Effect of sexual and seasonal differences in faecal steroid analysis in birds: optimalization of sample collection

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In the last decade many attempts were made on faecal steroid analysis, as a non-invasive method. Birds' faeces contain metabolites of steroid breakdown eliminated both from the intestines and the kidneys, resulting in a special situation.
For the precise analysis determination of the passage time of fodder in studied species is the first step. In mallards, intestinal passage time of marked (coloured) fodder and testosterone (T) turnover were examined by faecal steroid analysis in the reproductive and postrefractory period. In the latter, the discharge of coloured fodder began 36 and 56 minutes after ingestion in males and females, respectively. During reproduction period the discharge began later, 93 and 112 minutes after ingestion in males and females, respectively. The differences between sexes and seasons were significant. Total passage time was similar in both periods and sexes.
Knowledge of the frequency of defecation is also advisable. In postrefractory period the frequency of defecation of males (16,0±2,72/ 8 hours) was higher than that of females (13,5±1,20/ 8 hours, p=0,01), however, in reproductive cycle in this respect there were no differences between sexes (9,48±1,48/ 8 hours in males and 11,2±3,11/ 8 hours in females).
Following intraperitoneal T injection, faecal samples had been collected for 8 hours and T levels were measured using RIA. In the postrefractory period, there were no significant differences in T levels between sexes. The first T peaks appeared at 1-2 hours, the second one at 2,6-3,3 hours after loading.. During reproduction period in females the T excretion was similar to the data of postrefractory period, while in males the peak values were shifted to the autumnal values. In females, a slighter T peaks appeared in both periods. The duration of response to T loading was about 5 hours in both periods and sexes.
For an optimal sample collection it is important to have control values, to collect samples always at the same time, to know the frequency of defecation, the passage time and seasonal and sexual differences as well.

Keywords: faecal steroid analysis, passage time, sexual-, seasonal differences, mallard

Introduction

Non-invasive hormone measures must be carefully validated for the effective research and analyses. Validity is especially important for the endangered species (indigenous domestic animals as well). In mammals, faecal hormones measures have been extensively validated (Wasser and Hunt, 2005). The biological equivalence of faecal steroids is different in mammals and birds. In case of mammals there are two possible forms of excretion: bile-chimus-faeces axis and urine (Shires et al, 1987). In birds - being vertebrates with cloaca, faeces contains metabolites of steroid breakdown eliminated both from the intestines and the kidneys, resulting in a special situation. In mammals one of the basic problems is the different excretion ratios of certain steroid metabolites into urine and faeces (Sturkie, 1976), and in addition, relatively to urinal excretion, chimus-faeces excretion routes can show a considerable delay in mammals.

In mammals, the steroid equivalent peaks appear in approximately 5 hours in the urine and in 12–18 hours in the faeces after their appearance in the blood, in addition, there is a slight difference in the physiological mechanisms of their excretion (Miller et al, 1991; Schwarzenberger et al, 1996).

The percentage of excreted faecal and urinal steroids strongly depends on the animal species and on the type of the steroid. In case of testosterone (T) high deviation was found (14-89 %) excreted in mammalian faeces: 14% in pigs (Palme et al, 1996) and rodents (Busso et al, 2004), 28% in horse, 44% in sheep (Palme et al, 2004), and the highest (89%) in North American river otter (Gross, 1992).

Relatively few studies validated the hormone measures for avian species (Tell, 1997; Frigerio et al, 2001; Goymann et al, 2002; Kelemen et al, 2003; Nakagawa et al, 2003). As far back as the fifties faecal steroid analysis was used for sex-determination in monomorphic birds (Hurst et al, 1957; Wasser and Hunt, 2005), but problems concerning methods were not solved until recently. In birds, the mechanism of excretion is different from that of mammals, because the end-products are eliminated through both urine and chimus and are mixed in the cloaca. The intestinal food transport is fast: it takes only a few hours (2–4 hours) in geese (Hirschenhauser et al, 2000) and in domestic fowl (Dansky and Hill, 1952). Concerning urine production and excretion, experimental data in hens show that the effect of water load appears in 15–20 minutes, it reaches a peak value after about 80 minutes and decreases to the basic level after 120 minutes (Shires et al, 1987). Therefore, there could be no more than 1-2 hours of delay between the appearance of steroid quantity excreted by bile into the chimus, and steroid quantity excreted via urine into the faeces. Infusions of radiolabelled hormones showed that the northern spotted owls excreted most hormones within 6 hours (Wasser and Hunt, 2005)

There are only a few data concerning the complex study of passage time and the kinetic of steroid excretion in birds. Above the precise validation the optimalization of the sample collection is essential for the diagnosis of the real physiological status.

Material and methods

Animals

10 female and 10 male mallards were studied in the postrefactory (October) and in the reproductive (May) period. Birds were kept outdoors and fed and watered *ad libitum*. 2 days before the treatment ducks were put into the experimental box. Before the treatments birds were starved for 15 hours having free access to water. The ground of the box was covered with PE foil to make it easier to clean and prevent the loss and mixing of faecal samples. The intestinal passage-time of fodder was determined before T loading.

The passage-time of fodder

A small quantity of fodder was soaked in water, coloured with artificial food-colorant for human use (E102-tartrazin, synthetic yellow, E133-Brillant Blue, E211-Sodium benzoate, E330-citric acid, non-toxic, non-laxative) and force fed. After force-feeding mallards were continuously watched during the purging time. The apparition of the first coloured faeces and non-coloured faeces was noted.

Testosterone loading

On the next day, after the passage-time experiment mallards were injected with T solution (cca.100x physiological dose: 5µgT/2ml solution) intraperitoneally. Pure T (Sigma T1500) was diluted in 100µl ethanol abs. then diluted 100 times using physiological saline. After the T injection in an 8 hours period all faecal samples were collected in PE tubes. Two samples were used as control: the first (basic data) one was collected before T injection, the second one 24 hours after injection. The Control II level was necessary to check this value corresponds to the first endogen, physiological level (Control I).The samples were deep-frozen and stored at -20°C until use. Faecal T concentrations were measured by RIA.

Method of faecal testosterone analysis

The thawed, homogenizated faecal samples were dried at 37 °C until body-balance within 24 hours, preventing the altering effects of differences in the water content of samples. Then, samples were extracted (20 min) with diethyl-ether three times. To reduce the quantity of lipids 50-150 µl, 1% sodium dodecyl sulphate (SDS) was added to faecal samples before their extractions (Kelemen et al, 2003). 0,5 mg aliquot of dried faecal samples was used in triplets for the determination of steroids. T RIA by *Jallageas* [1975] was used for the determination of T equivalents. Antibody was developed against BSA-testosterone-3-hemisuccinate in the Laboratory of Peter Sharp, in Edinburgh. The sensitivity of the T assay was 5 pg/tube, the intra- and interassay reproducibility were 6–9 and 11–14% in CV. The specificity of the antibody was tested by cross reactions and results covered a wide range and group specificity of the applied polyclonal antibody.

The bound radioactivity (cpm) was measured by LKB-WALLACK scintillation spectrometer. To evaluate the steroid recovery of the extractions and lipid emulgeations 20µl (4000 cpm/g faeces), ^{3}H-T was added to the samples and 1/5 part of faeces extracts were used to calculate recovery. Data were compared using ANOVA and paired t-tests ($p \leq 0,05$). All statistical analysis were done with NCSS 6.0 for Windows. To quantify T excretion data were integrated for calculation the under curve area using Microcal Origin Version 3.5

The above mentioned method was used in several species: muscovy duck, domestic goose, domestic fowl, guinea fowl and great bustards, common cuckoo, collared flycatcher and dipper, as well.

Results and discussion

In the mallard study using non-invasive faecal steroid analyses, the testosterone (T) turnover was examined. The experiments were carried out in October -in the postrefractory period- and in the reproduction cycle, in May.

Passage-time of fodder in the postrefractory period

For the precise analysis determination of the passage time of fodder in studied species is the first step.

1. table Passage-time of fodder in the postrefractory period

	Postrefractory period		
	Appearance of coloured faeces (min) after ingestion	**Purging time (min)**	**Retention time (min)**
male	35,6±2,70*	253,8±3,03	218,2±3,35*
female	56,0±3,16*	253,6±4,16	197,6±5,68*

*: $p \leq 0,001$ (t-test)

Feeding of coloured fodder was carried out at 9.00 AM="Control I." time.

In the postrefractory period the discharge had began 36 (35,6±2,70) minutes after ingestion in males, and 56 (56,0±3,16) minutes in females. This difference was statistically verifiable ($p<0,01$). The total purging process ended at the same time in both sexes: in males 253,8±3,63 in females 253,6±4,16 minutes ($p=0,94$) respectively.

The total passage time of coloured fodder was about 254 min. in both sexes, but defecation of coloured fodder started 20 min. earlier in males than females. The duration of total discharge was significantly shorter in females ($p<0,01$).

Passage-time of fodder in the reproductive cycle

2. table Passage-time of fodder in the reproductive cycle

	Reproductive cycle		
	Appearance of coloured faeces (min) after ingestion	**Purging time (min)**	**Retention time (min)**
male	92,7±25,82[a]	248,8±17,24	156,0±22,89[b]
female	111,8±18,20[a]	242,6±17,30	130,8±12,76[b]

a: p=0,05 (t-test) b: p=0,01

During reproduction the discharge of coloured fodder began 92,7±25,82 minutes after the ingestion in males and 111,8±18,20 minutes in females. In both sexes, the discharge started two times later than in the postrefractory period. The total purging time in both sexes was similar: 250,0±16,37 minutes in males and 242,6±17,30 minutes in females. The time between apparition and total discharge of coloured faeces was significantly shorter in females, similarly to the postrefractory period.

Knowing the chimus-faeces passage the determination of speed of the steroid excretion is the second objective of interpretation of faecal steroid analysis. In chicken -the most studied avian species- appearance of marked fodder was about 1,6-2,6 hours (Dansky and Hill, 1952), the total retention time was estimated to 5-9 hours (Denbow, 2000). From physiological aspect retention time is more expressive than detecting the time of first appearance (Denbow, 2000).

Retention time prolongs with age within a species (Shires et al, 1987), and with increasing environmental temperature as well. Few data are available concerning to waterfowl: Hirschenhauser et al. (2000) found that coloured fodder appeared 2-4 hours after ingestion in geese, but they did not study the time of retention.

In the present study two parameters of food passage were examined: time of appearance, total passage time (purging) and retention time. Faecal excretion of intraperitoneal T loading was interpreted after determining these parameters.

In both studied periods coloured fodder appeared earlier in males than in females, but the difference was more considerable in the postrefractory period. In both sexes coloured fodder appeared two times later during reproduction than in the postrefractory period. Retention time (total passage time) was 243-254 min. regardless of periods and sexes. These retention times are 1-5 hours shorter than the values found in domestic fowl by Denbow, (2000). The purging period in mallard is relatively short and it depends on sex and on the actual levels of sexualsteroids. About sex-dependent excretion speed there are no published data so far.

Frequency of defecation

While in postrefractory period the frequency of defecation of males (16,0±2,72/ 8 hours) was higher than that of females (13,5±1,20/ 8 hours, p=0,01), in reproductive cycle it was similar in both sexes, that is 9,48±1,48/ 8 hours in males and 11,2±3,11/ 8 hours in females. Generally in postrefractory period both the males (p<0,01) and the females (p<0,05) defecated more frequently than in reproductive cycle.

Knowing the frequency of defecation is very important for the sample collection, since it is a season depending factor.

Testosterone loading

Faecal samples were collected just before T injection since catching of birds defecation can be mostly provocated (control I). On the different figures (Fig1-4) points represent average values of at least 5 defecations of 10-10 ducks within 10 minutes. In the postrefractory period (Fig 1) a strength increase of faecal T content was observable in 1-2 hours after T loading in males, while in females the increase was lower. The duration of response to steroid loading was 5 hours in both sexes. In physiological T levels (control I) no differences were found between sexes, however- according to the

measurements of under curve area- T loading resulted in 25% more T excretion in males than females. During 5 hours after T loading cc. 77% of the total detected T equivalents (8 hours) were excreted in both sexes (76,74% in males and 76,50% in females).

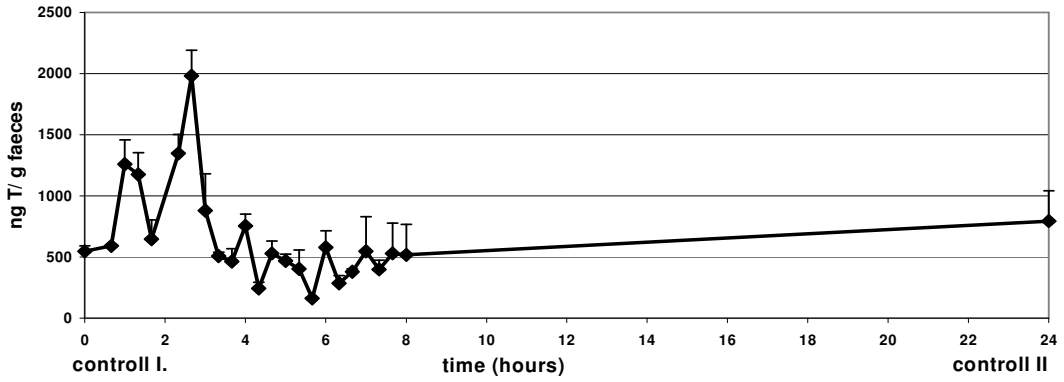

Figure 1. Fecal testosterone concentrations of male mallards in the postrefractory period

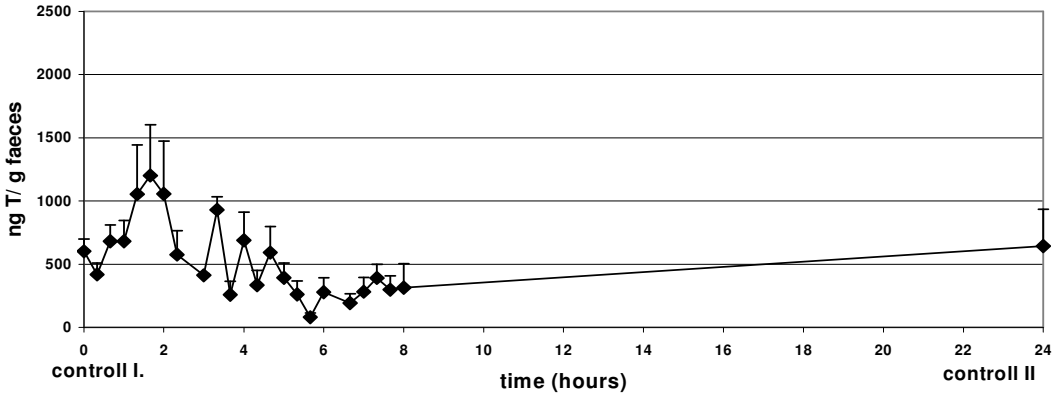

Figure 2. Fecal testosterone concentrations of female mallards in the postrefactory period

During reproduction cycle, 2 hours after T loading a fluctuating increase of T was detected in males. In females the faecal basic T level (control I) and the faecal T level during the excretion period (1,5-2 hours later) were lower than in males. In both sexes the duration of response was 5 hours (72,27% in males and 71,82% in females of the total examined period). Certainly, the physiological T levels of drakes were significantly higher ($p<0,01$) than in females. This parameter can be used for sexing as well similarly to northern spotted owls (Wasser and Hunt, 2005).

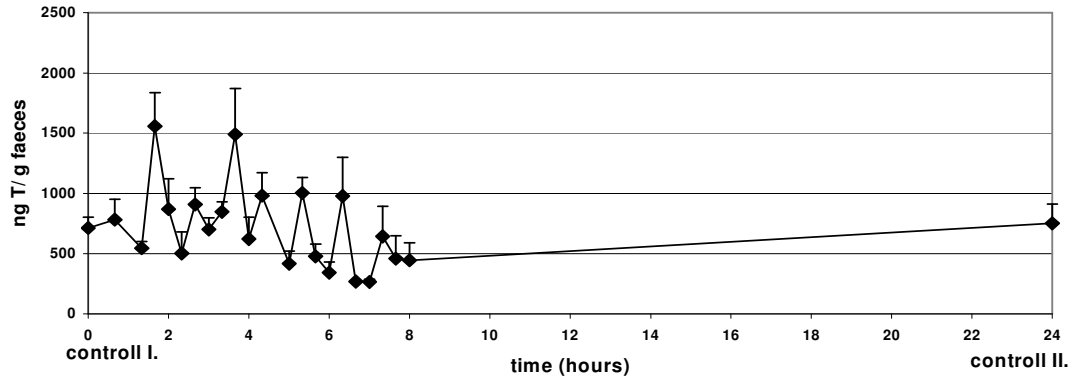

Figure 3. Faecal testosterone concentrations of male mallards in the reproductive cycle

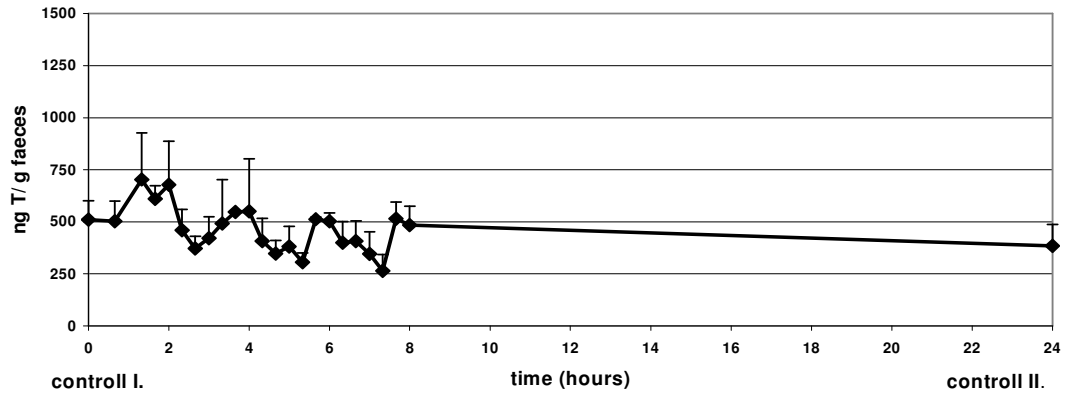

Figure 4. Fecal testosterone concentrations of female mallards in the reproductive cycle

In both sexes an early, intensive and a latter, lengthened period of T excretion was observable. In males the time of early, intensive excretion was longer and peaks of measured T levels were higher during the sexually inactive period than that of sexual activity. Diurnal changes of T turnover were studied in great bustard (Biczó et al, 2000) and time and position of faecal T peaks and the shift of these peaks during the seasons were detected. Same study was carried out in barred owls, moreover in this species the faecal steroid analysis was suitable for identification of gender and breeding stage (Wasser and Hunt, 2005).

In the present experiment, effects of T loading could be detected for 5 hours in postrefracter and in sexually active mallards as well. No differences in intensive excretion period could be observed between sexually active and inactive females. The period of intensive excretion after T loading appeared earlier in males than in females, but total excretion finished at the same time, 5 hours after injection in both sexes.

It can be concluded that the character of testosterone excretion shows a strong correlation with the passage of fodder-chimus-faeces in both sexes, in the reproductive as well as the postrefractory period. Testosterone excretion curves show higher peaks and more intensive fluctuation in drakes than in females in both studied seasons. Control I values show the endogen T levels. In males the postrefracter Control I values are significantly lower ($p < 0.01$) than that of during reproductive cycle. The results support the earlier study by Péczely and Kovács, (1998) whereas the endogen plasma T levels are higher in sexually active drakes**.** There is an inverse relationship between changes of endogen T level

and the character of excretion curve of the injected T in males. It may be supposed, that both the retention- and excretion character of T are influenced by the actual endogen androgen levels, but effects of another regulatory factors are also possible.

As a conclusion, the present study supports the idea that the correct sample collection for the non-invasive faecal steroid analysis is a crucial criterion. For an optimal sample collection it is important to have control values, to collect samples always at the same time, to know the frequency of defecation, the passage time and seasonal and sexual differences as well.

References

BICZÓ,A., TARCSAI,G., KELEMEN,K., PÉCZELY,P. (2000): Relationship between faecal sexual steroid content and intensity of display behaviour of male great bustard. (*Otis tarda) VII. International Symposium on Avian Endocrinology 11,11*

BUSSO JM, PONZIO M F, DABBENE V, DE CUNEO, M F, RUIZ R D (2004):Assessment of urine and fecal testosterone metabolite excretion in *Chinchilla lanigera* males. *Anim. Reprod Sci* **86**: 339-351.

DANSKY L.M., HILL, F. W. (1952): Application of the chromic oxide indicator method to balance studies with growing chickens. *J. Nutr*. **47(3)**:449-59.

DENBOW M (2000): Gastrointestinal Anatomy and Physiology Chapter 12. In Whittow, C. G (ed.) *Sturkie's Avian Physiology* pp. 299-325.

FRIGERIO, D., E. MÖSTL & K. KOTRSCHAL. (2001):. Excreted metabolites of gonadal steroid hormones and corticosterone in greylag geese (*Anser anser*) from hatching to fledging. *Gen. Comp. Endocrinol.* **124:** 246–255.

GOYMANN, W., E. MÖSTL & E. GWINNER. (2002): Non-invasive methods to measureandrogen metabolites in excrements of European stonechats, *Saxicola torquata rubicola*. *Gen. Comp. Endocrinol*. **129:** 80–87.

GROSS T S (1992): Development and use of faecal steroid analyses in several carnivore species. In W. Scaftenaar, R. M., Buiter and S. J. Dielman (eds) *The First International Symposium on Faecal Steroid Monitoring in Zoo Animals*, Rotterdam, 62-67.

HIRSCHENHAUSER, K., MÖSTL, E., PÉCZELY,P., WALLNER, B., KOTRSCHAL, K. (2000) Seasonal relationships between plasma and fecal testosterone in response to GnRH in domestic ganders. *Gen.Comp.Endocr.*, **118**: 262-272.

HURST,R.O., KUKSIS,A. AND BENDELL,J.F. (1957): The separation of oestrogens from avian droppings *Can.J.Biochem.Phasiol* **35**: 637-640.

JALLAGEAS, M. (1975): Interactions reciproques testo-thyroidiennes chez le Canard male. Indcidenés sur les cycles endocriniens annuels. *These*, Université de Montepelier, France.

KELEMEN, K, PÉCZELY, P, SZŐKE, ZS, LADJÁNSZKY, V (2003):A comparative methodical study of the fecal steroid analysis on birds: looking for a valid method of testosterone determination. *Acta Biol. Hung.* **54.(3-4):** 285-98.

MILLER, W.M., HOBBS, N.T., SOUSA, M.C. (1991) Detectin stress responses in Rocky Mountain Bighorn sheep (*Ovis canadensis canadensis*) reliability of cortisol concentrations in urine and feces. *Can.J.Zool.*, **69**: 15-24.

NAKAGAWA, S., E. MÖSTL & J.R. WAAS. (2003): Validation of an enzyme immunoassay to measure faecal glucocorticoid metabolites from Adelie penguins (*Pygoscelis adeliae*):a non-invasive tool for estimating stress? *Polar Biol.* **26:** 491–493.

PALME R, RETTENBACHER, S., HACKL, R. GHAREEB, K., MÖSTL, E. (2004): Nonivasive evaluation of adrenal activity in chicken *Proc. 8th International Symposium on Avian Endocrinology*, Phoenix AZ, USA, P55.

PALME, R., FISCHER, P., SCHILDORFER, H., ISMAIL, M.N. (1996): Excretion of infused 14C steroid hormones via faeces and urine in domestick livestock. *Anim.Reprod.Sci*., **43**: 43-63.

SCHWARZENBERGER, F., MÖSTL, E., PALME, R., BAMBERG, E., (1996) Faecal steroid analysis for non-invasive monitoring of reproductive status in farm, wild and Zoo animals. *Anim.Reprod.Sci.,* **42**: 513-526.

SHIRES, A., THOMPSON J.R, TURNER, B.V., KENNEDY, P.M, GOH, Y. K. (1987): Rate of passage of corn-canola meal and corn-soybean meal diets through the gastrointestinal tract of broiler and white leghorn chickens. *Poult. Sci.* **66**: 289-298.
STURKIE, P.D. (1976) *Avian Physiology*, 3.ed., Springer Verlag, New York Heidelberg Berlin pp.186-195.
TELL, L.A. (1997): Excretion and metabolic fate of radiolabeled estradiol and testosterone in the cockatiel (*Nymphicus hollandicus*). *Zoo Biol*. **16:** 505–518.
WASSER SK, HUNT KE. (2005): Noninvasive measures of reproductive function and disturbace in barred owl, great horned owl, and northern spotted owl *Ann. N.Y. Acad. Sci.* **1046**: 1-29.