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Faecal steroid analysis for non-invasive monitoring of reproductive status in farm, wild and zoo animals

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Abstract

Non-invasive faecal oestrogen and progesterone metabolite evaluations are well established approaches for monitoring reproductive function in a variety of mammalian species. The route of excretion of steroid hormone metabolites varies considerably among species, and also between steroids within the same species. Steroid concentrations in faeces exhibit a similar pattern to those in plasma, but have a lag time, which depending upon the species, can be from 12 h to more than 2 days. Faecal steroid metabolites in mammals are mainly unconjugated compounds. Faecal oestrogens consist predominantly of oestrone and/or oestradiol-17α or -17β. Therefore, specific oestrogen antibodies or antibodies against total oestrogens can be used for their determination. Progesterone is metabolised to several 5α- or 5β-reduced pregnanediones and hydroxylated pregnanes prior to its faecal excretion. Therefore, relevant antibodies for their determination show considerable cross-reactivities with several pregnane metabolites, whereas specific progesterone antibodies are less suitable. Faecal oestrogen evaluations have been used as reliable indicators of pregnancy in several ungulate and some primate species. They have also been used to determine the preovulatory period in carnivores, corpus luteum activity in New World primates, and to diagnose cryptorchidism in horses. Faecal progesterone metabolite analysis has been successfully used for monitoring corpus luteum function and pregnancy, abortion, seasonality and treatment therapies in an ever expanding list of species.

Keywords: Faecal steroids; Non-invasive monitoring; Oestrogens; Progesterone metabolites; Reproductive hormones

1. Introduction

There is a critical need to develop strategies to assess accurately the endocrine status of wild- and/or zoo animals. The determination of the reproductive status is one of the

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most important factors for effective management, and efforts to use assisted reproductive techniques (artificial insemination, in vitro fertilisation and/or embryo transfer) depend on the knowledge of the basic reproductive physiology of a given species. Artificial insemination in particular, for which the exact timing of insemination is the major limiting factor, would be much easier if oestrus could be determined.

For a successful investigation of reproductive-endocrine relationships, the collection of repeated samples for hormonal evaluations is necessary. However, in most non-domesticated species, repeated blood sampling is not possible and, therefore, non-invasive urinary and faecal steroid metabolite evaluations are used. Although urine and faecal samples can be collected for assessing the reproductive status in captive exotic animals, difficulties in urine collection limit their use for the investigation in free-ranging animals. Therefore faecal samples are the most practicable choice for this purpose.

Non-invasive reproductive monitoring in non-domesticated species was originally developed using oestrogen determination in urine samples (for literature see Lasley and Kirkpatrick, 1991). Aside from reports on faecal steroid evaluations for the sex determination in monomorphic birds (Bercovitz et al., 1978), the first reports on faecal steroid analysis described oestrogen determination in pregnant women (Adlerkreutz and Martin, 1976) and in farm animals (Möstl et al., 1983; Möstl et al., 1984; Bamberg et al., 1984; Bamberg et al., 1986; Choi, 1987; Choi et al., 1985; Choi et al., 1987). Immuno-assays cope with the complex faecal sample matrix, thus making faecal steroid analysis practicable. This opened a new analytical dimension for the assessment of reproductive status. Faecal steroid analysis is now commonly used and accepted as a diagnostic tool and as a means to study the fundamental reproductive endocrinology in farm, wild and zoo animals. Pregnancy diagnosis through faecal steroid analysis is already a standard procedure for mares (Palme et al., 1989), black rhinoceroses and okapis (F. Schwarzenberger, unpublished observations, 1993).

Faecal steroid assays have mainly been used to study female reproduction and provide information regarding the oestrous cycle, pregnancy, abortion, puberty, reproductive behaviour, seasonality and the monitoring of treatment therapies. Due to the fact that the male germ cell production and hormonal secretion are less tightly coupled than those of the female (Lasley and Kirkpatrick, 1991), there are few reports describing non-invasive faecal steroid analysis in males. Faecal testosterone metabolites were analysed to study seasonality and social dominance in male muskoxen (Flood et al., 1992), and faecal oestrogen and testosterone analysis was used to determine the sex of adult giant pandas (Kubokawa et al., 1992). Faecal androgen excretion patterns have been investigated during puberty and before and after castration in the domestic boar (Palme and Möstl, 1993), and faecal oestrogen analysis was used for diagnosing cryptorchidism in horses (Palme et al., 1994).

2. Administration of radioactively labelled steroids

The administration of radioactively labelled steroid hormones (progesterone, oestrogens, androgens, cortisol) in domestic and non-domestic species has been used to determine the route of excretion, the time course of excretion and the type of metabolic

endproducts of steroids in urine and faeces (Table 1). Radioinfusion studies have contributed to our understanding of the enterohepatic circulation of steroid hormones and have indicated that the excretion of steroids into the gut is mainly through bile, but they have also shown that a small proportion of the circulating steroids is secreted through the mucosa of the large intestine (Shille et al., 1990). Furthermore, radioinfusion studies have provided useful results in testing the efficiency of currently used extraction methods (Palme et al., 1996), and have shown that steroids might be unevenly distributed in the faecal balls of horses, swine and elephants (Palme et al., 1996; Wasser et al., 1996).

The route of excretion varies considerably among the species, as well as between steroids within the same species (Table 1). The delay time between the circulation of steroids in plasma and their appearance in urine samples is rather short (less than 5 h), but faecal steroid metabolites have an appreciable lag time which approximately correlates with the time necessary for the intestinal passage of bile to the rectum (Palme et al., 1996). The lag time of faecal steroids is about 12–24 h in ruminants and about 24 to over 48 h in animals which are hindgut fermenters (horse, pig, rhino, elephant, primates; Table 1). In non-ruminant species, studies on the rate of passage of food particles can provide an estimate of the lag time of faecal steroid metabolites. The food passage in ruminants is longer than the passage time of faecal steroids, since bile enters the intestine after the foregut. The lag time is affected by the digestibility of the forage, which influences the rate of passage of digesta. However, the final amount of radioactivity excreted in the faeces of nutritionally restricted and supplemented groups of sheep was similar (Adams et al., 1994).

After their administration, radioactively labelled steroids in the plasma are rapidly conjugated and excreted into the bile and urine. They are de-conjugated in the intestine and, in most species, voided faeces contain a higher percentage of free than conjugated steroids (Table 1). The type of metabolic endproducts of steroids in the faeces were identified by radioinfusion studies (for literature see Table 1) and studies using chromatographic and immuno-assay techniques (Bamberg et al., 1986; Kirkpatrick et al., 1991; Schwarzenberger et al., 1991; Schwarzenberger et al., 1992; Schwarzenberger et al., 1993a; Schwarzenberger et al., 1993b; Schwarzenberger et al., 1995; Schwarzenberger et al., 1996a; Schwarzenberger et al., 1996b; Heistermann et al., 1993; Möstl et al., 1993; Palme and Möstl, 1993; Palme and Möstl, 1993; Shideler et al., 1993a; Shideler et al., 1993b; Brown et al., 1994; Wasser et al., 1994; Wasser et al., 1996; Graham et al., 1995). In essence, these studies indicated that faecal oestrogens consist mainly of oestrone, oestradiol-17α and -17β. Oestrogens are endproducts of steroid metabolism and, therefore, the compounds in plasma and faeces are similar. In contrast, progesterone is extensively metabolised prior to its faecal excretion and several studies indicated that its faecal metabolites consist of several 5α- and 5β-pregnanes (pregnanediones and mono- and dihydroxylated pregnanes; Schwarzenberger et al., 1991; Schwarzenberger et al., 1992; Schwarzenberger et al., 1993a; Schwarzenberger et al., 1993b; Schwarzenberger et al., 1995; Schwarzenberger et al., 1996a; Schwarzenberger et al., 1996b; Heistermann et al., 1993; Möstl et al., 1993; Shideler et al., 1993a; Shideler et al., 1993b; Brown et al., 1994; Wasser et al., 1994; Wasser et al., 1996; Graham et al., 1995).

3. Faecal oestrogen determination

In general, in most studies on faecal oestrogen determinations, unconjugated oestrone and/or oestradiol-17α or -17β were measured using specific oestrogen antibodies or antibodies against total unconjugated oestrogens. These methods proved to be reliable indicators for pregnancy diagnosis in those species in which the foeto-placental unit is the source of large quantities of oestrogens. Faecal oestrogen evaluations for pregnancy determination were used in domesticated hoof stock (Möstl et al., 1983; Möstl et al., 1984; Bamberg et al., 1984; Bamberg et al., 1986; Choi, 1987; Choi et al., 1985; Choi et al., 1987; Sist et al., 1987; Palme et al., 1989; Desaulniers et al., 1989; Bamberg and Schwarzenberger, 1990; Schwarzenberger et al., 1991; Holtz, 1992), and in feral mares (Kirkpatrick et al., 1990; Kirkpatrick et al., 1991; Lucas et al., 1991). Consecutively, faecal oestrogen analysis was applied to non-domesticated ungulate and primate species and successful pregnancy diagnosis was reported in the red buffalo, yak, Grevy's zebra and Nubian ibex (Safar-Hermann et al., 1987), in the muskox (Desaulniers et al., 1989), in the caribou (Messier et al., 1990), in the gorilla, orang-utan, Mhorr gazelle, Przewalski's mare and Malaysian tapir (Bamberg et al., 1991), in the yellow baboon (Wasser et al., 1991), in the sable antelope (Chapeau et al., 1993), in the bison (Kirkpatrick et al., 1993), and in the cynomologus monkey (Shideler et al., 1993a). However, faecal oestrogen determination for the purpose of pregnancy diagnosis proved to be less effective in the moose (Monfort et al., 1993), and unsuccessful in the Nile hippopotamus (Safar-Hermann et al., 1987), the black rhinoceros, the giraffe and the okapi (F. Schwarzenberger, unpublished observations, 1993).

Although some studies reported the determination of the preovulatory oestrogen peak in mares, these methods proved to be less successful as compared to pregnancy determination; peak concentrations of faecal oestrone conjugates during the follicular phase were very low (less than 2.0 $ng\,g^{-1}$; Sist et al., 1987; Barkhuff et al., 1993). Determination of the preovulatory oestrogen peak in faecal samples was unsuccessful in the moose (Monfort et al., 1993) and in cows (F. Schwarzenberger, unpublished observations, 1996). Possible reasons for the failure to detect the preovulatory oestrogen peak in ungulates are the low oestrogen concentrations in the plasma (only $pg\,ml^{-1}$), and the fact that the main route of oestrogen excretion in some species is via urine (Palme et al., 1996). Therefore, assay blanks interfere with the measurement of the low faecal concentrations. For a reliable analysis of the preovulatory oestrogen peak in faecal samples, more rigorous extraction and clean up procedures of the samples and sensitive assays would be necessary.

In contrast to those in ungulates, oestrogens in carnivores are predominantly excreted into the faeces (Shille et al., 1984; Shille et al., 1990; Gross, 1992; Brown et al., 1994) and, therefore, the determination of the preovulatory oestrogen surge in feline and canine species proved to be a reliable indicator of ovulation (Gross, 1992; Brown et al., 1994; Brown et al., 1995; Graham et al., 1995; Czekala et al., 1994; Wasser et al., 1995). In addition, ovulation in the domestic dog is characterised by a preovulatory surge of faecal androgens (E. Möstl, unpublished observations, 1996). A preovulatory faecal oestrogen peak was reported in some primate species (cynomologus monkey, Shideler et al., 1993a; Shideler et al., 1993b; yellow baboons, Wasser et al., 1991). In

New World primates, however, faecal oestrogen determination is rather a measure of the luteal phase than of the preovulatory oestrogen peak. New World primates have maximal blood and urinary levels of oestrogens during the luteal phase of their oestrous cycle, when oestrogen values are 10-fold higher than during the follicular phase. Studies in Goeldi's monkeys, marmorset and tamarin species proved that this unusual oestrogen secretory pattern of the corpus luteum is also reflected in faecal oestrogen values (Heistermann et al., 1993; Pryce et al., 1994; Ziegler et al., 1996).

Faecal oestrogen determination in the male can be applied to those species, which produce high amounts of oestrogens in the testis, like the stallion and the boar. Faecal oestrogen values of mature stallions can reach values comparable to those of pregnant mares (Bamberg et al., 1986) and faecal oestrogen determination has been shown to be a reliable indicator of cryptorchidism in horses (Palme et al., 1994).

4. Faecal progesterone metabolites

Faecal progesterone metabolite analysis has been successfully used for monitoring corpus luteum function and pregnancy, abortion, puberty and seasonality in an ever expanding list of species including primates (Wasser et al., 1988; Wasser et al., 1991; Wasser et al., 1993; Wasser et al., 1994; Heistermann et al., 1993; Shideler et al., 1993a; Shideler et al., 1993b; Stavisky et al., 1995; Ziegler et al., 1996); bovine species (Desaulniers et al., 1989; Bamberg and Schwarzenberger, 1990; Kirkpatrick et al., 1993; Larter et al., 1993; Larter et al., 1994; Matsuda-Motomura et al., 1995; Schwarzenberger et al., 1996a; mares (Bamberg and Schwarzenberger, 1990; Schwarzenberger et al., 1991; Schwarzenberger et al., 1992; Kirkpatrick et al., 1991; Barkhuff et al., 1993); swine (Bamberg and Schwarzenberger, 1990; Sanders et al., 1994; Hulten et al., 1995); caribous (Messier et al., 1990); rhinoceroses (Bamberg and Schwarzenberger, 1990; Schwarzenberger et al., 1993a; Schwarzenberger and Walzer, 1995; Schwarzenberger et al., 1994; Schwarzenberger et al., 1996b; Berkeley, 1994); felids (Gross, 1992; Möstl et al., 1993; Czekala et al., 1994; Brown et al., 1994; Brown et al., 1995; Graham et al., 1995); okapis (Schwarzenberger et al., 1993b); moose (Monfort et al., 1993; Schwartz et al., 1995); mink (Möstl et al., 1993); vicunas (Schwarzenberger et al., 1995); oryx (Shaw et al., 1995); wolves (Wasser et al., 1995) and elephants (Wasser et al., 1996). In addition, faecal progesterone metabolites were used to monitor the success of reproductive treatment therapies (Kirkpatrick et al., 1995; Schwarzenberger and Walzer, 1995).

It has been assumed that changes in diet and fluctuating water content between faecal samples influence faecal steroid concentrations and thus make indexing to a compound excreted at a constant rate like creatinine in urine, necessary. Studies specifically addressing this question (Shideler et al., 1993a; Wasser et al., 1993) concluded that indexing of faecal steroids is not necessary. In some species like carnivores and elephants (Brown et al., 1994, Brown et al., 1995; Wasser et al., 1996), however, lyophilisation of faecal samples prior to analysis and expressing steroid concentration per gram of dry faeces is advantageous; after desiccation, undigested bones or plant material can be removed. We conclude from our long term experience with several herbivorous species kept in different zoos and thus not on standardised diets, that in

Table 1
Studies on the excretion of radioactively labeled steroids in mammalian species

Species	Administered hormone [a]	% excreted in faeces [b]	Peak excretion (h after administration)	% unconjugated in faeces	Reference
Sheep	E2	74	18	97	Adams et al. (1994)
	P4	77	11.2	99	Palme et al. (1996)
	E1	87	10.8	100	
	T	44	13.4	98	
	C	28	11.4	95	
Horse	P	75	23.8	97	Palme et al. (1996)
	E1	2	20.4	n.d. [c]	
	T	28	22.2	82	
	C	41	26	77	
Pig	P	34	57–113	96	Palme et al. (1996)
	E1	4	20–80	99	
	T	14	24–49	98	
	C	7	21–100	94	
Domestic cat	E2	98	24–48	22	Shille et al. (1984)
	E2	97	24–72	50	Shille et al. (1990)
	P4	97	12–24	22	Brown et al. (1994)
	E2	97	11–21	55	
	C	86	22.3	13	Graham and Brown (1996)
Siberian polecat	E2	93	n.d.	61	Gross (1992)
	P4	93	n.d.	66	
	T	93	n.d.	70	
North American river otter	E2	89	n.d.	71	Gross (1992)
	P4	89	n.d.	46	
	T	89	n.d.	63	
White rhino	E2 and P4 [d]	59	24–48	92	Hindle and Hodges (1990)
Eld's deer	T	24	12–24	n.d.	Monfort et al. (1995)
African elephant	P4	55	48–50	80	Wasser et al. (1996)
	E2	5	48–50	80	
Slow lori	E2	93	24–48	59	Perez et al. (1988)
Ring tailed lemur	E2	16	24–48	100	Perez et al. (1988)
Cotton top tamarin	E1	43	24–48	11	Ziegler et al. (1989)
	E2	13	24–48	15	
	P4	95	24–48	65	
Macaque	E2	45	24–56	100	Shideler et al. (1993b)
	P4	58	32–56	100	
Yellow baboon	DHEA	14	45	n.d.	Wasser et al. (1993)
	E2	10	37	80	Wasser et al. (1994)
	P4	40	37	80	

these species, desiccation of faecal samples is not necessary for studying faecal progesterone metabolites (Schwarzenberger and Walzer, 1995; Schwarzenberger et al., 1991; Schwarzenberger et al., 1992; Schwarzenberger et al., 1993a; Schwarzenberger et al., 1993b; Schwarzenberger et al., 1994; Schwarzenberger et al., 1995; Schwarzenberger et al., 1996a; Schwarzenberger et al., 1996b). Moreover, Wasser et al. (1993); Wasser et al. (1994) concluded that considerable seasonal changes in the diet of free-ranging animals, like baboons, do not significantly affect the quantification of faecal steroids. Additionally, Ziegler et al. (1996) concluded that fluid removal from the faeces does not effectively alter steroid profiles, since steroid concentrations between frozen and lyophilised faecal samples are highly correlated.

Several studies comparing plasma and faecal steroid values during the oestrous cycle indicated a delay of faecal concentrations compared to that in plasma. The delay time was about 2 days in mares (Schwarzenberger et al., 1992), in primate species (*Macaca fascicularis*, Shideler et al., 1993a; yellow baboons, Wasser et al., 1994; callitrichids, Ziegler et al., 1996) and in black rhinoceroses (Berkeley, 1994); it was 12 h between milk progesterone and its faecal metabolites in cows (Schwarzenberger et al., 1996a). Faecal progesterone metabolites after parturition did not decrease to basal values until 3–4 days (Schwarzenberger et al., 1991; Schwarzenberger et al., 1993a; Schwarzenberger et al., 1996b). The difference in the excretion time of steroids between the oestrous cycle and post partum is probably caused by the very high concentrations present during pregnancy and by the enterohepatic circulation, which retards the excretion.

Studies using chromatographic techniques in combination with immuno-assays indicated that progesterone is metabolised to several 5α- and 5β-reduced pregnanes (pregnanediones and mono- and dihydroxylated pregnanes) prior to its excretion into the faeces (Kirkpatrick et al., 1991; Schwarzenberger et al., 1991; Schwarzenberger et al., 1992; Schwarzenberger et al., 1993a; Schwarzenberger et al., 1993b; Schwarzenberger et al., 1995; Schwarzenberger et al., 1996a; Schwarzenberger et al., 1996b; Heistermann et al., 1993; Möstl et al., 1993; Shideler et al., 1993a; Brown et al., 1994; Wasser et al., 1994; Graham et al., 1995). Unmetabolised progesterone is barely present, if at all, in the faecal samples. Faecal pregnanes have either a 20-oxo, a 20α-OH (OH = hydroxyl) or a 20β-OH group. The number of metabolites and the series (5α- or 5β-pregnanes), to which the faecal pregnanes belong varies between species, i.e. faecal pregnanes of mares and rhinos belong to the 5α-series, whereas those of okapis belong to the 5β- and those of cows to both the 5α- and 5β-pregnane series (Schwarzenberger et al., 1991; Schwarzenberger et al., 1992; Schwarzenberger et al., 1993b; Schwarzenberger et al., 1996a).

Notes to Table 1:

[a] P4 = progesterone; E1 = oestrone; E2 = estradiol-17β; T = Testosterone; C = Cortisol; DHEA = Dehydroepiandrosterone.

[b] The remaining proportion to 100% was recovered from urine.

[c] n.d. = not determined.

[d] Combined application and determination of faecal E2 and P4 metabolites.

The large number of progesterone metabolites present in the faeces explain why several assay systems can be used for their measurement. In general, antibodies for the analysis of faecal progesterone metabolites identify a certain C20 position (20-oxo, 20α-OH, 20β-OH) of the pregnane molecule, i.e. progesterone antibodies cross-react with 20-oxo-pregnanes. Since progesterone is not present in the faecal samples, progesterone assays used to measure faecal steroid values did not report progesterone, but values of cross-reacting 5α-and 5β-reduced pregnane metabolites containing a 20-oxo-group (Desaulniers et al., 1989; Wasser et al., 1988; Wasser et al., 1991; Wasser et al., 1993; Wasser et al., 1994; Wasser et al., 1995; Wasser et al., 1996; Messier et al., 1990; Kirkpatrick et al., 1991; Kirkpatrick et al., 1993; Gross, 1992; Barkhuff et al., 1993; Larter et al., 1993; Larter et al., 1994; Monfort et al., 1993; Möstl et al., 1993; Schwarzenberger and Walzer, 1995; Schwarzenberger et al., 1993a; Schwarzenberger et al., 1995; Schwarzenberger et al., 1996a; Schwarzenberger et al., 1996b; Shideler et al., 1993a; Shideler et al., 1993b; Brown et al., 1994; Brown et al., 1995; Czekala et al., 1994; Berkeley, 1994; Sanders et al., 1994; Graham et al., 1995; Hulten et al., 1995; Matsuda-Motomura et al., 1995; Schwartz et al., 1995; Stavisky et al., 1995; Ziegler et al., 1996). Since different extraction methods and assays with different cross-reactivities were used, values between these studies are difficult to compare. Cross-reactivities with pregnanes could give indications about comparability of results and therefore should be published in any study on faecal progesterone metabolite analysis.

Schwarzenberger et al. (1996a) and Schwarzenberger et al. (1996b) compared 3 enzyme-immunoassays with different degrees of specificity and the results indicated that the most suitable assay(s) show considerable cross-reactivities with several 20-oxo-pregnanes. The authors concluded that the combined measurement of pregnane metabolites in an assay concurrently detecting several metabolites containing a similar C20 group of the steroid is superior to one that only detects a limited number of progesterone metabolites. Specific progesterone antibodies are less suitable, since they quantitatively underestimate the actual amount of faecal 20-oxo-pregnanes considerably. The authors strongly suggest that antibodies for faecal steroid analysis should be raised against 5α- or 5β-pregnane immunogens conjugated at the 3-position. These antibodies have high cross-reactivities with pregnanes sharing a similar C20 group. For this reason the assays are termed group-specific. They quantify total immunoreactive pregnane metabolites and can be generally applied to a wide range of species. The improved specificity for faecal pregnane metabolites improves sensitivity, accuracy, reliability and practicability of the assay.

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