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ORIGINAL ARTICLE

Excreted corticosterone metabolites differ between two galliform species, Japanese Quail and Chicken, between sexes and between urine and faecal parts of droppings

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Abstract Japanese Quail (*Coturnix japonica*) are a common avian laboratory model, and measuring corticosterone metabolites (CM) non-invasively from Quail droppings would be of broad interest. Using the enzyme immunoassay suitable for measuring CM in droppings of Chicken (*Gallus domesticus*) and other galliform species, we tested the CM measured in Quail droppings after stimulation with adrenocorticotropic hormone. An ACTH challenge indicated that the assay did not detect major amounts of CM in Japanese Quail excreta. Therefore, we aimed at testing whether steroid metabolism and clearance differed between closely related species. We compared the CM excretion patterns in male and female Japanese Quail and Chicken. After intravenous injection with [^{3}H]-labelled corticosterone, we collected all droppings for 24 h, including caecum excreta. Males and females of both species excreted radioactive CM with two peaks. The first radioactivity-peak presumably was from uric acid (Quail 45/55 min; Chicken 120/75 min post-injection, males/females respectively), whereas the second peak probably represented faecal excretion (180/210 min; 240/140 min, respectively). Several highly polar radioactive CM, but no corticosterone, was separated by chromatography in droppings of both species. Number and identity of CM considerably differed between males and females, as well as between urine and faecal excretion. The nature of excreted CM in Quail droppings remains unresolved. However, we show that knowing the effective time for dropping collection is essential for interpreting stimulus-specific CM responses, and mixing samples from rapid urine and slower faecal excretion should be avoided.

Keywords Non-invasive methods · Faecal hormones · Steroid · Glucocorticoids · *Coturnix japonica* · *Gallus domesticus*

Zusammenfassung

Unterschiedliche Ausscheidungsmetaboliten von Kortikosteron bei zwei Galliformen-Arten, Japanwachteln und Hühner

Japanwachteln (*Coturnix japonica*) sind beliebte Laborvögel und die Möglichkeit, Kortikosteron-Metaboliten (CM) nicht-invasiv aus Wachtellosung zu messen, ist von großem Interesse. Wir haben die Messung von CM in der Losung von Japanwachteln unter Anwendung jenes Enzymimmunoassays, der für Haushühner (*Gallus domesticus*) und andere Galliformen-Arten geeignet ist, getestet. Ein Test mit dem Auslöserhormon Adrenocorticotropin deutete darauf hin, dass der Assay im Kot von Japanwachteln keine

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der hauptsächlich ausgeschiedenen Metaboliten misst. Um zu untersuchen, ob die Metabolisierung und Ausscheidung von Kortikosteron bei nahe verwandten Arten ähnlich ist, wurden die CM-Ausscheidungsmuster von männlichen und weiblichen Japanwachteln und Hühnern verglichen. Nach intravenöser Injektion von [^{3}H]-markiertem Kortikosteron wurde 24 h lang jede abgegebene Losung inklusive Blinddarm-Ausscheidungen gesammelt. Männchen und Weibchen beider Arten schieden radioaktive CM in Form von zwei Ausscheidungs-Peaks aus. Für den ersten Peak war vermutlich der Ausscheidungsweg über Harnsäure verantwortlich (Japanwachteln 45/55 min; Hühner 120/75 min *post injectionem*, Männchen/Weibchen respektive), während der zweite Peak die Ausscheidung von CM über Kot darstellte (180/210 min; 240/140 min). Chromatographisch konnten wir mehrere hochpolare radioaktive CM aber kein Kortikosteron auftrennen. Anzahl und Identität der ausgeschiedenen CM unterschied sich zwischen Männchen und Weibchen, sowie zwischen Harnsäure- und Kot-Ausscheidung. Die Identität der von Japanwachteln hauptsächlich ausgeschiedenen CM bleibt offen. Wir zeigen jedoch, dass das Vermischen von Proben, die CM aus dem schnelleren Harnsäure-Exkretionsweg und/oder CM aus langsamerer Kotausscheidung beinhalten, vermieden werden sollte. Um reizspezifische CM-Veränderungen sinnvoll interpretieren zu können, ist es deshalb wesentlich, die effektive Zeitspanne für das Sammeln von Losungsmaterial zu berücksichtigen.

Introduction

Measuring glucocorticoid levels is of broad interest for investigating stress and stressors, particularly in the social domain. However, obtaining meaningful data especially by using blood samples has methodological and ethical limits. The handling of animals for blood sampling itself causes stress and in a social context interferes with ongoing behavioural interactions. In Bar-headed Geese (*Anser indicus*), repeated sampling of individuals resulted in already elevated corticosterone levels in anticipation of being caught and handled (Dittami 1981). Furthermore, number, volume and time intervals between blood samples are limited by body size. A non-invasive alternative to blood sampling is measuring excreted corticosterone metabolites (CM) from individual droppings (Möstl et al. 2005; Palme et al. 2005). Collection of droppings does not require handling, and the sampling procedure may be organised with minimum disturbance of the animals' behaviour in most species. Glucocorticoids are secreted by the adrenal glands and are quickly eliminated from the blood. They are mainly metabolised by the liver and excreted as conjugates via urine and bile. In the gut, most of the CM are deconjugated by bacteria and partially reabsorbed (enterohepatic circulation). Steroids which are not reabsorbed are eliminated via faeces (Taylor 1971). Bacteria in the microflora of the intestines and the caeca may be very active in metabolising steroid hormones. Conjugated steroid metabolites have a higher polarity than deconjugated molecules, are more hydrophilic and can be measured in urine and faeces (Florkin and Stotz 1963; Lexen et al. 2008). In birds, renal and faecal excreta are jointly excreted through the cloaca and, therefore, CM in droppings of birds are of renal and faecal origin and cannot be separated entirely by hand (Klasing 2005). Levels of excreted steroid metabolites are integrative measures over time and reflect changes of circulating corticoids during an earlier period. The delay times depend on gut-passage time and/or urine production (uric acid in birds) and vary considerably between species. In the faeces of mammals, steroid metabolites were present mainly in an unconjugated form, whereas in urine as conjugates (Palme et al. 1996). A recent study in Snow Geese (*Chen caerulescens atlantica*) showed extremely rapid excretion of CM after capture in the wild (Legagneux et al. 2011). Such rapid CM changes in response to a stressful event assumingly represent excretion via urine. For improving the precision of sampling and choice of immunoassay, we still need a better understanding of a species' time patterns underlying excretion. Optimal integrative measures over time should be based on either specific urinary or faecal excreta and hence, knowing when to collect samples is essential for using hormone metabolite measures in behavioural endocrinology.

Non-invasive measures of glucocorticoid metabolites have advanced over the last decade and have been successfully used in an increasing number of species. CM can be measured in the droppings of Chicken, *Gallus domesticus*, and two Grouse species (Tetraonidae) with the cortisone EIA (enzyme immune assay) measuring glucocorticoid metabolites with a 3,11-dione structure (Chicken: Rettenbacher et al. 2004; Capercaillie, *Tetrao urogallus*: Thiel et al. 2005; Black Grouse, *Tetrao tetrix*: Baltic et al. 2005; Arlettaz et al. 2007). There is growing awareness that metabolism and excretion of CM may differ even between closely related species (Palme et al. 2005) and between sexes (Touma et al. 2003; Goymann 2005). In the present study, we tested the suitability of the cortisone EIA for measuring CM in Japanese Quail, *Coturnix japonica*, droppings. The initial physiological challenge test with adrenocorticotropic hormone (ACTH) for validating the cortisone EIA in Japanese Quail droppings produced negative results (see Electronic Supplementary Material). Therefore, we employed the CM identified in Chicken droppings by Rettenbacher et al. (2004) as reference for

comparing polarity and elution patterns of excreted CM in Japanese Quail droppings. We used radioactively labelled corticosterone to track (1) the time course of CM excretion, and (2) the number and (chemical) identity of labelled CM in urine and faecal droppings from males and females of two Galliform species, Japanese Quail and domestic Chicken.

Materials and methods

Study animals

All birds were kept solitarily in cages with about 2 m^2 of space for Chicken and 1 m^2 for Japanese Quail. We tested 14 Chickens and 14 Japanese Quails, 7 males and 7 females of each species. Female Chickens (bantams, mean body mass ± SE: 1.3 ± 0.5 kg) came from our own breeding program, male Chickens (Dorkings, 2.4 ± 0.9 kg) and Japanese Quails (males: 285.0 ± 107.7 g; females: 269.6 ± 101.9 g) came from local private breeders. The female Chickens and all Japanese Quails were acclimated to individual cages for 3 days and male Chickens for 2 weeks. All animals had a wooden floor with sawdust litter; sawdust was removed during periods of dropping collection. Food and water were supplied ad libitum at all times, both species having the same grain mix diet, but grain for Quails was finer milled. Artificial daylight was set from 0800 to 2200 hours throughout all phases of the experiment.

Administration of radio-labelled corticosterone in Chicken and Japanese Quail

After collecting a control dropping, we injected birds intravenously with [^{3}H]-corticosterone. Each bird received an injection of 1.7 MBq of [^{3}H]-corticosterone (NET-399; [1, 2, 6, 7- ^{3}H (N)]-corticosterone; specific activity: 2,830.5 GBq/mmol; New England Nuclear, Dreieich, Germany) dissolved in 0.5 ml of 0.9 % (w/v) NaCl solution into the Vena cutanea ulnaris (Rettenbacher et al. 2004). Seven birds of same sex and species were treated and observed per trial; the sequence was male Chicken, female Chicken, female Quail, and male Quail. In all trials, we injected the birds between 0900 and 1000 hours to avoid bias due to diurnal variation of glucocorticoid levels or metabolism. After treatment, we collected all droppings and noted the time when defecation was observed until lights-off (2200 hours, ≈12 h post-treatment) and one sample 24 h after the injection. We used small plastic bags for collecting the Chicken excreta and 2-ml commercial plastic tubes (Eppendorf) for Quail droppings. We also noted and sampled caecum faeces (Chicken: 6 male and 5 female; Quail: 1 male and 7 female) to test the CM composition of caecum excreta. Caecum excreta have a different consistency, smell and colour than non-caecum faeces. A total of 481 dropping samples were collected and stored at −20 °C.

Extraction of excreted CM and radioactivity

In the laboratory, droppings were defrosted rapidly at 40 °C, homogenised manually and 0.1 g of each dropping was extracted in 60 % methanol and vortexed for 30 min (Thermomixer, Eppendorf, Germany). After centrifugation (15 min, 2,500*g*), we mixed 100 µl of the supernatant with 12 ml scintillation fluid (Quicksafe A, No. 100800; Zinsser Analytic, Maidenhead, UK) to measure radioactivity in a liquid scintillation counter (Tri-Cab 2100 TR; Packard Instruments, Meridien, CT, USA) for 5 min while running a quench compensation program. The remaining 1.5 ml of 60 % methanol extracts were evaporated at 40 °C under nitrogen and re-suspended in 100 µl of 100 % methanol.

Furthermore, we tested two methods to deconjugate the excreted CM: enzymatic deconjugation (using *β*-glucuronidase/arylsulphatase) and chemical deconjugation (using sulphuric acid) prior to TLC analyses. Six samples, which contained high values of radioactivity, were selected for the deconjugation tests. For the enzymatic and chemical deconjugation, 0.1 g of the selected samples were extracted with methanol as described above. The extracts were acidified with 10 µl concentrated hydrochloric acid and diluted with 10 ml water. Non-polar compounds were separated from polar compounds using reversed-phased chromatography with Sep-Pak C18 cartridges. The cartridges were washed three times with 10 ml water and eluted with 4 ml of 100 % methanol. All eluates were tested for radioactivity, including the first aqueous phase to control for background radiation (Palme et al. 1997). The methanol eluate was evaporated at 40 °C under a stream of nitrogen and re-dissolved in 1.5 ml ethyl-acetate. The 1.5-ml solvents were divided into three 500-µl parts: one part was used for the enzymatic deconjugation, one for the chemical splitting and one part remained untreated as control. After the deconjugation treatment, all samples were evaporated one more time at 40 °C under nitrogen. For the enzymatic deconjugation, we added 500 µl ß-Glucuronidase/Arylsulphatase (Merck EC 3.2.1.31 and EC 3.1.6.1) diluted 1:100 in 0.2 mol/l sodium-acetate-buffer (pH 4.8). The incubation lasted overnight at 40 °C. For chemical deconjugation, we refilled the vial with 4 ml ethylacetate and 10 µl hydrochloric acid. After 1 h, 10 µl 5 % sodium bicarbonate were added to stop the chemical reaction. After enzymatic or chemical treatments, all samples were purified one more time through Sep-Pak C18

cartridges, evaporated and re-suspended in 100 µl of 100 % methanol.

Characterisation of CM in Chicken and Japanese Quail excreta

We used thin layer chromatography (TLC; Palme et al. 1997) for separating structurally different compounds of the excreted CM metabolites and ß-imaging to detect substances directly on the TLC plate. Based on excretion profiles of excreted radioactivity (Fig. 1), we chose samples with peak radioactivity from one representative individual for each sex and species—one first radioactivity excretion peak sample and one second radioactivity excretion peak sample—to compare CM in urine and in faeces. We also selected samples deposited just before and after a peak (24 samples in total). Then, 50-µl spots of extracts of these samples were applied to TLC plates (TLC Silica gel 60 F_{254}) using a Linomat (CAMAC Muttenz, Switzerland) along a standardised starting line. In addition, extracts of both enzymatically and chemically deconjugated samples were compared. As reference, a standard solution containing corticosterone, progesterone, cortisol, estrone sulphate and estrone glucuronide was applied three times per TLC plate.

TLC plates were developed consecutively in three mobile phases: (A) chloroform:methanol:water (10:5:2), (B) chloroform:acetone (20:5), and (C) chloroform:acetone (20:1). Total distance travelled by solvent A was 6 cm, 10 cm for solvent B and 16 cm for solvent C. The radioactivity of all spots on the TLC plates (i.e. [^{3}H]-labelled CM) was visualised in a beta-imager (Biospace Lab, France). The reference solution contained no radioactivity; after running through the three different solvents, we manually labelled the bands of the reference solution with radioactivity under ultraviolet light to enable visualisation with the beta-imager.

To refine and reconfirm the patterns obtained by TLC, we also used reversed-phase high-performance liquid chromatography (RP-HPLC as in Hirschenhauser et al. 2000; Touma et al. 2003; Rettenbacher et al. 2004) for characterising the different metabolites in droppings containing peak radioactivity from urine or faecal parts of the droppings for each sex and species (i.e. four samples per species). In contrast to TLC, RP-HPLC provides a better resolution of the separated fractions based on the polarity of mobile phases, and HPLC is more accurate than the manual application of reference and test extracts on TLC plates. Flow rate was 1 ml per min, 90 fractions were collected in total from each sample (three fractions per min). Each HPLC fraction was tested for the presence of radioactivity, as well as immunoreactivity with the cortisone EIA (Rettenbacher et al. 2004; Baltic et al. 2005; Thiel et al. 2005).

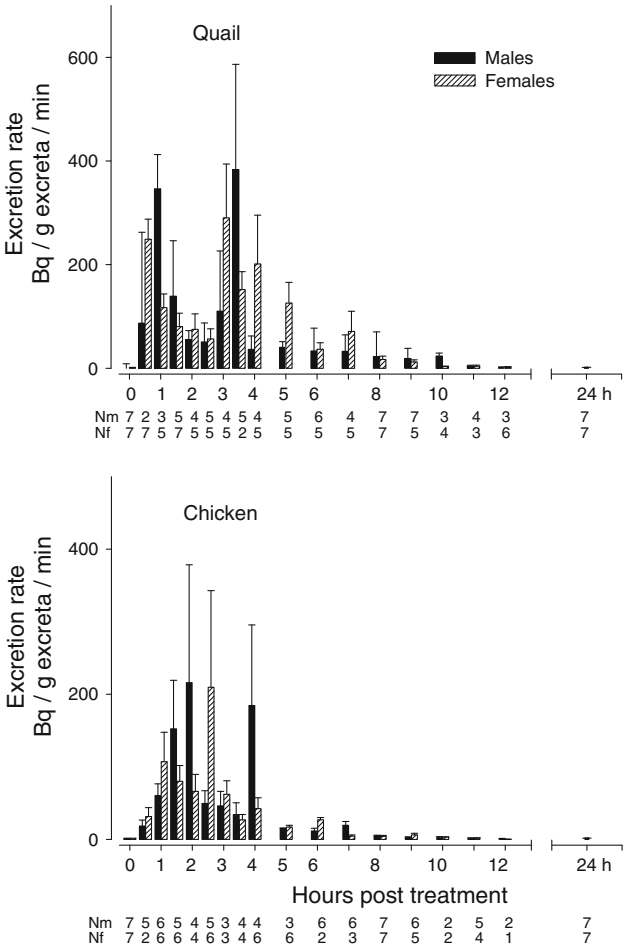


Fig. 1 Excretion patterns of radioactivity (Bq/g) per minute in male (*filled bars*) and female (*hatched bars*) Japanese Quail, *Coturnix japonica*, and Chicken, *Gallus domesticus*. *Bars* mean ± SE per 30-min intervals until 4 h after treatment with [^{3}H]-corticosterone, at hourly intervals from 4 to 12 h, and after 24 h. Sample sizes vary due to individual defecation rates and are given underneath the *x*-axis (*Nm* number of males, *Nf* number of females)

Statistics

Excretion patterns of radioactivity were controlled for individual defecation intervals by dividing the radioactivity excreted in a sample through the time elapsed (in minutes) since last dropping (Goymann et al. 2006; Goymann and Trappschuh 2011). Because treatments of the seven birds in the mornings of experimental days were spread over up to 60 min, we assorted individual samples according to minutes (hours) post-treatment. We calculated the sum of radioactivity excreted per 30 min until 4 h post-treatment (which is the assumed major excretion interval with regard to androgens; Hirschenhauser et al. 2008). Between 5 and 12 h post-treatment, we used sum of radioactivity excreted per hour. The excretion pattern of radioactivity was then plotted over the day as mean Bq/g excreta from *n* individuals per 30 min or h (Fig. 1). For comparing individual

delay times of corticoid metabolism, we calculated the interval between treatment and first and second peak of excreted radioactivity. Individual peak excretion was defined as Bq/g excreta greater than the individual mean + 1SD (i.e. the sum of excreted radioactivity within the 12 h post-treatment divided through the number of droppings deposited per bird). Delay times between injection and peak excretion satisfied the assumptions of normal distribution (Shapiro–Wilk test, Quail: $W = 0.97$; $n = 14$; $P = 0.8$; Chicken: $W = 0.89$; $n = 14$; $P = 0.1$) and males were compared with females with t tests, and Pearson's correlation tests were used to test the co-variation between body mass and excretion times. Statistical analyses were conducted using the SPSS for Windows 15.0.1 and SigmaStat 11.1.0. All probabilities are given two-tailed and means presented with standard errors (SE).

Results

Excretion patterns of radioactivity in Chicken and Japanese Quail

Males and females of both species excreted the radioactivity in a biphasic pattern over 24 h (Fig. 1). For simplicity, we will refer to the second radioactivity peak radioactive peak as 'faecal excretion'. In Quail, the delay between treatment and first peak of excreted radioactivity was 45 ± 5 and 55 ± 8 min (males and females, respectively). Faecal excretion occurred after 180 ± 25 min in male Quails and 210 ± 12 min in female Quails. Thus, excretion times of Quails were similar in males and females for both first and second peak (first radioactive peak: $t_{7,7} = 0.9$; $P = 0.4$; second radioactive peak: $t_{7,7} = 0.6$; $P = 0.6$). In Chickens, first radioactivity peaks occurred after 120 ± 10 min in males and 75 ± 12 min in females. Second radioactive peaks were excreted after 240 ± 26 min in male Chickens and after 140 ± 22 min in female Chickens. Statistically, male and female Chickens had similar excretion times for the first radioactivity peak ($t_{7,7} = 1.2$; $P = 0.2$), while the faecal excretion took significantly longer in males than in females ($t_{6,7} = 2.3$; $P = 0.04$). Body mass was negatively correlated with excretion time for first radioactivity peaks: larger Chickens had a faster urine excretion than smaller-sized ones ($r_{14} = -0.6$; $P = 0.026$). However, body mass did not explain the different faecal excretion times in male and female Chickens (male: $r_7 = 0.1$; $P = 0.9$; female: $r_7 = -0.3$; $P = 0.6$). In Japanese Quail, body size was not related with either first or second radioactivity peak excretion time ($r_{14} = -0.04$; $P = 0.9$; $r_{14} = -0.1$; $P = 0.8$).

Caecum droppings contained a wide and inconsistent range of radioactivity (median: 2,492 Bq/g; ranging from 665 to 16,956). In Japanese Quail, the majority (≈80 %) of these caecum droppings were deposited during afternoons, while in Chicken, caecum deposition was spread over the day (54 % a.m., 46 % p.m.). Some of the caecum droppings contained high levels of radioactivity, exceeding any of the peaks measured in non-caecum faeces excreta. There were great differences of radioactivity between caecum and non-caecum droppings. This was particularly evident when they were deposited within a short defecation interval: e.g. in six cases, caecum material was excreted within 15 min after a non-caecum dropping and we observed an excretion rate of 268 Bq/g/min (median; range from 92 to 1,696) compared with 6.5 Bq/g/min in the non-caecum samples deposited 15 min before (ranging 0.2–13). In addition, caecum excreta may have been retained inside the caeca for an unknown period of time: an extreme example was seen from a male Chicken at 24 h post-treatment, which deposited a caecum dropping within 5 min after a non-caecum dropping. The non-caecum sample held no radioactivity, while the caecum dropping still contained 1,204 Bq/g.

Characterisation of CM in Chicken and Japanese Quail excreta

Extraction with Sep-Pak C18 cartridges was effective as the first aqueous eluate contained no radiation and, thus, all excreted [^{3}H]-marked CM were in the methanol phase. All separated CM were characterised by high polarity and none of the samples contained corticosterone itself (Fig. 2). Distinct TLC bands appeared best in the solvent A containing methanol. After mobile phase A, no additional bands were resolved by the acetonic mobile phases B and C. Enzymatic and chemical deconjugation procedures did not change the distribution of separated metabolites in the TLC images. First and second radioactivity peak droppings eluted with similar numbers of qualitatively different CM (Fig. 3). Droppings contaminated with caecum faeces generally eluted with a higher number of bands than first and second radioactivity peak samples. Thus, caecum excreta contained more radioactive-labelled compounds/metabolites than non-caecum droppings. In both species, as well as in males and females, first and second peak of excreted radioactivity droppings shared only a few compounds with identical polarity (Fig. 2). Thus, the first and second radioactivity peaks were composed of different CM, which indicates that different metabolites are excreted in urine and faeces. In the female Quail, for example, the first but not second radioactivity peak sample eluted with similar polarity as estrone-glucuronide (Fig. 2). The remaining radioactive compounds were not comparable

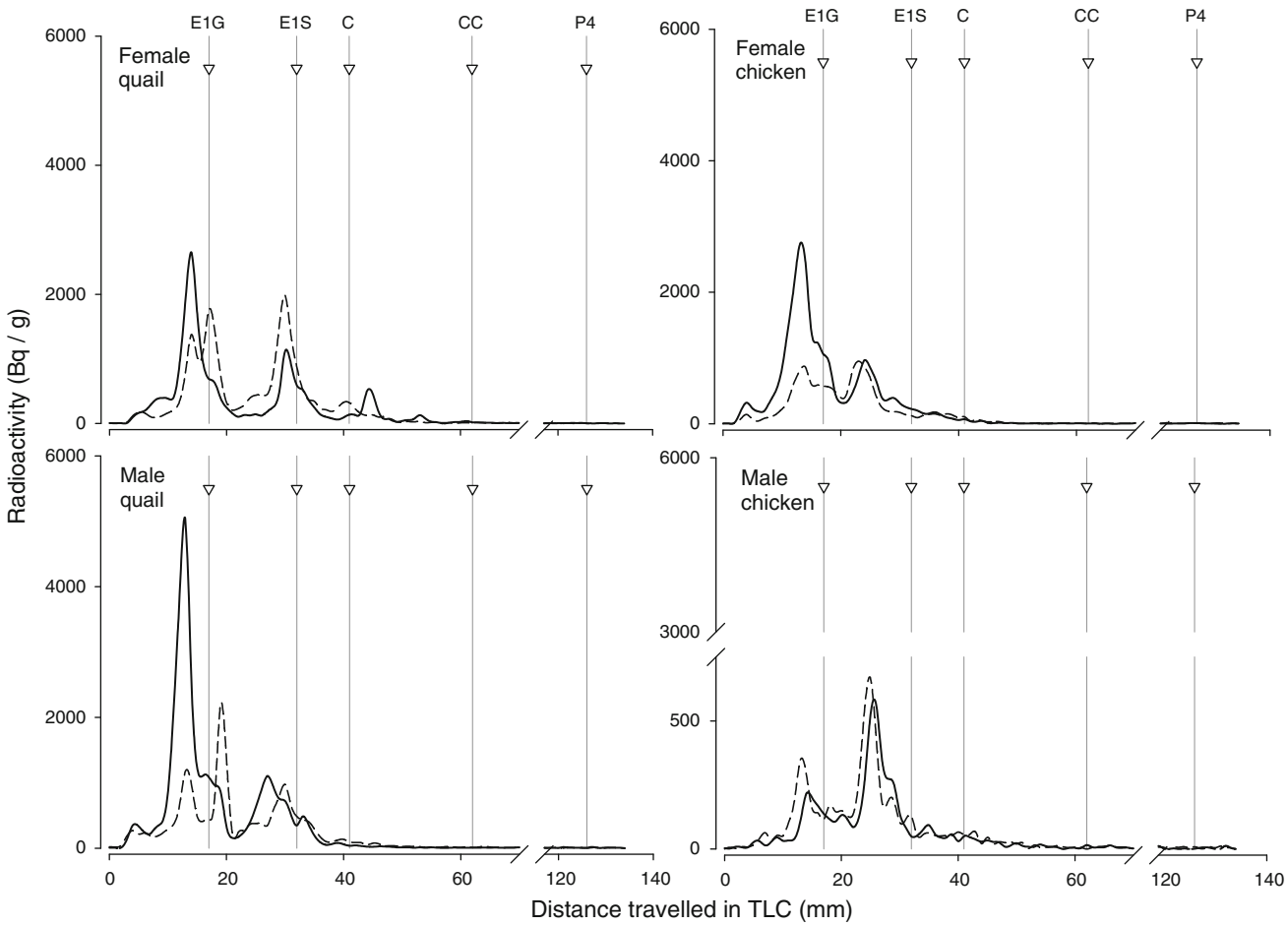


Fig. 2 2D-Radiograms showing the presence of radioactivity, radioactive-labelled CM as separated by thin layer chromatography (*TLC*). Metabolites with different polarities travelled different distances on the TLC plate. As representative examples, two droppings (first and second radioactivity peaks) from one male and one female of each species are shown. *Hatched lines* show the radioactive CM from droppings with a first radioactivity peak, i.e. presumably from urinary excretion. *Full lines* show the radioactive CM from droppings with second radioactivity peak, i.e. from faeces and urine. *Triangles* mark distances eluted by known compounds in the reference solution (E_1G estrone-glucuronide, E_1S estrone-sulphate, *C* cortisol, *CC* corticosterone, P_4 progesterone)

with any of the known standards and clearly differed between first and second radioactivity peak samples.

The TLC patterns were reconfirmed and refined by the HPLC separation of Quail and Chicken droppings. In addition to sex-specific differences, HPLC revealed the different CM in the 'urine' and 'faeces' parts of Quail and Chicken droppings more clearly than TLC (Fig. 3a, b). Only in the female Chicken, did urine and faeces droppings contain fractions, in which CM immunoreactive with the 'cortisone-assay' (i.e. glucocorticoid metabolites with a 3,11-dione structure) co-occurred with the presence of radioactivity (Fig. 3b). In droppings of male and female Japanese Quail, the major radioactive CM were not measured by the 'cortisone-assay' (Fig. 3a), and this was similar in droppings from male Chicken (Fig. 3b). Some immunoreactivity in the vicinity of the position where corticosterone should elute was present in all samples (Fig. 3a, b); however none of those matched with radioactivity (Figs. 2 and 3).

Discussion

Corticosteroid metabolism varies between species and, therefore, it cannot be predicted whether an assay tested in one species, e.g. in domestic Chickens, also measures meaningful proportions of excreted CM in another, even closely related, species, e.g. Japanese Quails. Using the 'cortisone-assay' suitable for Chickens and other galliform species did not prove valid with Japanese Quail excreta. The presented radiometabolism experiments show that (1) both species excreted the radioactivity in a biphasic pattern, probably representing urinary and faecal origin; (2) CM of extremely high polarity were excreted in the

droppings of both species, but no corticosterone itself; and (3) the two closely related species, as well as both sexes, excreted different numbers and identities of CM in droppings from urinary and faecal origin (containing first and second peaks of radioactivity). Hence, in addition to validating that an assay measures major metabolites in the dropping material, knowing the effective time for collecting droppings is crucial for interpreting results. Our results also show that mixing samples from urine and faecal pathways is not recommended.

Excretion patterns of radioactivity

Glucocorticoid metabolite levels in excreta represent integrated measures over a longer period of time. The length of the integral depends on the defecation frequency of the study organism (Goymann 2005). Depending on the research question, a good knowledge of gut passage times is desirable to choose the effective time interval for collecting samples, best reflecting the hormone response of interest, as well as for interpreting results. Mammals treated with radio-labelled cortisol, rapidly excreted urine with peak radioactivity already during treatment or in the first sample thereafter, while faecal excretion of peak radioactivity took considerably longer (e.g. 12 h in sheep, 48 h in pigs; Palme et al. 1996). Mice, *Mus musculus f. domesticus*, excreted CM after 2 h in urine and after 8 h in faeces (Touma et al. 2003). In birds, uric acid and faeces are excreted together through the cloaca. Therefore, we cannot exclude a peak radioactivity excretion into the cloaca prior to the first dropping we were able to collect. Yet, in no case did the very first dropping collected after treatment with [^{3}H]-marked corticosterone contain much radioactivity. In the two galliform species studied, first radioactivity peaks were excreted between 30 and 60 min after treatment, while the second radioactivity peak was in the range of 3 to 4 h. Compared with mice, galliform birds excreted urine and faeces relatively rapid.

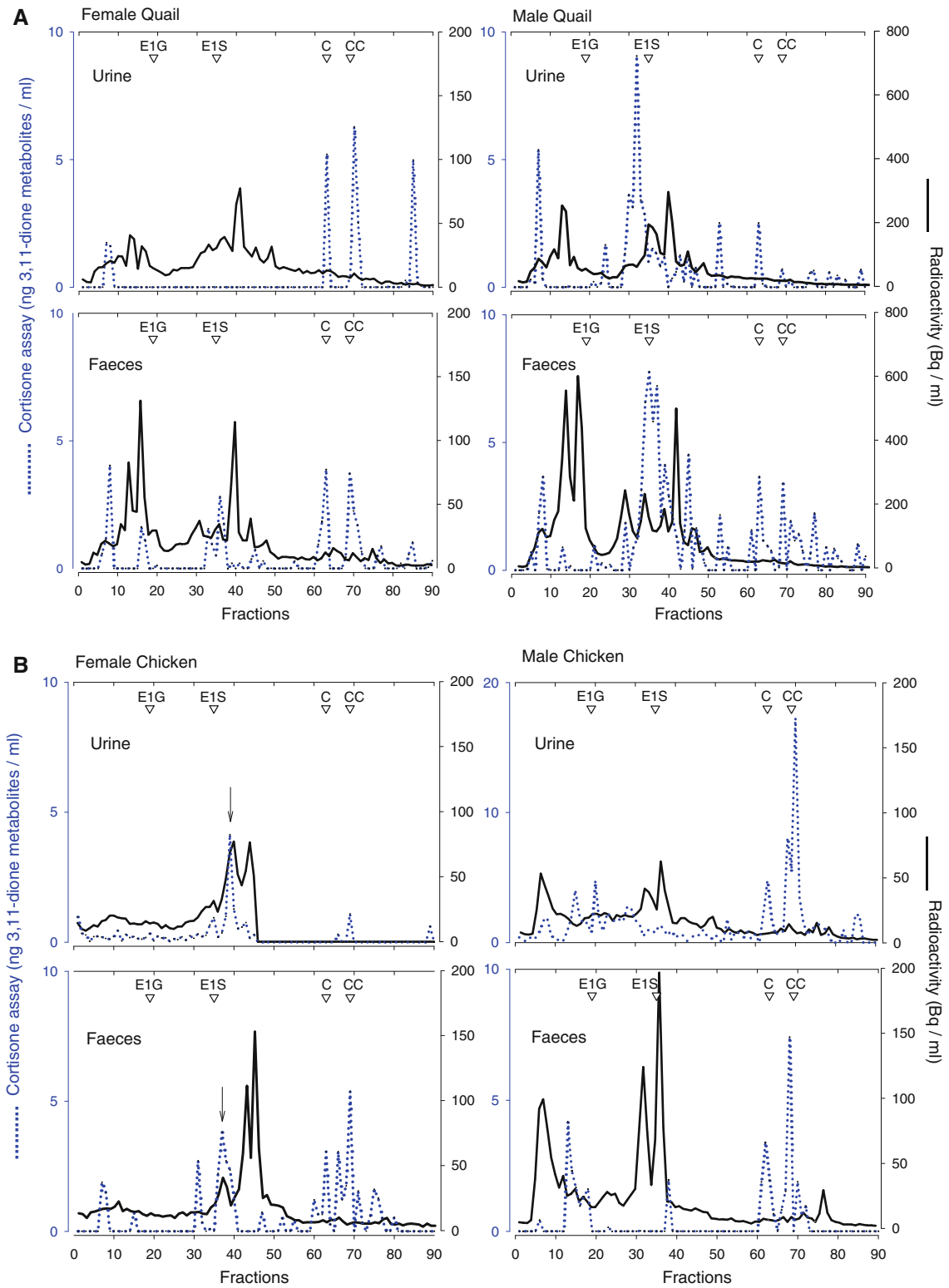
The excretion patterns of radioactivity after injecting [^{3}H]-marked corticosterone clearly showed two major peaks. Domestic Geese, *Anser domesticus*, also excreted major CM after releaser hormone (ACTH) treatment with two distinct peaks over the day (Kotrschal et al. 2000); a seasonal variation of clearance rates could not be excluded in that study and remains to be studied. Careful manual separation of white uric acid parts from Chicken droppings confirmed rapid CM excretion via urine and a slower faecal pathway (Rettenbacher et al. 2004). Because bird faeces and urine are joined in the cloaca, taken strictly, avian faecal material is always contaminated with uric acid (Klasing 2005). Thus, in our study, the first peaks of excreted radioactivity were probably of pure renal origin (with a non-radioactive faecal part), whereas the second radioactivity peak represented faecal excretion combined with urine. For the practical aspect of collecting droppings, our results suggest choosing either samples from the faster excretion route, i.e. containing CM of pure urinary origin, or droppings containing CM from combined faecal and urinary origin. Mixing of the two pathways is not recommended as we found different major CM excreted in the two pathways. The two routes of CM excretion have been supposed as potential source for biased metabolism rates in other bird species (Hiebert et al. 2000; Hirschenhauser et al. 2000; Goymann et al. 2002). Here, we demonstrate in two closely related species that the metabolites excreted in urine and faeces are indeed not identical.

In addition to differences between first and second radioactivity peak droppings, we show sex-specific patterns of gut passage time (i.e. delay times between treatment and peaks of radioactivity excreted in droppings) in Chickens. Chicken males had slower gut passage times than females, while male and female Japanese Quail had rather similar clearance rates (Fig. 1). Body mass did not explain different faecal excretion rates, while urinary excretion (i.e. first radioactivity peak) was faster in larger Chickens. Although speculative, the different clearance rates in Chickens imply that females might handle stress load in a different way than males. The regular feeding behaviour of Quails and Chickens in our laboratory setting and their relatively frequent defecation rates (2.0 $\pm$ 0.7 per h in Quails and 2.8 $\pm$ 0.9 per h in Chickens) is advantageous compared with seasonally varying feeding periods of other species, e.g. passerines (Goymann et al. 2002; Goymann and Trappschuh, 2011) or the rather irregular feeding events in owls (Wasser et al. 1997). The 180-min gut passage time in male Quails corresponds well with the excretion of androgen metabolites in the droppings of male Quail in a previous study based on edible dye (Hirschenhauser et al. 2008).

We were also interested in between-species comparisons, to test the relationship between body size and CM clearance in the two galliform species. We expected longer gut passage times in the larger Chickens, and faster excretion in the smaller-sized Japanese Quails (with assumingly smaller inner organs). Males met our expectation, as the large Chicken males took longer for excreting peak radioactivity than the small-sized Quail males. However, faecal excretion was even faster in the large female Chickens (143 $\pm$ 19 min) than in the much smaller Quail females (180 $\pm$ 25 min). This illustrates that, when comparing species, gut passage time is not a simple function of the body size.

Characterisation of CM in Chicken and Japanese Quail excreta

Based on different polarities, a large number of different metabolites eluted from all samples tested with TLC



(Fig. 2). This included samples from males and females, as well as first and second radioactivity peak samples, from both, Japanese Quails and Chickens. HPLC reconfirmed that radioactive corticosterone was not present in the droppings of either species and there were no labelled metabolites even in the proximity of corticosterone (Figs. 2 and 3). In all the samples investigated, the EIA of the separated HPLC fractions produced a peak at the position where the corticosterone should elute (Fig. 3a, b); however, there was no matching with radioactivity and no

◀ **Fig. 3** Immunograms based on HPLC separation of Japanese Quail **(a)** and Chicken **(b)** droppings containing first ('Urine', *upper row*) and second peak radioactivity ('Faeces', *bottom row*) after injection of [^{3}H]-corticosterone (Fig. 1) to visualise the difference between CM excreted in urine or faeces parts of droppings. 'Urine' and 'faeces' droppings from (**a**) the female Quail (*left column*) were from 50 to 215 min after injecting [^{3}H]-corticosterone, 45 and 220 min after treatment from the male Quail (*right column*); (**b**) 70 min and 180 min from the female Chicken (*left column*) and 100 and 185 min after treatment from the male Chicken (*right column*). Urine and faeces droppings were from same individuals to allow direct comparison of the CM from different excretion pathways. *Black line* is the radioactivity per fraction, i.e. indicating excreted metabolites deriving from the injected [^{3}H]-corticosterone. *Blue axis* and *dotted lines* show the immunoreactivity per fraction measured with the 'cortisone-EIA' (Rettenbacher et al. 2004). *Triangles* mark distances eluted by known compounds in the reference solution (*E_1G* estrone-glucuronide, *E_1S* estrone-sulphate, *C* cortisol, *CC* corticosterone). The small *arrows* mark where immunoreactivity and radioactivity matched in the female Chicken's urine and faeces

radiolabelled metabolite was even in the vicinity of the corticosterone (Figs. 2 and 3). Rettenbacher et al. (2004) achieved similar results using HPLC separation of Chicken droppings; our experiments add a comparative aspect by comparing two closely related species in the same study. Using a corticosterone assay has worked with Chicken droppings before (Dehnhard et al. 2003), probably due to its cross-reactivities with other steroid metabolites (Rettenbacher et al. 2004). However, our results underscore that, in the case of the Quail, the use of a specific corticosterone immunoassay will not cover the major amount of excreted CM, which will result in a sub-optimal signal-to-noise ratio and, thereby, increase the likelihood of a type II error. The polar nature of glucocorticoids makes a distinction between conjugated and non-conjugated types of metabolites difficult (Hirschenhauser et al. 2005). The autoradiograms of the TLC suggested extremely high polarities of the CM in droppings of both species, which may confirm that corticosterone was either entirely converted into highly polar (yet unknown) metabolites or fully conjugated in both species. However, neither chemical nor enzymatic deconjugation changed elution patterns, which indicates that the excreted metabolites were not conjugates but other polar glucocorticoid metabolites. This was different in the studies of Capercaillie, *Tetrao urogallus*, and Black Grouse, *Tetrao tetrix*, which also revealed a number of unidentified CM but with lower polarity (Baltic et al. 2005; Thiel et al. 2005).

Sex-specific metabolism of corticosterone was found in both species studied. This may well be common in galliforms, because sex-specific patterns have been reported before in Chicken and Black Grouse (Rettenbacher et al. 2004; Baltic et al. 2005) as well as in one passerine bird (European Stonechat, *Saxicola torquata*; Goymann et al. 2002). In other birds, such as Greylag Geese (*Anser anser*) and Adélie Penguins (*Pygoscelis adeliae*), the excreted profile of CM was similar in males and females (Nakagawa et al. 2003; Möstl et al. 2005). Thus, a sex-specific metabolisation difference may be common, but is not a universal phenomenon and definitely needs be tested as part of validation routines before the method is applicable for comparisons between males and females of a species.

First and second radioactivity peak samples, i.e. urine and faeces droppings, contained considerably different metabolites (Figs. 2 and 3). This emphasises that the choice of a standardised effective sampling period is crucial for yielding meaningful results with this method in avian species. For example, the rapid CM changes recently reported in Snow Goose droppings (6–10 min after capture; Legagneux et al. 2011) were probably of urinary origin. The rapid CM changes may bear methodological/logistic limits similar to using blood samples and take the 'faecal method' issue to an unexpected turn. Hence, to benefit from the integrative nature of excreted CM over time (Goymann, 2005), one should target droppings of faecal origin. Beyond doubt, there are numerous studies showing that biologically meaningful data can be achieved by measuring CM with other glucocorticoid immunoassays (RIA or ELISA) and using commercial reagents (e.g. Wasser et al. 2000; Dehnhard et al. 2003; Washburn et al. 2003; Fraisse and Cockrem 2006; Goymann et al. 2006; Lèche et al. 2011). However, biologically meaningful patterns of excreted CM in droppings may emerge when the assay has high cross-reactions with other major corticosterone metabolites rather than with corticosterone itself. We claim that, for studies in the field of behavioural endocrinology, it is desirable to unravel and develop more specific CM measures for faecal (not urine) excretion. It may also be relevant for explaining the degree of metabolisation how long the excreted material was retained inside the animal's gut (Lexen et al. 2008). We report a large number of various radioactive CM in caecum faeces droppings from both species. Caecum material has been exposed to microbes for a longer time and, thus, presumably glucocorticoid compounds may have been metabolised to a higher degree than in non-caecum faeces (Klasing 2005). In addition, CM measurements from caecum faeces remain unpredictable as long as we do not know how long the dropping material had been deposited (and subjected to microbial degradation) inside the caeca before being excreted. To our knowledge, this is the first study to show that in practice collection of caecum droppings should be avoided, as they contain a large number of different CM and potentially extended retention times compared with non-caecum faeces.

Although we still do not know how to measure CM in Japanese Quail excreta, this study provides useful details on the nature and variability of CM in their excreta. In general, we unravel important aspects on the benefits and

limits of measuring glucocorticoid metabolites in avian droppings. The great potentials of the non-invasive method are worth the required analytical effort, and the presented results might serve as basis for developing an assay specifically measuring CM from faecal excretion.

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Conflict of interest The authors declare that they have no conflict of interest.

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Electronic Supplementary Material

'Cortisone-assay' does not measure major glucocorticoid metabolites in Japanese quail excreta

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Excreted corticosterone metabolites differ between two galliform species, Japanese Quail and Chicken, between sexes and between urine and faecal parts of droppings

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'Cortisone-assay' does not measure major corticosterone metabolites in Japanese quail excreta

Measuring corticosterone metabolites (CM) in droppings with the 'cortisone assay' has been thoroughly validated in three galliform species, i.e. chicken (*Gallus gallus*, Rettenbacher et al. 2004), capercaillie (*Tetrao urogallus*; Thiel et al. 2005) and black grouse (*Tetrao tetrix*; Baltic et al. 2005). The cortisone assay measures glucocorticoid metabolites with a 3,11-dione structure (Rettenbacher et al.). However, in droppings of another galliform bird, the Japanese Quail (*Coturnix japonica*), initial physiological validation tests for measuring corticosterone metabolites revealed that the cortisone assay did not measure parameters of adrenocortical activity.

In a physiological challenge test adrenocortical activity and the secretion of corticosterone is stimulated by administering adrenocorticotropic hormone (ACTH). The increased circulating corticosterone must be metabolized and excreted (Möstl et al. 2005). If the immunoassay used measures relevant parameters of adrenocortical activity in droppings of Japanese Quail we expected to find increased levels of excreted CM after ACTH-treatment. Here we tested whether the 'cortisone-assay' measured biologically meaningful estimates of the CM excreted in Japanese quail droppings (Fig. S1ab). We also tested a second assay against tetrathydrocorticosterone, which is known to measure major CM in other bird species (Quillfeldt and Möstl 2003).

Both EIA tested, the cortisone and the tetrahydrocorticosterone assay, failed to detect the expected increased levels of CM in the droppings of ACTH-treated Japanese Quail. Throughout ten hours after treatment the measured CM levels of birds injected with ACTH did not differ from control birds (Fig. S1). In contrast to our expectation, based on the cortisone assay females in the control group had even higher CM levels throughout the sampling day than the ACTH-treated group (Table 1; Fig. S1a). In male Japanese Quail ACTH treatment had no significant effect on CM levels as measured with the cortisone assay (Table 1; Fig. 1b). The tetrahydrocorticosterone assay resulted in similar patterns (Fig. S1cd). Male CM showed one peak 120 min after ACTH, which was higher than in the control group (Table 1); however, 180 min post-treatment male Quail from the control group had higher CM levels than the ACTH treated males and overall the pattern did not appear satisfying for a validated assay (Fig. S1d).

Dropping production was similar in females treated with ACTH (22.0 ± 1.6 droppings in 10 h) or Ringer solution (23.3 ± 1.0 droppings in 10 h; t-test: $t_{7,7}$ = -1.1; P = 0.3). Male Japanese Quail produced significantly more droppings after ACTH treatment (16.4 ± 1.3 droppings in 10 h) than after control treatment (11.4 ± 1.0 droppings in 10 h; paired t-test: $t_{5,5}$ = -5.0; P = 0.008). Thus, in male Quail the ACTH-treatment had an overall effect on the birds' dropping production; however, female Japanese Quail produced more droppings in general and there was no effect of ACTH-treatment.

Methods:

ACTH treatment in Japanese Quail

We intra-peritoneally injected male (N = 7) and female (N = 7) Japanese Quails with 0.2 mg ACTH (Synacten, Ciba-Geigy, Basel; Rettenbacher et al. 2004) in 50 µl saline solution, control birds received 50 µl saline Ringer solution (N = 5 males and 7 females). Five males were used for both treatments aiming at within-individual controls. The five males were treated with saline solution at a first 'control' day and with ACTH the next day. Female control group birds were not identical with the individuals in the ACTH-group but groups were also treated on different days. Directly before

treatments we collected a first dropping from each individual for baseline levels. Injections took place between 10:00 and 10:45 a.m. and we collected all droppings until 10 h after treatment, noted the time and stored samples at -20°C until further processing in the lab. Aliquots of 0.1 g of the excreta were extracted and processed with a) the cortisone enzyme immunoassay (EIA) following the protocol as published elsewhere (Rettenbacher et al. 2004; Baltic et al. 2005; Thiel et al. 2005) and b) the tetrahydrocorticosterone EIA as in Quillfeldt and Möstl (2003).

We assorted the CM-data from both assays individually to time intervals of 30 min post-treatment and calculated mean CM ± SE per 30-min interval. CM data passed the Shapiro-Wilk normality test in female Quail (cortisone $P = 0.939$, tetrahydrocortisone $P = 0.624$) and after square-root transformation in male Quail (cortisone $P = 0.246$, tetrahydrocortisone $P = 0.283$). The effects of treatments with ACTH or saline solution were tested using Two-Way Repeated Measures ANOVA with post-hoc Holm-Sidak adjustments for multiple pairwise comparisons.

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Table 1: Results of the two-way repeated measures ANOVA for the ACTH-effects on the excretion of CM in Japanese Quail droppings. Pairwise comparisons at 120 and 270 min post-treatment refer to Fig. S1. Note that in female Quail, CM levels were higher in the control group than in the ACTH-treated group.

	Cortisone assay		Tetrahydrocorticosterone assay	
	Male Quail	Female Quail	Male Quail	Female Quail
Repeated factor (within-subjects effect)	Time from treatment			
	$F_{26,104} = 2.3$ $P = 0.001$	$F_{26,102} = 1.6$ $P = 0.054$	$F_{26,102} = 2.0$ $P = 0.006$	$F_{26,102} = 0.9$ $P = 0.571$
Independent factor (between-subjects effect)	ACTH or control treatment			
	$F_{1,4} = 8.7$ $P = 0.035$	$F_{1,4} = 25.0$ $P = 0.007$[a]	$F_{1,4} = 7.5$ $P = 0.052$	$F_{1,4} = 0.2$ $P = 0.656$
Interaction of the two factors				
	$F_{26,104} = 1.4$ $P = 0.119$	$F_{26,102} = 1.6$ $P = 0.063$	$F_{26,104} = 2.1$ $P = 0.005$	$F_{26,102} = 1.1$ $P = 0.394$
Pairwise comparisons (Holm-Sidak)	120 min after ACTH-treatment		120 min after ACTH-treatment	
	$t_5 = 1.7$ $P = 0.103$	$t_3 = 2.6$ $P = 0.011$[a]	$t_5 = 2.3$ $P = 0.027$	
	270 min after ACTH treatment		180 min after ACTH treatment	
	$t_5 = 1.8$ $P = 0.081$	$t_3 = 2.9$ $P = 0.005$[a]	$t_5 = 2.3$ $P = 0.022$[a]	

[a] Superscript marks comparisons in which the CM levels of control group were higher than CM of the ACTH-treated group

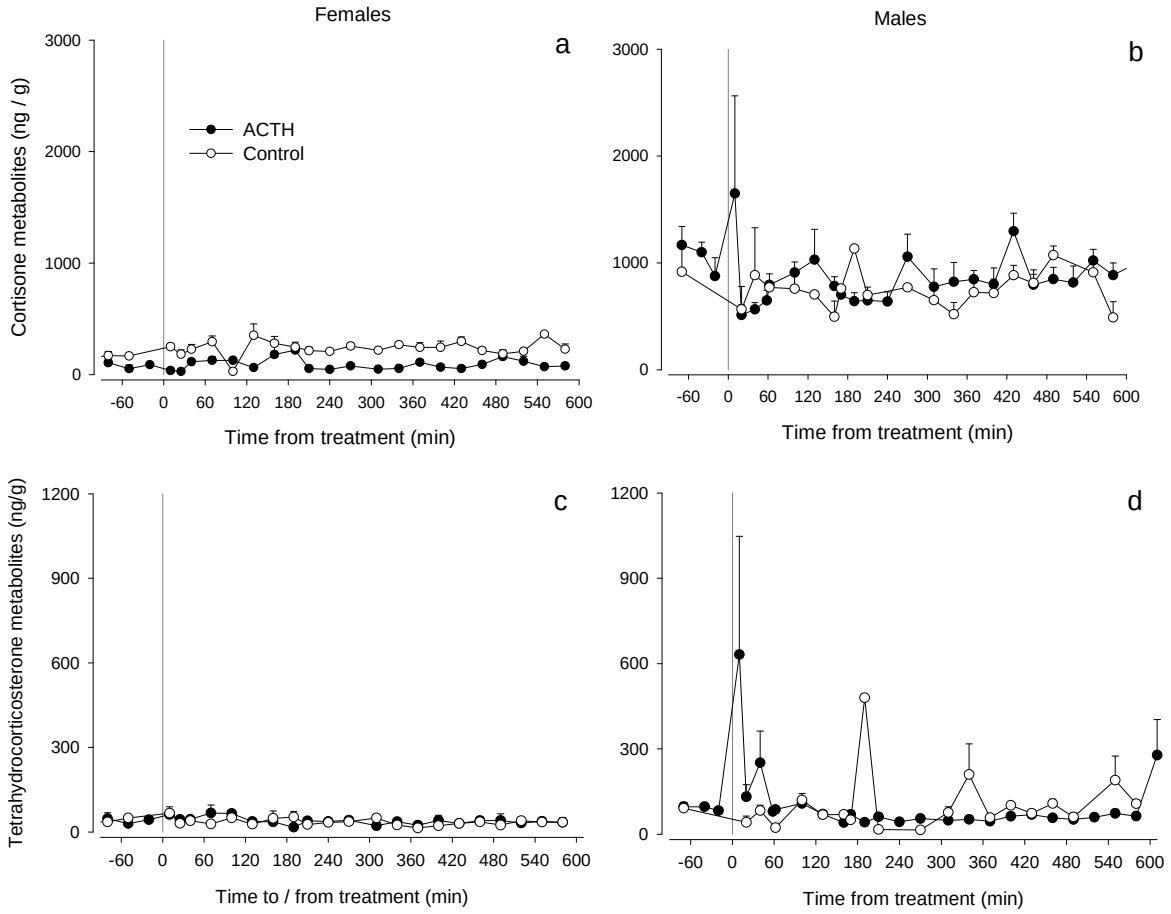

Figure S1. Patterns of excreted CM in droppings of female (left column) and male (right column) Japanese Quail as measured with the 'cortisone-assay' (a,b, upper row) and tetrahydrocorticosterone-assay (c,d, bottom row). Birds were treated with ACTH at time "zero" (indicated by the grey vertical line). Plotted are means + SE per 30 min. The administration of ACTH to Japanese Quails was permitted and approved by the Austrian commission for the protection of animal welfare (BMBWK 66.066/14-BrGT/2005).