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Faecal Oestrogens and Progesterone Metabolites in Mares of Different Breeds During the Last Trimester of Pregnancy

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Contents

Non-invasive pregnancy diagnosis in mares by measuring faecal oestrogens has been performed over years with great accuracy. However, results have indicated breed-related differences in the amount of excreted steroids during late pregnancy. Therefore faecal samples were collected during the last 4 months of pregnancy of Thoroughbred (n = 10), New Forest pony (n = 9), Shetland pony (n = 10) and Iceland pony mares (n = 11). Concentrations of oestrogens, 20α-hydroxy- and 20-oxopregnanes were measured using enzyme immunoassays. Breed differences concerning both levels (though significant only in case of oestrogens) and time course of measured steroids were observed. There was a highly significant time effect ($p < 0.00001$) and an interaction between time and breeds ($p < 0.02$) for all steroids measured, suggesting that the time effect differs for different breeds. Oestrogen concentrations showed a decrease towards parturition, whereas in 20α-hydroxy- and 20-oxopregnane levels a pronounced increase was found 2 and 1 months, respectively, before parturition. A breed effect was only significant ($p = 0.001$) when comparing oestrogen concentrations and was mainly due to Iceland ponies, which had the lowest concentrations especially during the last 2 months of pregnancy. An almost significant ($p = 0.06$) breed effect was found for 20-oxopregnanes. In Iceland mares an additional increase in faecal pregnane content was already observed earlier, reaching maximum levels before the 60th day ante-partum (a.p.), followed by a decrease until the 30th day a.p. The ratio of 20-oxopregnanes to oestrogens in the samples was significantly higher ($p < 0.006$) in Iceland ponies in comparison with any other breed throughout all months before parturition. The breed differences observed in the amounts of oestrogens and/or progestagens present during late pregnancy may demonstrate micro-evolutionary changes in the endocrine system of a species.

Introduction

Oestrogens and progestagens are produced by the equine placenta from about day 50 onwards. Whereas oestrogen concentrations peak between days 150 and 270 and decline until parturition, progestagens steadily increase with advancing gestation and reach maximum levels in the month before parturition (Ginther 1992; Hoffmann et al. 1996). As both groups of steroids (although to a different extent) are excreted via urine and faeces (Palme et al. 1996), these changes are reflected in the concentrations of metabolites in the excreta (Lasley et al. 1990; Schwarzenberger et al. 1991; Schuler 1998).

Pregnancy diagnosis, which is routinely carried out by rectal palpation and/or ultrasonography, may for different reasons (Schuler 1998) rely on non-invasive methods, such as determinations of steroid hormones (or their metabolites) in urine (Lasley et al. 1990; Schuler 1998) or faeces (Möstl et al. 1983). Such a confirmation of pregnancy in mares by measuring faecal oestrogens (and progesterone metabolites) has been performed over the years with great accuracy (Palme et al. 1989). However, the results have indicated breed-related differences in the amount of excreted oestrogens during late pregnancy. This was underlined by Schwarzenberger et al. (1991) who investigated faecal oestrogen and progestagen concentrations in Lipizzan, Thoroughbred and Trotter mares. The later for example showed significantly higher oestrogen concentrations than the other two. Therefore faecal samples were collected during the last trimester of pregnancy of four different breeds (largely differing in body mass) to evaluate patterns of oestrogen and progestagen excretion. The inclusion of Iceland ponies, which have been bred in isolation for centuries, may also demonstrate micro-evolutionary changes in the endocrine system of a species.

Materials and Methods

Animals and sampling

Thoroughbred (group Tb; n = 10), New Forest (Nf; n = 9), Shetland pony (Sh; n = 10) and Iceland pony mares (Ic, n = 11), all kept by private owners, were used for this experiment. The mares were between 3.5 and 18 years old. Both primi- and multiparous (up to 13th) mares were included within each breed group. As the mares were kept by private owners, the type of housing and the feeding regimen could not be standardised. With the exception of group Ic, which were maintained in outdoor paddocks, the mares were kept in individual boxes, but were allowed to range free and graze at different times. For all mares water was available *ad libitum*. Their diet consisted of grass, supplemented with hay, oat, carrots and a vitamin/mineral mixture. Mares in groups Sh and Ic were kept in groups together with a stallion for between 7 and 20 days and therefore the actual pregnancy length could not be evaluated. In the other groups (Tb and Nf) this was 335 ± 5 and 347 ± 9 days, respectively.

Faecal samples were collected every second week starting around day 220 of the pregnancy until parturition. They were frozen within 4 h and stored at −24°C until analysis.

Analysis of faecal steroids

Portions (0.5 g) of wet faeces of each sample were utilized for the determination of oestrogens or progestagen metabolites. Unconjugated oestrogens were

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determined by an enzyme immunoassay (EIA) following extraction with KOH–chloroform–*n*-hexane as described by Palme and Möstl (1994). After extraction of another 0.5 g of each faecal sample with methanol/water, the amounts of 20α-hydroxy- and 20-oxopregnanes were measured by EIAs as previously described (20α-hydroxyprogesterone- and 5β-20-one-EIA, respectively; Schwarzenberger et al. 1991, 1996). Intra- and interassay coefficients of variations were 12.4 and 14.3% (oestrogens), 12.5 and 15.8% (20α-hydroxypregnanes) and 9.6 and 14.6% (20-oxopregnanes), respectively. The sensitivity of the EIAs was 0.2; 0.5 and 5.7 pg/well, respectively.

Statistical analysis

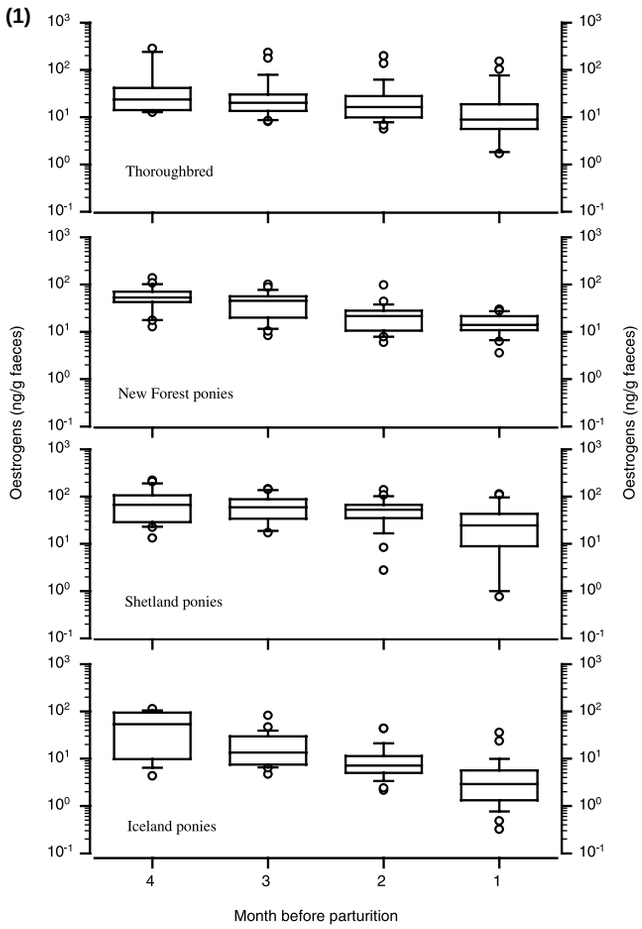
The actual day of parturition was defined as day 0. In each mare, samples were related to this actual date (days before parturition). The samples within each breed were grouped monthly before parturition. Parametric tests were used throughout. Concentrations of the steroids were log $x + 1$ transformed, when they did not meet requirements for normality. To examine a time and breed effect of steroid concentrations a repeated measures analysis of variance (ANOVA) was used with steroid concentrations as the dependent variable, breed as the independent variable and month as the repeated factor. A Newman–Keuls test was used for pairwise *post hoc* comparisons for both factors (time and breed). Furthermore, to examine the monthly changes separately for each breed, simple pairwise comparisons were carried out for the four successive months and therefore the p-values were Bonferroni-corrected to avoid type I error rate (Wright 1992). A strict application of this method was used ($p_{critical}$ divided by the number of tests) although it severely reduced the power of the test (Chandler 1995). Hence the critical value was $\alpha = 0.016$ for these comparisons.

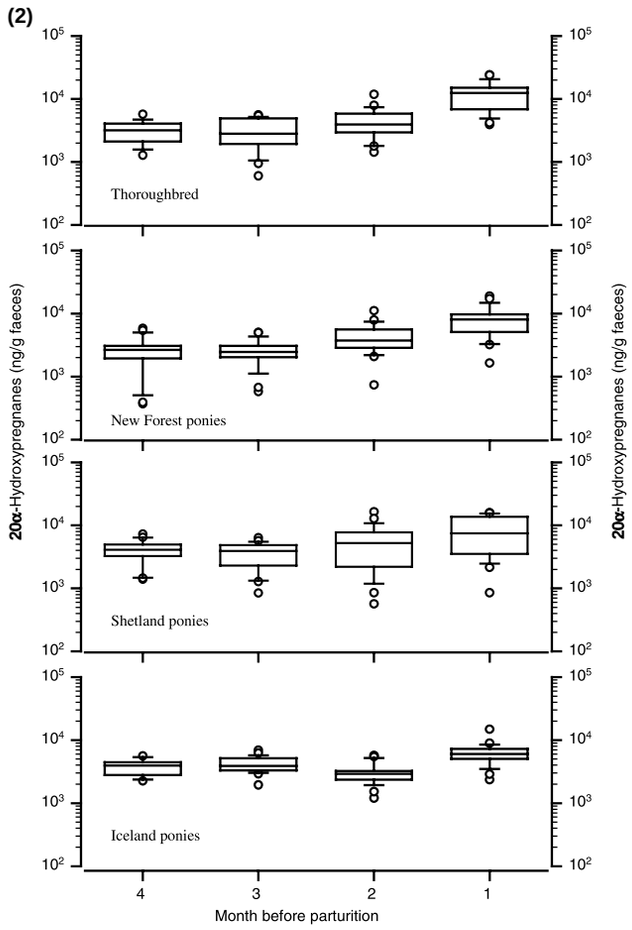
Results

Breed differences concerning both levels and time course of measured steroids were observed. There was a highly significant time effect for all steroids measured (repeated measures ANOVA: for all three steroids $p < 0.00001$; Table 1; Figs 1–3). Pairwise *post hoc* comparisons suggested that for oestrogens there appeared to be a steady but significant decrease over the 4 months (Newman–Keuls test: $p < 0.05$ for all comparisons). For the 20α-hydroxypregnanes this time effect was due to a significant change 2 months before parturition (Newman–Keuls test: $p < 0.05$ comparing months 3 and 4 with months 1 and 2, respectively) and for the 20-oxopregnanes there was a change 1 month ante-partum (a.p.) (Newman–Keuls test: $p < 0.05$ comparing months 2–4 with month 1).

Table 1. Amounts (minimum, maximum and median of samples of all animals of a given breed) of the different groups of measured steroids present in the faeces of Thoroughbred (Tb), New Forest (Nf), Shetland (Sh) and Iceland pony (Ic) mares during the last 4 months of pregnancy

Steroid (ng/g faeces)	Breed		Month 4	Month 3	Month 2	Month 1
Oestrogens	Tb	Min	12.6	8.1	5.6	0.1
	Nf		12.8	8.4	6.0	3.6
	Sh		13.2	17.2	2.8	0.1
	Ic		4.3	3.6	1.2	0.3
	Tb	Max	283.0	232.0	197.0	151.0
	Nf		139.0	101.0	98.0	30.0
	Sh		219.0	146.0	139.0	114.0
	Ic		113.2	82.4	43.6	35.6
	Tb	Med	26.2	20.2	15.8	8.8
	Nf		53.2	45.6	21.8	15.4
	Sh		66.4	59.0	52.8	24.5
	Ic		35.9	13.4	7.1	3.0
20α-hydroxypregnanes	Tb	Min	1275	600	1425	3905
	Nf		370	575	735	3200
	Sh		1395	840	200	850
	Ic		2240	1940	1200	2880
	Tb	Max	5700	5550	7900	23850
	Nf		5800	5000	11100	18850
	Sh		7250	6350	16350	15850
	Ic		5600	6900	5700	10300
	Tb	Med	3205	2803	3760	10000
	Nf		2655	2460	3745	8300
	Sh		4088	3930	5225	7475
	Ic		3590	3910	2920	6050
20-oxopregnanes	Tb	Min	615	525	1200	1445
	Nf		600	830	1330	4755
	Sh		1005	745	120	430
	Ic		5250	5700	915	3675
	Tb	Max	12300	18300	17050	180000
	Nf		9500	8350	15850	31150
	Sh		14800	17500	30000	30000
	Ic		22250	59500	42050	84000
	Tb	Med	2793	2263	4783	21350
	Nf		3325	3110	4965	10600
	Sh		5725	5100	6125	12375
	Ic		10800	13400	5900	14000





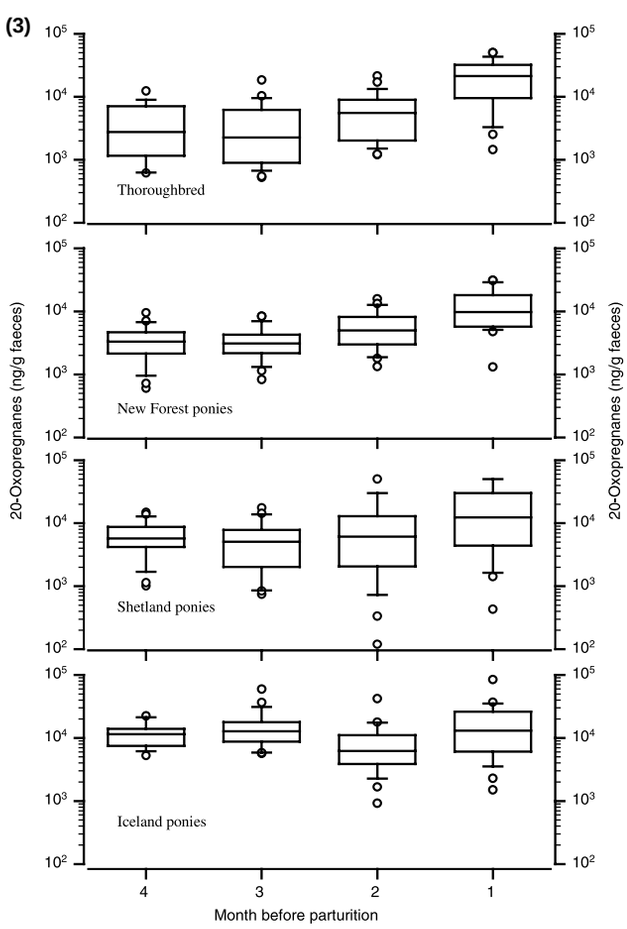


Fig. 1. Concentrations (note the logarithmic scale!) of oestrogens (ng/g faeces) in mares of different breeds during late pregnancy
Fig. 2. Concentrations (note the logarithmic scale!) of 20α-hydroxypregnanes (ng/g faeces) in mares of different breeds during late pregnancy
Fig. 3. Concentrations (note the logarithmic scale!) of 20-oxopregnanes (ng/g faeces) in mares of different breeds during late pregnancy

A significant breed effect was detected only in case of oestrogens (repeated measures ANOVA: $F = 6.7$; d.f. = 3, 147; $p = 0.001$), an almost significant breed effect was detected for 20-oxopregnanes (repeated measures ANOVA: $F = 2.6$; d.f. = 3, 147; $p = 0.06$) but no breed effect was found for 20α-hydroxypregnanes (repeated measures ANOVA: $F = 6.7$; d.f. = 3, 147; $p > 0.5$). *Post hoc* pairwise comparisons suggest that this breed difference was mainly due to breed Ic, having lower oestrogen concentrations than the other breeds (Newman–Keuls test: $p < 0.05$ for all), which seemed most pronounced for the last 2 months of pregnancy (Fig. 1) and Sh having higher levels than Th mares (Newman–Keuls test: $p < 0.05$). Most important, however, there was an interaction between time and breed for all steroids ($p < 0.02$ for all) suggesting the time effect to be different for different breeds. Therefore pairwise comparisons were carried out separately between successive months before parturition for each breed. In the last month ante-partum all breeds showed an increase in pregnane content. Groups Tb, Nf and Ic (the latter only in case of 20α-hydroxypregnanes) had statistically significant ($p < 0.003$) higher values in comparison with the second month ante-partum (Figs 2, 3). In Iceland mares an additional increase of progestagens concentrations was already observed earlier, reaching maximum levels before the 60th day (3rd month) a.p., followed by a decrease until about the 30th day (2nd month) a.p (Figs 2 and 3). During the latter, lower concentrations in comparison with the third month ($p < 0.003$ for 20α-hydroxypregnanes and $p < 0.015$ for 20-oxopregnanes) were found. Interestingly, the ratio of 20-oxopregnanes to oestrogens in the samples was significantly ($p < 0.006$) higher in Iceland ponies in comparison with any other breed throughout pregnancy.

Discussion

Pashen and Allen (1979) demonstrated that oestrogen concentrations in the blood of mares during the second half of the pregnancy depend on the presence of fetal gonads as the major source of C19 precursors. Therefore the rise (peaking between day 150 and day 270) and fall of oestrogen concentrations parallel the enlargement and subsequent regression of the fetal gonads (Ginther 1992). In the blood of pregnant mares it has been well documented that in contrast to oestrogens, the progestagen concentrations steadily increase, reaching peak values during the last month before parturition (Holtan et al. 1991; Ginther 1992).

These changes were well reflected by the concentrations of oestrogens and progestagens (20-oxo- and 20α-hydroxypregnanes) in the faeces of the four different breeds investigated. However, breed differences concerning both levels (though significant only in the case of oestrogens) and time course of measured steroids were observed. This resulted in significantly different amounts of faecal oestrogens and progestagens present at certain months.

During late pregnancy Barnes et al. (1975) also found higher plasma oestrogen concentrations in pony mares in comparison with Thoroughbred mares, but they did not explain or account for the variation. Schwarzenberger et al. (1991) found higher faecal oestrogen concentrations in Trotter mares, than in Lipizzan or Thoroughbred mares during the period between week 12 and week 2 before parturition. As differences of faecal oestrogen concentrations between Lipizzan and Trotter mares were around five-fold, this result indicates a major effect of breed specificity. However, no differences in faecal 20α-progestagen concentrations were found in those breeds. Similar, lower oestrogen concentrations were found in Thoroughbred mares and also in Iceland ponies. In the case of the Iceland ponies these lower levels may be related to the fact that this breed has been bred in isolation for centuries (Greil and Osborne 1970). As the fetus plays an important role in oestrogen production (Ginther 1992), differences of the (relative or absolute) mass of fetal gonadal tissue between breeds may result in higher or lower oestrogen levels.

Different diets and thus fibre contents could be excluded as a source of the observed differences between breeds as the three different groups of steroids were measured with distinct levels and time courses. For example, in Iceland pony mares in contrast to the other breeds lower oestrogen concentrations were measured during the 2 months before parturition, but 20-oxopregnane levels were not different. In addition the 20-oxopregnane : oestrogen ratio was highest in Iceland ponies throughout the whole 4 months. For the same reasons different body mass and bulk of faeces, which might 'dilute' the steroids produced, was not responsible for the observed differences. Or, if it were so in one case (e.g. high amounts of oestrogens in group S) the differences would have been masked in another case (e.g. progestagens during that period, which did not differ).

It has been demonstrated (Holtan et al. 1991; Ginther 1992) that a complex mixture of type and quantity of progestagens is present in the blood of mares especially during late pregnancy. In the faeces Palme et al. (1997) detected several reduced metabolites (pregnanes) derived from infused ^{14}C-labelled progesterone. As steroids in animals are metabolized and partly excreted into the gut, each metabolite present in the blood of pregnant mares may itself serve as a precursor of others, thus leading to an even more complex mixture in the faeces. One must be aware that results of a determination of steroid metabolites in the faeces by EIAs largely depends upon the cross-reactions of the antibody used (Palme et al. 1997) and thus will not yield absolute values. As the EIA used for measuring 20α-hydroxy-progestagens shows lower cross-reactivities with pregnanes, than the one for 20-oxoprogestagens, the generally higher values of the later are not necessarily a reflection of higher absolute concentrations. Therefore, especially in the case of the progestagens measured in the present study, changes in the concentration of pregnanes during pregnancy reflect changes in quantity and/or type of formed metabolites.

Breed differences observed between the amounts of oestrogens and/or progestagens present during late gestation may demonstrate micro-evolutionary changes in the endocrine system of a species. If they are attributed to differences in the production of these steroids, the metabolism or the excretion needs to be further elucidated.

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