0.5 ml dextran-coated charcoal. After 14 min incubation with charcoal, samples were spun (3600*g*, 10 min, 4 °C) and supernatants decanted into scintillation vials. After adding 4 ml scintillation liquid, vials were counted to an accuracy of 2–3% in a Beckman LS6000 β-counter.

Serial dilutions (1:5 up to 1:80) of extracts yielded displacement curves parallel to standard testosterone (standard: $y = 4.72x + 1.46$; samples: $y = 5.14x + 49.11$; the slopes of the two curves do not significantly differ from each other, $t = 0.284$, $p = 0.79$). The assay detection limit was 0.03 ng/ml and intra- and interassay coefficients of variation for pooled extracts of stonechat excrements were 1.0% ($N = 4$) and 10.9%, respectively. In each assay buffer blanks were analyzed to test for contamination. Samples were assayed in duplicates and concentrations were expressed as nanogram per gram dried excreted droppings.

2.7. High performance liquid chromatography (HPLC)

The number and relative proportions of immunoreactive androgen metabolites in stonechat droppings were determined via HPLC. A methanol extracted sample (1 ml) of the [^{3}H]T injected male, containing the highest amount of radioactivity was diluted with double-distilled water (10 ml) and pre-purified using a Sep-Pac C-18 matrix column (Waters, Milford, MA, USA). Columns were then washed with ddH_2O (2×5 ml), and eluted with methanol (4 ml). The methanol was

evaporated to dryness under a stream of nitrogen, the sample resuspended in 50% methanol, and reversed-phase chromatography performed (Novapak C-18 column, 3.9 × 150 cm; eluent: 10% methanol for 5 min, then linear increase to 100% methanol in 35 min; flow rate 1 ml/min, 3 fractions/min). Ninety-five 330 μl fractions were collected, dried under a stream of nitrogen, reconstituted in sodium acetate buffer (0.2 M, pH 4.8) with β-glucuronidase/arylsulfatase (1:100), to cleave conjugated steroids. Then vials were sealed and incubated for 16 h at 39 °C. To determine which fractions contained metabolites of [^{3}H]T, radioactivity of 100 μl subsamples of each fraction were counted in a Beckman LS6000 β-counter in duplicates after incubation. To test whether the T-RIA was able to recognize these metabolites, 20 μl subsamples of each fraction were quantified with the T-RIA in duplicates, as described above. The 20 μl-subsamples used for the T-RIA contained a maximum radioactivity of 500 dpm, which was less than 4% of the total radioactivity (13,000 dpm) added during the radioimmunoassay procedure.

2.8. Statistics

As fecal androgen data did not follow a normal distribution, we used non-parametric statistical tests. The Friedman tests with post hoc multiple comparisons were calculated with a custom made computer program (by J. Lamprecht and B. Knauer, MPI Seewiesen, Germany) following Conover (1980). Results were considered significant at the 0.05% level and all *p*-values refer to two-tailed tests.

3. Results

3.1. Radiolabeled testosterone metabolism

Recovery of [^{3}H]testosterone radioactivity injected to a male stonechat was 96.8%. Metabolites of i.p. injected [^{3}H]testosterone ([^{3}H]T) were excreted mainly within 3–6 h after the injection and radioactivity returned to background levels after 47.8 h (Fig. 1). There were also background levels of radioactivity in the 70.8 h sample.

HPLC separation of the excrement pool containing the highest amount of radioactivity (=3 h sample) showed, that [^{3}H]T was completely metabolized in excrements, as testosterone, which should elute in fraction 78, did not contribute a radioactive peak (Fig. 2). Instead, there were two major metabolites of [^{3}H]T, eluting in fractions 42 and 52, as well as several minor metabolites adjacent to these fractions (Fig. 2). These metabolites showed a chromatographic mobility of polar substances.

Subsequent analysis of the HPLC fractions with the testosterone radioimmunoassay (T-RIA) showed a high

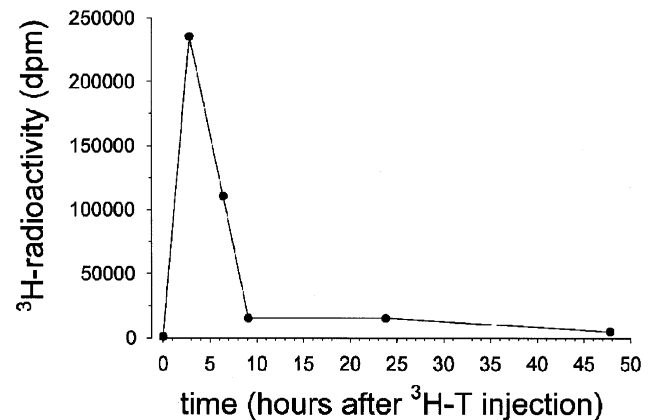

Fig. 1. Time course of excretion of i.p. injected [^{3}H]testosterone in a male European stonechat.

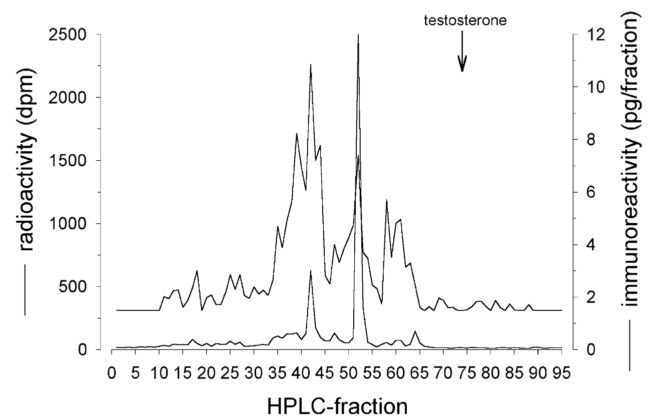

Fig. 2. HPLC profile of metabolites of i.p. injected [^{3}H]testosterone (grey line) and immunoreactive substances measured with the T-RIA (black line). The two major (grey line, fraction 42 and 52) and the several adjacent minor peaks represent excreted metabolites of [^{3}H]testosterone. The T-RIA crossreacted with the two major and several minor fractions containing [^{3}H]testosterone metabolites (black line). The arrow indicates in which fraction standard testosterone elutes.

cross reactivity of the T-RIA with the two major and several minor metabolites of [^{3}H]testosterone (Fig. 2), indicating that the T-RIA picked up substances that qualitatively represent metabolized androgens. The T-RIA showed one further major peak that did not correspond with a major [^{3}H]testosterone metabolite.

3.2. Experiment 1: GnRH-challenge of androgen production

Excreted androgen metabolite levels measured with the T-RIA significantly increased after challenging seven males with GnRH (Friedman test, $\chi^2 = 15.31$, $p = 0.02$, Fig. 3). Post hoc comparisons revealed that samples taken 1.3 and 2.7 h after the GnRH challenge had significantly higher excreted androgen levels than samples taken before the GnRH challenge or samples taken later

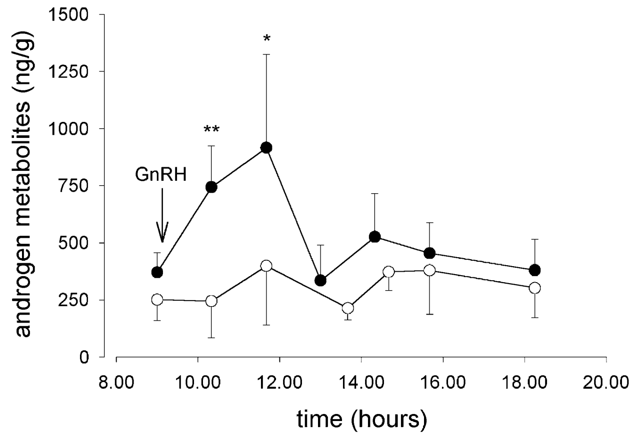

Fig. 3. Profile of mean (±SEM) excreted androgen metabolite concentrations in seven male European stonechats injected at 09:00 h with GnRH (closed circles) and six males injected with saline (open circles, ** $= p < 0.02$; *** $= p < 0.01$), measured with the testosterone RIA.

than 2.7 h after the challenge. Androgen levels of control birds injected with saline did not significantly change (Friedman test, $\chi^2 = 10.88$, $p = 0.1$, Fig. 3). These results indicate that the T-RIA provides a quantitative measure of androgen metabolites.

3.3. Experiment 2: seasonal changes in androgen metabolite excretion

As expected, there was a significant seasonal increase in excreted androgen metabolite concentrations (Friedman test, $\chi^2 = 26.93$, $p < 0.0001$, Fig. 4). Post hoc comparisons revealed that the samples collected in February, March, and April had significantly higher androgen metabolite concentrations than the samples taken in early and late January.

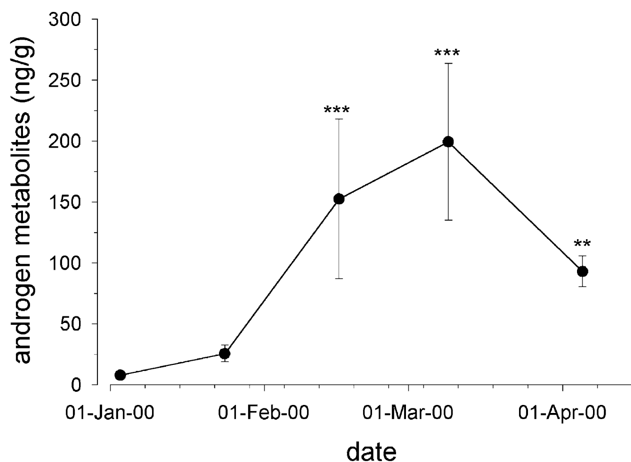

Fig. 4. Seasonal profile of mean (±SEM) excreted androgen metabolite concentrations of nine male European stonechats, measured with the testosterone RIA (** $= p < 0.01$; *** $= p < 0.001$).

4. Discussion

The aim of this study was to investigate whether excreted androgen metabolites of European stonechats reflect the gonadal production of these androgens.

HPLC and subsequent RIA of HPLC fractions confirmed a qualitative measure of testosterone metabolites in the excrements of a male stonechat. It is not possible to draw a general conclusion from a sample size of just one bird. But the bird used in this treatment represented an average, two-year-old male stonechat of our captive population. It is thus likely that the amount and type of testosterone metabolites excreted by this male are representative.

The HPLC analysis showed, that [^{3}H]testosterone injected to this male was metabolized and excreted in the form of two major and several minor metabolites. Further analysis of the HPLC fractions with the T-RIA confirmed, that the antibody used in this study detected two major and several minor metabolites of [^{3}H]testosterone in the excrements of the male. The chromatographic mobility of these metabolites suggested that they are polar substances and thus most likely represent conjugated steroids. Testosterone itself did not contribute to the [^{3}H]testosterone metabolite excretion, nor to the T-RIA peak, suggesting that it had been completely metabolized. One major peak of the immunoassay did not correlate well with metabolites of [^{3}H]testosterone. Possibly the T-RIA picked up metabolites of adrenal androgens (e.g., dehydroepiandrosterone), gonadal testosterone precursors (e.g., androstenedione) or testosterone metabolites (e.g., 5α-dihydrotestosterone). Hence, it is appropriate to refer to the measurements of the T-RIA as 'androgen metabolites' rather than 'testosterone metabolites'.

Two lines of evidence suggest that the T-RIA provided a quantitative measure of excreted androgen metabolite levels: firstly, administration of exogenous GnRH led to a significant increase in these androgen metabolite levels in seven male stonechats, whereas there was no change in androgen metabolite levels in six individuals injected with saline. Secondly, androgen metabolite levels showed the seasonal increase that is typically found in males of all temperate bird species, including stonechats (Gwinner, unpublished data; Wingfield and Farner, 1980; Wingfield et al., 1990).

The time-lag between a plasma hormone peak and its appearance in bird excrements depends on kidney and gut passage times and the rate of liver metabolism. Excreted androgen metabolite levels in male European stonechats peaked within 1.3 and 2.7 h after administration of GnRH. Also, the major proportion of radioactivity in the [^{3}H]T treated bird was excreted within the first 3 h after i.p. injection, suggesting that excreted androgen metabolites integrate plasma hormone levels of less than 1–3 h in stonechats. Peak excretion of androgen metabolites after a challenge with GnRH was

3–6 h in domestic geese (Hirschenhauser et al., 2000a). In rufous hummingbirds (*Selasphorus rufus*) steroid metabolite concentrations in cloacal fluid represent pooled plasma levels of these hormones of 30 min (Hiebert et al., 2000a). In domestic and greylag geese peak excretion of corticosteroid metabolites was within 2 h after i.v. administration of [^{3}H]corticosterone (Krawany, 1996). In the northern spotted owl (*Strix occidentalis caurina*; Wasser et al., 1997) excreted corticosteroid levels peaked 12 h after an ACTH challenge. Carnivorous owls may feed only once or twice a day and thus defecation rate may also be low. In contrast, herbivorous geese or small birds with a high metabolic rate, such as passerines or hummingbirds, feed more continuously throughout the day, and thus may defecate at a higher rate than owls. As a consequence, clearance of steroid metabolites may be faster in species with a higher defecation rate.

In plasma, total osmolality remains relatively constant. Thus, hormone concentrations determined in plasma can be used directly and expressed in Mole or as nanogram per milliliter plasma. In urine or feces, however, water content is likely to vary with the hydration state of the animal. It is thus necessary to control for the water content of the samples. In the present study we freeze–dried (lyophilized) samples before extraction and used the dry weight of the samples as a reference for hormone levels to control for differences in water content. This method is commonly used to prepare fecal samples of birds and mammals for hormone analysis (e.g., Goymann et al., 1999; Monfort et al., 1998; Wasser et al., 1997; Whitten et al., 1998). In rufous hummingbirds, however, (Hiebert et al., 2000a) found that creatinine is a more suitable reference in cloacal fluid than dry weight. In contrast to hummingbirds it was not feasible to separate the urinary and fecal fraction of excrements in stonechats. As the fecal fraction represents a significant part of the total excrements in most birds creatinine may not be as suitable as a reference as it is in the rather special case of rufous hummingbirds. Also, we were not able to trace detectable levels of creatinine in stonechat droppings (Goymann, unpublished data).

Typically, plasma androgen levels are highly variable, both between and within subjects. Researchers have been puzzled by this variability and have developed theories such as the 'challenge hypothesis' (Wingfield et al., 1990) that led to a better understanding of these differences. Also our measure of androgen metabolite levels during the GnRH challenge and the seasonal profile showed a high variability between individuals (see error bars in Figs. 3 and 4). Most of these differences are likely to represent 'true' differences of androgen metabolite levels between and within individuals. However, apart from actual differences in androgen metabolite levels between or within individuals other factors may contribute to some of the variability of androgens measured in excrements. For example, differences in metabolic rate and hence gut passage time, or changes in the relative proportion to which the renal and intestinal pathways contribute to an actual sample, may introduce additional variability. Further studies are necessary to address these issues. To cope with this potential variability of androgen metabolite levels when conducting a field study, it may be advisable to collect and analyze a larger number of samples.

In conclusion, the T-RIA presented in this paper reflects androgen excretion patterns of male European stonechats. To our knowledge this study represents the first validation of a non-invasive androgen assay in a passerine bird. Other studies have shown that an immunoassay that detects steroid metabolites in one species also worked in related taxa (Goymann et al., 1999; Monfort et al., 1998; Wasser et al., 2000). We thus hope that the assay described in this paper also may be useful for researchers working with passerine birds, other than stonechats.

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References

Balthazart, J., 1983. Hormonal correlates of behavior. In: Farner, D.S., King, J.R., Parker, K.C. (Eds.), Avian Biology, vol. VII. Academic Press, San Diego, pp. 221–365.

Bercovitz, A.B., Collins, J., Price, P., Tuttle, D., 1982. Noninvasive assessment of seasonal hormone profile in captive bald eagles (*Haliaeetus leucocephalus*). Zoo Biol. 1, 111–117.

Canoine, V., Gwinner, E., 2002. Seasonal differences in the hormonal control of territorial aggression in free-living European stonechats. Horm. Behav. 41, 1–8.

Cavigelli, S.A., 1999. Behavioural patterns associated with fecal cortisol levels in free-ranging female ring-tailed lemurs, *Lemur catta*. Anim. Behav. 57, 935–944.

Cavigelli, S.A., Pereira, M.E., 2000. Mating season aggression and fecal testosterone levels in male ring-tailed lemurs (*Lemur catta*). Horm. Behav. 37, 246–255.

Conover, W.J., 1980. Practical Non-Parametric Statistics. Wiley, New York.

Cramp, S., 1988. Handbook of the Birds of Europe, the Middle East and North Africa. Oxford University Press, Oxford.

Creel, S., Creel, N.M., Mills, M.G.L., Monfort, S.L., 1997. Rank and reproduction in cooperatively breeding African wild dogs—behavioral and endocrine correlates. Behav. Ecol. 8, 298–306.

Goymann, W., East, M.L., Wachter, B., Höner, O.P., Möstl, E., Van't Hof, T.J., Hofer, H., 2001. Social, state-dependent and environmental modulation of fecal corticosteroid levels in free-ranging female spotted hyaenas. Proc. R. Soc. London B 268, 2453–2459.

Goymann, W., Möstl, E., Van't Hof, T., East, M.L., Hofer, H., 1999. Noninvasive fecal monitoring of glucocorticoids in spotted hyenas, *Crocuta crocuta*. Gen. Comp. Endocrinol. 114, 340–348.

Graham, L.H., Brown, J.L., 1996. Cortisol metabolism in the domestic cat and implications for non-invasive monitoring of adrenocortical function in endangered felids. Zoo Biol. 15, 71–82.

Graham, L.H., Brown, J.L., 1997. Noninvasive assessment of gonadal and adrenocortical function in felid species via fecal steroid analysis. Z. Saeugetierk. 62 (Suppl. 2), 78–82.

Gwinner, E., Koenig, S., Haley, C., 1995. Genetic and environmental factors influencing clutch size in equatorial and temperature zone stonechats (*Saxicola torquata axillaris* and *S. t. rubicola*): an experimental study. Auk 112, 748–755.

Gwinner, E., Rödl, T., Schwabl, H., 1994. Pair territoriality of wintering stonechats: behavior, function and hormones. Behav. Ecol. Sociobiol. 34, 321–327.

Hiebert, S.M., Ramenofsky, M., Salvante, K., Wingfield, J.C., Gass, C.L., 2000a. Noninvasive methods for measuring and manipulating corticosterone in hummingbirds. Gen. Comp. Endocrinol. 120, 235–247.

Hiebert, S.M., Salvante, K.G., Ramenofsky, M., Wingfield, J.C., 2000b. Corticosterone and nocturnal torpor in the rufous hummingbird (*Selasphorus rufus*). Gen. Comp. Endocrinol. 120, 220–234.

Hirschenhauser, K., Möstl, E., Kotrschal, K., 1999a. Seasonal patterns of sex steroids determined from feces in different social categories of greylag geese (*Anser anser*). Gen. Comp. Endocrinol. 114, 67–79.

Hirschenhauser, K., Möstl, E., Kotrschal, K., 1999b. Within-pair testosterone covariation and reproductive output in greylag geese (*Anser anser*). Ibis 141, 577–586.

Hirschenhauser, K., Möstl, E., Peczely, P., Wallner, B., Dittami, J., Kotrschal, K., 2000a. Seasonal relationships between plasma and fecal testosterone in response to GnRH in domestic ganders. Gen. Comp. Endocrinol. 118, 262–272.

Hirschenhauser, K., Möstl, E., Wallner, B., Dittami, J., Kotrschal, K., 2000b. Endocrine and behavioural responses of male greylag geese (*Anser anser*) to pairbond challenges during the reproductive season. Ethology 106, 63–77.

Hultén, F., Zhang, B.R., Dalin, A.-M., 1995. Applying a progesterone assay to fecal samples collected from sows during the oestrous cycle. Reprod. Dom. Anim. 30, 101–105.

Jurke, M.H., Czekala, N.M., Lindburg, D.G., Millard, S.E., 1997. Fecal corticoid metabolite measurement in the cheetah (*Acinonyx jubatus*). Zoo Biol. 16, 133–147.

Ketterson, E.D., Nolan, V., 1992. Hormones and life histories: an integrative approach. Am. Nat. 140 (Suppl.), S33–S62.

Kirkpatrick, J.F., 1990. Pregnancy determination in uncaptured feral horses based on steroid metabolites in urine soaked snow and free steroids in feces. Can. J. Zool. 68, 2576–2579.

Kirkpatrick, J.F., Bancroft, K., Kincy, V., 1992. Pregnancy and ovulation detection in bison (*Bison bison*) assessed by means of urinary and fecal steroids. J. Wildl. Dis. 28, 590–597.

Kotrschal, K., Hirschenhauser, K., Möstl, E., 1998. The relationship between social stress and dominance is seasonal in greylag geese. Anim. Behav. 55, 171–176.

Krawany, M., 1996. "Die Entwicklung einer nicht-invasiven Methode zum Nachweis von Steroidhormonmetaboliten im Kot von Gänsen." Doctoral thesis, University of Veterinary Medicine, Vienna, Austria.

Lee, J.V., Whaling, C.S., Lasley, B.L., Marler, P., 1995. Validation of an enzyme immunoassay for measurement of excreted estrogen and testosterone metabolites in the white-crowned sparrow (*Zonotrichia leucophrys oriantha*). Zoo Biol. 14, 97–106.

Lucas, Z., Raeside, J.I., Betteridge, K.L., 1991. Non-invasive assessment of the incidences of pregnancy and pregnancy loss in the feral horses of Sable Island. J. Reprod. Fertil. Suppl. 44, 479–488.

Miller, M.W., Hobbs, N.T., Sousa, M.C., 1991. Detecting stress responses in Rocky Mountain bighorn sheep (*Ovis canadensis canadensis*): reliability of cortisol concentrations in urine and feces. Can. J. Zool. 69, 15–24.

Monfort, S.L., Mashburn, K.L., Brewer, B.A., Creel, S.R., 1998. Evaluating adrenal activity in african wild dogs (*Lycaon pictus*) by fecal corticosteroid analysis. J. Zoo Wildl. Med. 29, 129–133.

Möstl, E., Choi, H.S., Wurm, W., Ismail, N., Bamberg, E., 1984. Pregnancy diagnosis in cows and heifers by determination of oestradiol-17a in feces. Br. Vet. J. 140, 287–291.

Möstl, E., Meyer, H.H.D., Bamberg E., von Hegel, G., 1987. Oestrogen determination in feces of mares by enzyme immunoassay on microtitre plates. In: Proceedings of the Symposium on the Analysis of Steroids, Sopron, Hungary, pp. 219–224.

Palme, R., Möstl, E., 1993. Biotin-streptavidin enzyme immunoassay for the determination of oestrogens and androgens in boar feces. Proceedings of the 5th Symposium on the Analysis of Steroids, Szombathely, Hungary, pp. 111–117.

Palme, R., Möstl, E., 1997. Measurement of cortisol metabolites in feces of sheep as a parameter of cortisol concentration in blood. Z. Saeugetierk. 62 (Suppl. 2), 192–197.

Rödl, T., 1995. The wintering of territorial stonechat pairs, *Saxicola torquata*, in Israel. J. Ornithol. 136, 423–425.

Rödl, T., 1999. Environmental factors determine numbers of overwintering European stonechats, *Saxicola rubicola*: a long-term study. Ardea 87, 247–259.

Rödl, T., Flinks, H., 1996. Nutrition of Stonechats (*Saxicola torquata*) and Mourning Wheatears (*Oenanthe lugens*) wintering sympatrically in Israel. Ecol. Birds 18, 107–126.

Scheuerlein, A., 2000. Control of reproduction in a tropical bird, the stonechat (*Saxicola torquata axillaris*). PhD thesis, University of Munich, Munich, Germany.

Scheuerlein, A., Nitsche, G., 1994. Brutbestand und Verbreitung des Schwarzkehlchens *Saxicola torquata* im bayerischen Voralpenland. Ornithologischer Anzeiger 33, 19–26.

Scheuerlein, A., Van't Hof, T., Gwinner, E., 2001. Predators as stressors? Physiological and reproductive consequences of predation risk in tropical stonechats (*Saxicola torquata axillaris*). Proc. R. Soc. London B 268, 1575–1582.

Schoech, S.J., Mumme, R.L., Wingfield, J.C., 1996. Delayed breeding in the cooperatively breeding Florida Scrub-jay (*Aphelocoma coerulescens*) — Inhibition or the absence of stimulation. Behav. Ecol. Sociobiol. 39, 77–90.

Schwabl, H., 1996. Environment modifies the testosterone levels of a female bird and its eggs. J. Exp. Zool. 276, 157–163.

Schwarzenberger, F., Möstl, E., Bamberg, E., von Hegel, G., 1992. Monitoring of *Corpus luteum* function by measuring pro-gestagens in feces of non-pregnant mares (*Equus caballus*) and Przewalski mares (*Equus przewalskii*). Anim. Reprod. Sci. 29, 263–273.

Silver, R., Ball, G.F., 1989. Brain, hormone and behavior interactions in avian reproduction: status and prospectus. Condor 91, 966–978.

Sousa, M.B.C., Ziegler, T.E., 1998. Diurnal-variation on the excretion patterns of fecal steroids in common marmoset (*Callithrix jacchus*) females. Am. J. Primatol. 46, 105–117.

Wallner, B., Möstl, E., Dittami, J., Prossinger, H., 1999. Fecal glucocorticoids document stress in female Barbary macaques (*Macaca sylvanus*). Gen. Comp. Endocrinol. 113, 80–86.

Wasser Samuel, K., Hunt Kathleen, E., Brown Janina, L., Cooper, K., Crockett Carolyn, M., Bechert, U., Millspaugh Joshua, J., Larson, S., Monfort Steven, L., 2000. A generalized fecal glucocorticoid assay for use in a diverse array of nondomestic mammalian and avian species. Gen. Comp. Endocrinol. 120, 260–275.

Wasser, S.K., Bevis, K., King, G., Hanson, E., 1997. Noninvasive physiological measures of disturbance in the northern spotted owl. Cons. Biol. 11, 1019–1022.

White, P.J., Garrott, R.A., Kirkpatrick, J.F., Berkeley, E.V., 1995. Diagnosing pregnancy in free-ranging elk using fecal steroid metabolites. J. Wildl. Dis. 31, 514–522.

Whitten, P.L., Brockman, D.K., Stavisky, R.C., 1998. Recent advances in noninvasive techniques to monitor hormone-behavior interactions. Yearb. Physiol. Anthropol. 41, 1–23.

Wingfield, J.C., Farner, D.S., 1980. Control of seasonal reproduction in temperate-zone birds. Prog. Reprod. Biol. 5, 66–101.

Wingfield, J.C., Farner, D.S., 1993. Endocrinology of reproduction in wild species. In: Farner, D.S., King, J.R., Parkes, K.C. (Eds.), Avian Biology Vol. IX. Academic Press, London and San Diego, pp. 163–327.

Wingfield, J.C., Ramenofsky, M., 1985. Testosterone and aggressive behavior during the reproductive cycles of male birds. In: Gilles, R., Balthazart, J. (Eds.), Neurobiology. Springer-Verlag, Berlin, pp. 92–104.

Wingfield, J.C., Hegner, R.E., Lewis, D.M., 1991. Circulating levels of luteinizing hormone and steroid hormones in relation to social status in the cooperatively breeding white-browed sparrow weaver, *Plocepasser mahali*. J. Zool. Lond. 225, 43–58.

Wingfield, J.C., Jacobs, J., Hillgarth, N., 1997a. Ecological constraints and the evolution of hormone/behavior interrelationships. Ann. NY Acad. Sci. 807, 22–41.

Wingfield, J.C., Vleck, C.M., Moore, M.C., 1992. Seasonal changes of the adrenocortical response to stress in birds of the Sonoran desert. J. Exp. Zool. 264, 419–428.

Wingfield, J.C., Whaling, C.S., Marler, P., 1994. Communication in vertebrate aggression and reproduction: the role of hormones. In: Knobil, E., Neill, J.D. (Eds.), The Physiology of Reproduction, second ed. Raven Press, New York, pp. 303–342.

Wingfield, J.C., Hegner, R.E., Dufty, A.M., Ball, G.F., 1990. The "challenge hypothesis": theoretical implications for patterns of testosterone secretion, mating systems, breeding strategies. Am. Nat. 136, 829–846.

Wingfield, J.C., Hunt, K., Breuner, C., Dunlap, K., Fowler, G.S., Freed, L., Lepson, J., 1997b. Environmental stress, field endocrinology and conservation biology. In: Clemmons, J.R., Buchholz, R. (Eds.), Behavioral Approaches to Conservation in the Wild. Cambridge University Press, Boston, pp. 95–131.

Ziegler, T.E., Sholl, S.A., Scheffler, G., Haggerty, M.A., Lasley, B.L., 1988. Excretion of estrone, estradiol and progesterone in the urine and feces of the female cotton-top tamarin (*Sanguinus oedipus oedipus*). Am. J. Primatol. 17, 185–195.