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Effect of ACTH (tetracosactide) on steroid hormone levels in the mare Part A: Effect in intact normal mares and mares with possible estrous related behavioral abnormalities

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Abstract

Ovariectomized mares and mares with inactive ovaries may show signs of estrus. The reason behind this phenomenon is not clear; however, steroid hormones of adrenal origin have been suggested. Moreover, aberrant adrenal hormone production has been implied as a reason why some intact mares may change behavior. In the present study, the effect of ACTH on plasma levels of cortisol, progesterone, androstenedione and testosterone was investigated in intact mares with normal estrous behavior ('controls', $n = 5$) and intact mares that according to their owners showed deviant estrous behavior ('problem' mares, $n = 7$). Blood samples were collected hourly from 12:00 h until 14:00 h the following day (half-hourly between 14:00 and 17:00 h) on two occasions (at two estruses), with saline or ACTH treatment (tetracosactide) at 14:00 h (saline treatment day or ACTH treatment day). ACTH treatment caused a significant increase in plasma levels of cortisol, progesterone, androstenedione and testosterone in all mares ($P < 0.05$). An overall significant difference in cortisol response to ACTH was found ($P < 0.05$), with 'problem' mares showing a significantly lower increase in cortisol levels 30 min to 3 h post ACTH treatment ($P < 0.001$). The 'problem' mares also

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showed a significantly higher increase than controls in progesterone levels in the same time period ($P < 0.05$). The reason for the reduced adreno-cortical reactivity, with a low cortisol response to the ACTH treatment, in the 'problem' mares is unknown, but may indicate a difference in adrenal function as compared to control mares.

Keywords: Mare; Estrous behavior; ACTH; Adrenal gland; Steroid hormones; Nymphomania; Adreno-cortical reactivity

1. Introduction

The hormonal mechanisms underlying estrous behavior in the mare are still not fully understood. It seems that, unlike most mammals, high estrogen levels of ovarian origin are not required for the mare to show symptoms of estrus. As many as 90% of ovariectomized mares and 80% of seasonally anovulatory mares have been shown to display at least 1 day of full estrous behavior during a 15 day period (Asa et al., 1980a). In another study, 35% of mares (8 of 23) demonstrated estrous signs after ovariectomy (Hooper et al., 1993). Asa et al. (1980b) showed that suppression of the adrenal gland by dexamethasone administration reduced the sexual behavior of ovariectomized mares, suggesting that the adrenal gland plays a part in estrous behavior of the mare. In humans and primates, the adrenal gland has been shown to produce androgens, such as testosterone, androstenedione and dehydroepiandrosterone (Baird et al., 1969; Resko, 1971; Hornsby and Aldern, 1984). In other mammals, such as the pig and ruminants, stimulation of the adrenal gland caused an increase in plasma progesterone concentrations (De Silva et al., 1983; Plotka et al., 1983; Watson and Munro, 1984; Bolaños et al., 1997; Tsuma et al., 1998). Treatment of ovariectomized mares with adrenocorticotrophic hormone (ACTH) led to an increase in plasma levels of testosterone, but not progesterone or estradiol (Watson and Hinrichs, 1989). Further, it has been demonstrated in vitro that the mare's adrenal gland can convert pregnenolone to testosterone and androstenedione (Silberzahn et al., 1984).

It is well known that the intensity of estrous symptoms in the intact mare varies, with some animals showing very strong and/or exaggerated symptoms (sometimes referred to as nymphomania). Such disturbed estrous behavior may lead to difficulties in the training and handling of the mare, particularly for those who wish to use the animal for competition purposes. It is difficult to assess the extent of the problem since there are a number of underlying causes, which can lead to symptoms resembling exaggerated estrous behavior, such as granulosa-theca cell tumors (Meagher et al., 1978), urogenital discomfort or submissive behavior (McDonnell, 1992). Also, the mare has a naturally long estrus, on average 6.5 days, with a diestrous period of approximately 14 days, which to the uneducated owner may seem abnormal. Although few cases of nymphomania were reported in equine clinics, approximately 200 Standardbred trotter mares per year competed in Sweden under progesterone treatment, when such treatment was still permitted to ease symptoms of nymphomania (Lindström, 2000). However, in Sweden, since 1998, the use of long-acting progesterone treatment is banned in competing horses in all equestrian disciplines. Also, short-acting progesterone treatment, such as altrenogest orally, is prohibited for use at competition in riding horses and has a recommended withdrawal time of 14 days in Standardbred racehorses. In most cases, mares are judged by their owners to show estrous-related behavioral problems. However, in order to determine if nymphomania exists, and if so, what the possible underlying causes are, such mares need to be studied by both careful clinical examination and hormone analysis. If the adrenal gland is capable of secreting androgens, then perhaps an aberrant secretion of

such androgens cause some mares to show exaggerated estrous symptoms or aggressive behavior. In females of several species, including the mare, androgens have been shown to affect sexual behavior (Everitt and Herbert, 1971; Myerson et al., 1973; Thompson et al., 1983; Tuiten et al., 2000). In this context, it has been suggested that impaired enzymatic transformations within the mare's adrenal gland may result in an increased production of testosterone (Roberts and Beaver, 1987) and be a cause of naturally elevated serum testosterone levels in mares showing aggressive behavior (Beaver and Amoss, 1982). Adrenal enzyme defects causing hyperandrogenism have been described in, e.g. humans (see Smikle, 1999).

The aim of the present study was to investigate the effect of ACTH treatment (tetracosactide, Synachten) on the plasma levels of cortisol, progesterone, androstenedione and testosterone in intact mares with normal estrous behavior (controls) and mares described as showing deviant estrous behavior ('problem' mares).

2. Materials and methods

All procedures were approved by the Ethical Committee for Experimentation with Animals, Sweden.

2.1. Animals, housing and management

From a survey using questionnaires sent to owners of 'problem' mares, mares were selected. Seven mares were used in the experiment ('problem' mares). Another three were excluded due to being impossible and dangerous to handle. Inclusion criteria were behavioral problems perceived by the owner to be related to the estrous cycle or very frequent/strong estrous signs. The histories of the problem mares were varied and are summarized in Table 2.1. Four mares had displayed their behavior during the entire ownership (range 1–3 years) (mares An, Ev, F and Z). The other three mares had begun their behavior with a change in environment or circumstance [e.g. change of trainer (mare Be), at start of training (mare C) and when lost a foal (mare W)]. Further details of the survey are published elsewhere (Hedberg et al., 2005). Five other mares used in the study had no history of abnormal behavior at estrus (controls). These control mares were

Table 2.1
History of behaviors in 'problem' mares as assessed by the owner; summary from a survey

	Mare An	Mare Be	Mare C	Mare Ev	Mare F	Mare W	Mare Z
Behavior							
Frequent urination/tail raise	×	×	×		×	×	
Uncooperative	×		×		×	×	×
Aggressive					×	×	×
Screaming, hitting hind-end against box wall				×			
Pressing against handler/cart	×	×					
Time aberrant behavior occurred							
Always			×*		×*		×*,**
Regular intervals	×			×		×*	
At competition		×					

* Behavior most frequent at exercise.
** Better when lactating.

all Swedish Standardbred trotter mares that had been used in training and racing, but not shown good enough athletic abilities to continue racing. The 'problem' mares were of the following breeds: New Forest (1), Gotlandsruss (1), Swedish Warmblood (2), Swedish Standardbred (2) and Swedish Standardbred/cold-blooded trotter cross (1). Mares weighed between 250 and 600 kg with an age span of 5–16 years. The experiment was performed in the spring and summer of 2003 (March–July). During the experimental study, the horses were kept at the Department of Clinical Sciences, Division of Comparative Reproduction, Obstetrics and Udder Health. They were stabled on straw in individual loose boxes indoors at night and were turned out during the day (approximately, 8 a.m. to 3 p.m.). Four of the 'problem' mares were lent to the department for a period of at least two estrous cycles. When turned out during the day, these client-owned mares were for safety reasons kept in individual pens (approximately 10 m × 12 m), but within sight and hearing contact of other horses. The department-owned mares were during the day turned out together in a larger field. During the test days, mares were kept stabled. All mares were fed three times daily with hay (morning, noon and evening) and once daily in the afternoon with a small amount of oats, sugar beet and minerals. Water was provided ad libitum.

2.2. Clinical examination

Before the experiment, the mares were subjected to a thorough clinical examination. This was done to rule out other possible causes of aberrant behavior in the 'problem' mares. The examination included a lameness examination (walking and trotting in a straight line on a hard surface and flexion tests of all limbs), back palpation, blood test [hemoglobin, hematocrit, total white cell count (with differential count) and fibrinogen] and urine sample (to check for bacterial growth). Urine was sampled using a sterile catheter inserted into the urethra and applied to a three-partitioned selective culture plate (SELMA, National Veterinary Institute, Uppsala, Sweden) which was incubated overnight at 37 °C before being checked for bacterial growth. A gynecological examination was also performed, including vaginoscopy. The mares that according to their owners were difficult to ride or drive also had their teeth checked. All twelve mares passed the above examinations and were therefore included in the experiment.

2.3. Experimental protocol

All mares were followed for two complete estrous cycles, the first serving as a control. Any deviant behavior during the study was carefully documented and related to estrous cycle stage.

2.4. Estrous detection and gynecological examination

Mares were teased with a stallion twice daily (before being turned out and when brought in). Estrous signs were scored as follows: +1 for standing in a straddled posture, +1 for flashing clitoris/urinating, +1 for raising tail, −1 for kicking, −1 for switching tail, and −1 for ears back. The mare was considered to be in estrus if she showed at least two positive scores. Mares were rectally examined every second to every fourth day with ultrasonography (6 MHz linear-array scanner, 485 Anser, Pie Medical, Netherlands) to determine follicle size, presence of edema in the uterus or presence of a corpus luteum. Edema in the uterus was graded as follows: grade one indicated a heterogenic appearance, but no defined endometrial folds; grade two indicated an increase in heterogenicity with some endometrial folds visible; grade three indicated extreme heterogenicity with large anechoic areas.

2.5. Blood sampling

Daily blood samples for progesterone analysis, as a complementary test of normal estrous cyclicity, were taken by jugular venipuncture into heparinized tubes, which were immediately centrifuged and the plasma stored at −18 °C. When a follicle size of 3 cm or larger was detected and the mare showed estrous signs and/or edema in the uterus, frequent blood sampling was performed. Behavioral estrous signs were not deemed necessary, since three of the mares (mares F, Z and Ae) often showed only weak signs when teased with the stallion. Frequent blood sampling was performed at two consecutive estruses for a 26-h period, the first serving as a control (saline treatment day). At the second estrus, ACTH [tetracosactide, Synachten (0.5 mg)] was injected intravenously (ACTH treatment day). For practical reasons, a specific day at estrus could not be chosen. The mares were sampled on day 1 ($n = 3$), 2 ($n = 5$), 3 ($n = 3$) or 5 ($n = 1$) of estrus when saline treated. One mare (mare Z) showed no estrous signs at teasing on the saline treatment day (estrous detection score of 0; plasma progesterone level <0.6 nmol/l), but had showed a score of +3 on the first day of estrus, the day before. When ACTH treated the mares were sampled on day 1 ($n = 4$), 2 ($n = 3$), 3 ($n = 2$) or 4 ($n = 1$) of estrus. Day of estrus could not be estimated for two mares (mares F and B) on the ACTH treatment day, although both mares had follicles ≥ 3 cm and uterine edema. Mare F tended to show no or weak estrus at teasing and had an estrous detection score of 0. However, her pre-treatment sample progesterone level was just above 3 nmol/l, suggesting some progesterone from a corpus luteum still present and indicating she was sampled too early. Mare B had an overall negative score when teased on the ACTH treatment day. Later analysis, however, showed low progesterone levels in the pre-treatment samples of mare B (≤0.8 nmol/l). For frequent blood sampling, an indwelling catheter (Milacath®, 14 gauge × 3.5 in., MILA International Inc.) was inserted into the jugular vein between 08:00 and 10:00 h [after a subcutaneous anesthetic injection of 1 ml xylocain (10 mg/ml) with adrenaline (5 μg/ml)]. Blood samples were then taken hourly from 12:00 h until 14:00 h (pre-treatment samples), thereafter every half-hour until 17:00 h and again hourly until 14:00 h the following day. After each sample, the catheter was flushed with approximately 5 ml of heparinized saline (0.5 IU/ml heparin solution) to avoid blood clotting in the catheter. In the second estrus studied, the sampling was done as above and, in addition, 2 ml (0.5 mg) of tetracosactide (Synachten®, 0.25 mg/ml, Novartis) diluted in saline to a volume of 5 ml was given via the jugular catheter immediately after the blood sample at 14:00 h. Tetracosactide is a synthetic polypeptide containing the first 24 of the 39 amino acids of naturally occurring ACTH. All blood samples were immediately transferred into heparinized vacutainer tubes, centrifuged and the plasma stored at −18 °C until assay.

2.6. Hormone assays

The daily blood samples were analyzed for progesterone content. All of the frequent blood samples (30 samples/mare/estrus) were analyzed for plasma content of cortisol, progesterone and androstenedione. Testosterone content was determined in the pre-treatment samples and in samples obtained during the first 8-h period after ACTH treatment in all mares (14 samples/mare/estrus). The analyses for cortisol, progesterone and androstenedione were performed at the Department of Biomedical Sciences and Veterinary Public Health, Swedish University of Agricultural Sciences, Uppsala, Sweden. The analyses for testosterone were performed at the Klinik für Geburtshilfe, Gynäkologie und Andrologie der Groß- und Kleintiere mit Tierärztlicher Ambulanz, Justus-Liebig-Universität, Giessen, Germany.

The concentration of cortisol in peripheral blood plasma was determined using a solid-phase radioimmunoassay (Coat-A-Count Cortisol, Diagnostic Products Corporation, Los Angeles, USA). Plasma samples of 25 μl were used and all samples run in duplicates. The kit was used according to the manufacturer's instructions with the following modification: to produce a new standard curve, standard B was diluted with standard A (human 0-plasma) which resulted in a standard curve with the following concentrations: 0, 13.8, 27.6, 138, 276, 552 and 1380 nmol/l. The relative cross-reactions of the antibody were 0.94% with corticosterone and 0.98% with cortisone. The inter- and intra-assay coefficients of variation for cortisol were as follows: 8.8% and 8.8% at 38.3 nmol/l; 8.6% and 9.2% at 83.7 nmol/l; 11.1% and 3.9% at 668.6 nmol/l. The minimal assay sensitivity of cortisol was 8.2 nmol/l.

The concentration of progesterone in peripheral blood plasma was determined using a solid-phase radioimmunoassay (Coat-a-Count Progesterone, Diagnostic Products Corporation, Los Angeles, USA). Plasma samples of 100 μl were used. The kit was used according to the manufacturer's instructions. The relative cross-reactions of the antibody were 0.9% with corticosterone, 0.0% with cortisol, and 0.1% with testosterone. The inter- and intra-assay coefficients of variation for progesterone were as follows: 16.1% and 4.3% at 3.5 nmol/l; 7.3% and 8.5% at 22.5 nmol/l; 23.3% and 6.4% at 54.8 nmol/l. The minimal assay sensitivity of progesterone was 0.15 nmol/l.

The concentration of androstenedione was analyzed using a liquid-phase radioimmunoassay (DSL-4200, Diagnostic Systems Laboratories, Inc., TX, USA). Plasma samples of 250 μl were used and extracted with 2.5 ml of ether. The procedure used has been described for ewes (Viñoles et al., 2003). Some modifications to the procedure were made: the mares' samples contained high concentrations of androstenedione and therefore the undiluted standard was used as the highest point on the curve giving a standard curve that ranged from 27 to 1725 pmol/l [the lowest point of the standard curve (14 pmol/l) was excluded]. Three controls were used and run in duplicates. One sample from a mare in the study, which contained a high level of androstenedione, was used as the high control (690 pmol/l). The high control provided with the kit was diluted 1:2 and used as a medium control (155 pmol/l). Diluting the medium control 1:4 made a low control (39 pmol/l). Parallelism of the standard curve with serial dilution of equine plasma samples was demonstrated. The inter- and intra-assay coefficients of variation were as follows: 28.6% and 10.3% at 53 pmol/l, 17.8% and 6.1% at 216 pmol/l; 10.7% and 0.1% at 622 pmol/l. The detection limit of the assay was 10.4 pmol/l.

The concentration of testosterone was analyzed using a liquid-phase radioimmunoassay. All samples were run in duplicates. Repeats were done when a variation greater than 10%, based on the c.p.m., was detected. A 0.1 ml sample of plasma was extracted twice with 2 ml of toluene and the pooled extracts were evaporated to dryness. After addition of 0.1 ml BSA–phosphate buffer the following radioimmunoassay was set up as a sequential assay (Strecker et al., 1979). The antiserum used was GI-testosterone-1 (28.03.1989/XIV), obtained after immunisation of rabbits with 4-androstene-11α,17β-diol 3-one-11-HS-BSA (dilution 1:100 000, KA = 0.312 l/nmol), with the following cross-reactions: dihydrotestosterone 47%, androstenedione 0.84%, 17-β-estradiol 0.04%, progesterone 0.02% and all other tested steroids < 0.01%. Of this antiserum, 0.4 ml was added to the samples and standards (5–640 fmol/tube in 0.1 ml BSA–phosphate buffer). The tubes were then placed in a water bath of 37 °C for 20 min, followed by a first over-night incubation (minimum 12 h) at 4 °C. ^{3}H testosterone (0.18 pmol = 0.47 kBqu per tube) was then added and the samples were again incubated for 45 min at 4 °C (second incubation). After separation of free and bound steroid by addition of 0.2 ml of ice-cold charcoal suspension (0.5% charcoal, 0.05% dextran) and centrifugation for 15 min at revolutions 2400 × g at

4 °C, 0.6 ml of the supernatant was removed to measure the radioactivity of the bound fraction. Absolute binding of ^{3}H testosterone was 17.4 ± 3.48%. The control samples run with each assay were the blank value, low positive (gelding) and high positive (stallion). The readings were corrected for the individual blank of each assay. The inter-assay coefficient of variation for the low positive control was 20.8% [0.055 ± 0.011 pg/ml (190 ± 40 pmol/l); $n = 9$] and 12.9% for the stallion plasma [0.616 ± 0.079 pg/ml (2136 ± 275 pmol/l); $n = 9$]. Based on the course of the calibration curves the assay allowed a clear distinction between 5 fmol/tube.

2.7. Dexamethasone-suppression test

When a mare had been sampled on the two occasions (i.e. the saline treatment day and the ACTH treatment day), a dexamethasone-suppression test was performed to evaluate hypothalamus–pituitary–adrenal (HPA) axis function. This test involved a blood sample taken with vacutainer from the jugular vein between 16:00 and 18:00 h (pre-sample) followed by an intramuscular injection of dexamethasone (Vorenvet®vet, 1 mg/ml, Boehringer Ingelheim Vetmedica) at a dose of 4 mg/100 kg body weight on day 1. On day 2, a second blood sample was obtained between 08:00 and 12:00 h. The blood samples were analyzed for cortisol content.

2.8. Statistical analyses

Statistical analyses were carried out using the SAS software (Version 8; SAS Institute Inc., Cary, NC, USA). For the hormone concentrations, repeated measurement analyses of variance were performed using the MIXED procedure. The observations were for the statistical analyses grouped into five time periods: 12:00–14:00 h (period one; pre-treatment samples); 14:30–17:00 h (period two); 18:00–23:00 h (period three); 24:00–05:00 h (period four); 06:00–14:00 h (period five). The analyses were performed for each treatment day (saline or ACTH), as well as for the difference between treatment days (ACTH-treated minus saline, within mare and sampling time). The fixed effects included in the statistical models were group of mares (control or 'problem'), time period (five periods), sampling time nested within time period, and the interaction between group of mares and time period. The time periods were chosen based on results from a pilot study performed at the Division of Comparative Reproduction, Obstetrics and Udder Health, where the same dose of ACTH as used in the present study caused an increase in cortisol level for at least 3 h (period two in the present study) (Dalin et al., 2002). Period four was defined because of negative feedback during this time period found in the pilot study. Least-squares means were estimated, and pair-wise tests of significance of the difference between Least-squares means were performed. Differences between morning and afternoon estrous detection scores for each mare group ('problem' and control), on both the saline- and ACTH treatment days, were analyzed with paired *t*-test. The pattern of different estrous behaviors was compared between the two mare groups during both estruses, using analysis of variance (GLM procedure), including the effect of mare group, but not treatment. Days that each mare showed each behavior ('tail raise', 'clitorial winking/urination', 'straddled posture', 'kicking', 'switching tail' and 'ears back') were expressed as percentage of total number of estrous days (days with each behavior × 100/total estrous days). Least-squares means were estimated, and pair-wise tests of significance of the difference between Least-squares means were performed.

3. Results

3.1. Clinical examination, cyclicity and dexamethasone suppression test

No signs of infection could be found in either the blood or urine samples. Except for minor reactions to the flexion tests of the limbs, none of the mares showed any musco-skeletal problems. The mares were all found to have normal ovarian cyclicity, with no pathological findings. The daily blood samples confirmed that all mares were cycling normally during the experimental period. One 'problem' mare (mare Be) had an estrous period of 17 days in her first cycle. This was, however, considered within normal limits for the time of year (March). The dexamethasone-suppression test to evaluate HPA function (performed for each mare after both frequent sampling periods were completed) showed normal suppression of the adrenal gland in all mares with pre-samples (range 64–220 nmol/l) and samples obtained after the dexamethasone injection (range < 10–13 nmol/l) all within the laboratory's normal reference ranges (<320 and <45 nmol/l, respectively).

3.2. 'Problem' mare behavior

At the clinic, during the experimental period, five of the seven mares showed deviant estrous behavior. These behaviors were different for each mare: one mare showed very intense, but regular, estrous signs and continually stood in a urine stance and winked her clitoris (which by some may be seen as normal) (mare Be); one mare screamed and hit her hind-end on the box-wall at estrus (mare Ev); one mare pressed against her handler and refused to move forward during the estrous phase of the cycle (mare An). Two mares (mare Z and mare F) often showed no or only weak estrous signs at teasing. The control mares showed no behavioral changes across the estrous cycle. One control mare (mare Ae) tended to show weak estrous signs at teasing.

3.3. Estrous detection scores and gynecological findings

For all mares, the mean sizes of the largest follicle were 4.1 cm (±S.D. 0.5) and 3.7 cm (±S.D. 0.5) on the saline and ACTH treatment days, respectively. Mean edema grade in the uterus was 1.4 (±S.D. 0.8) on the saline treatment day and 1.5 (±S.D. 0.7) on the ACTH treatment day. The mean estrous detection scores (±S.D.) for each mare group in the morning and afternoon on both sampling occasions, respectively, are shown in Table 3.1. Teasing in the afternoon was always performed in time period two, when also maximal hormone concentrations after ACTH treatment were found. No statistical differences between the morning and afternoon estrous detection scores were found in either mare group on either sampling occasion (t-test, $p > 0.2$). The proportion of behaviors displayed by each mare during both estrous phases studied (calculated as a percentage

Table 3.1
Mean (±S.D.) estrous detection scores in both mare groups on both sampling occasions

Mare group	Saline treatment day		ACTH treatment day	
	a.m.	p.m.	a.m.	p.m.
Control mares	2.0 (1.4)	2.2 (1.2)	1.4 (1.7)	2.1 (1.2)
'Problem' mares	3.0 (0.0)	2.4 (0.9)	1.2 (2.4)	1.4 (2.5)

Scored as follows: +1 for straddled posture, +1 for flashing clitoris/urinating, +1 for raising tail, −1 for kicking, −1 for switching tail, −1 for ears back.

Table 3.2
Proportion of different behaviors displayed by each mare group during the entire estrous periods (calculated as mean (±S.D.) percentage of total estrous days)

Behavior at teasing	Tail raise	Clitorial winking/ urination	Straddled posture	Kicking	Switching tail	Ears back
Saline treatment estrus						
Control mares	85.2 (29.3)	80.2 (40.5)	73 (37.3)	8.8 (9.4)	14.8 (29.3)	18.0 (36.5)
'Problem' mares	89.0 (20.2)	79.9 (22.1)	63.9 (40.5)	2.6 (6.8)	1.7 (4.5)	0.9 (2.3)
ACTH treatment estrus						
Control mares	77.6 (30.0)	76.8 (36.4)	42.2 (35.2)	13.8 (18.0)	26.2 (27.4)	22.6 (31.5)
'Problem' mares	87.7 (26.0)	78.3 (36.4)	68.6 (40.3)	9.7 (13.3)	6.4 (7.0)	1.6 (2.8)

of total number of estrous days) is shown in Table 3.2. There were no statistical differences in behavior between the mare groups (difference between Least-squares means, GLM procedure).

3.4. Hormone concentrations at the saline and ACTH treatment days

3.4.1. Cortisol

On the saline treatment day (control), there was a significant effect of time period ($P < 0.001$), showing that the cortisol levels changed over the sampling period (Fig. 3.1). No significant effect of mare group ('problem' versus control) or interaction between mare group and time period was found on the saline treatment day. For both groups of mares, the mean (±S.D.) concentrations of cortisol during the first 4 h after the saline and ACTH injection are shown in Fig. 3.2. After the ACTH treatment all mares ('problem' and control) showed a significant increase in cortisol from 30 min (337 ± 104 nmol/l) to 5 h post-injection (180 ± 77 nmol/l) ($P < 0.01$), with the highest average cortisol value at 2 h after ACTH treatment (407 ± 101 nmol/l). The negative feedback exerted by this cortisol increase after the ACTH injection resulted in lower cortisol values between

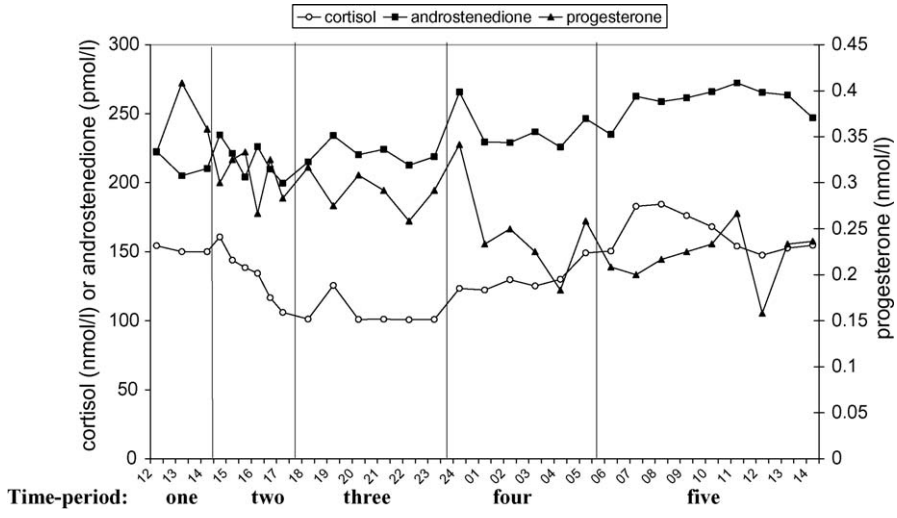

Fig. 3.1. Mean cortisol, progesterone and androstenedione values on the saline treatment day for all mares.

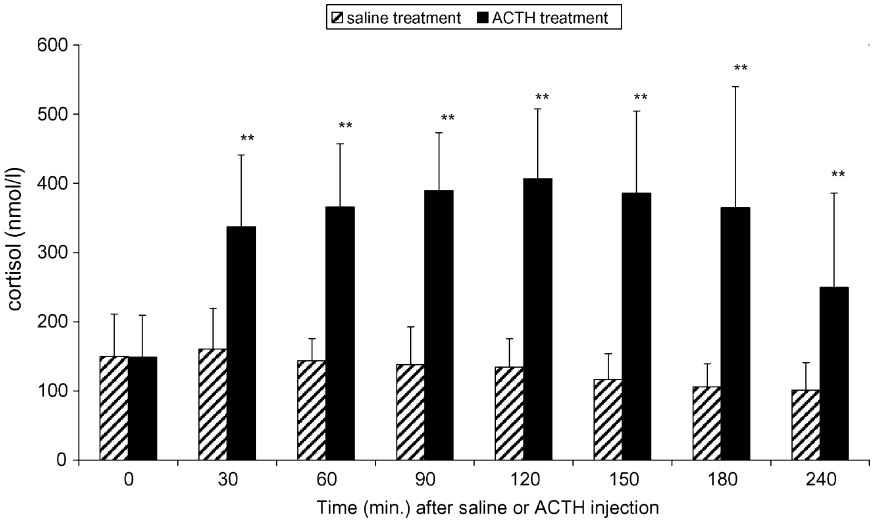

Fig. 3.2. Cortisol values (mean ± S.D.) on the saline- and ACTH treatment days (all mares). **Significant at $P < 0.01$.

10 and 13 h post-injection on the ACTH treatment day as compared to the saline treatment day ($P < 0.05$). An overall significant effect of mare group was found in the cortisol response to ACTH (i.e. difference between treatment days; $P < 0.05$), with control mares showing a greater response compared to 'problem' mares in time period two (30 min to 3 h post injection) ($P < 0.001$). This resulted in cortisol values on the ACTH treatment day being overall significantly higher in control mares as compared to 'problem' mares ($P < 0.05$). The interaction between mare group and time period further showed that control mares, compared with 'problem' mares, had significantly higher cortisol values in time period two (30 min to 3 h post injection) ($P < 0.001$) and three (4–9 h post injection) ($P < 0.05$) on the ACTH treatment day (Fig. 3.3). Upon scrutinizing the data, one mare in the control group (mare Ex) had visibly higher cortisol levels on the ACTH treatment day as compared to the other four control mares. Excluding her from the analyses resulted in no overall significant effect of mare group in the cortisol response to ACTH (difference between treatment days; $P = 0.07$). However, the control mares still showed a significantly greater increase in cortisol in time period two (difference between treatment days; $P < 0.001$). Also, the significantly different cortisol values on the ACTH treatment day between the two mare groups, described above, remained significantly different after excluding mare Ex.

3.4.2. Progesterone

On the saline treatment day, the mean progesterone level was low (≤0.4 nmol/l). However, there was a significant effect of time period ($P < 0.001$), showing levels changed with time (Fig. 3.1). There was also an overall significant interaction between mare group (control versus 'problem' mare) and time period on the saline treatment day ($P < 0.05$), suggesting the two groups of mares differed in progesterone pattern. The mean (±S.D.) concentrations of progesterone during the first 4 h after the ACTH treatment for all mares are shown in Fig. 3.4. The progesterone concentrations increased after the ACTH injection and were significantly higher, as compared to the saline treatment day, from 30 min (1.2 ± 1.1 nmol/l) to 4 h (0.7 ± 0.7 nmol/l) after the injection ($P < 0.05$). The highest average progesterone value (1.3 ± 0.7 nmol/l) was reached 90 min post-injection. Individual peak values on the ACTH treatment day ranged from 0.6 to 4.3 nmol/l,

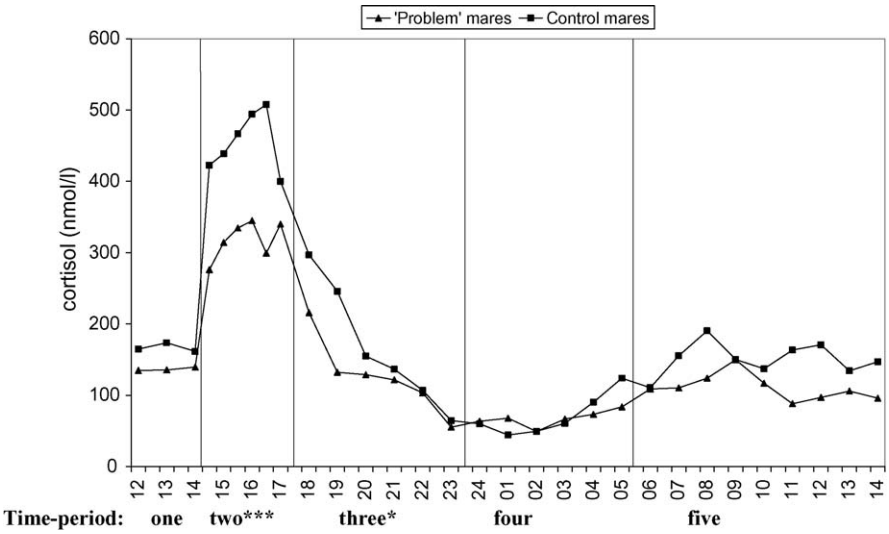

Fig. 3.3. Mean cortisol values on the ACTH treatment day for control and 'problem' mares. ACTH treatment given at the beginning of time period two. ***Significant at $P < 0.001$; *Significant at $P < 0.05$.

representing an increase from pre-injection values in the range of 0.5–1.2 nmol/l. The 'problem' mares showed a significantly higher response in progesterone levels as compared to controls in time period two (difference between treatment days; $P < 0.05$). Also, on the ACTH treatment day, an overall significant interaction between mare group and time period was seen ($P < 0.001$), with the 'problem' mares having significantly higher progesterone concentrations than controls in time period one (pre-treatment samples) and two ($P < 0.05$) (Fig. 3.5). The significantly higher pre-treatment sample levels were not significant when mare F (a 'problem' mare) was excluded from the analysis. This mare F had edema in the uterus and a follicle $\geq$3 cm at sampling, but was

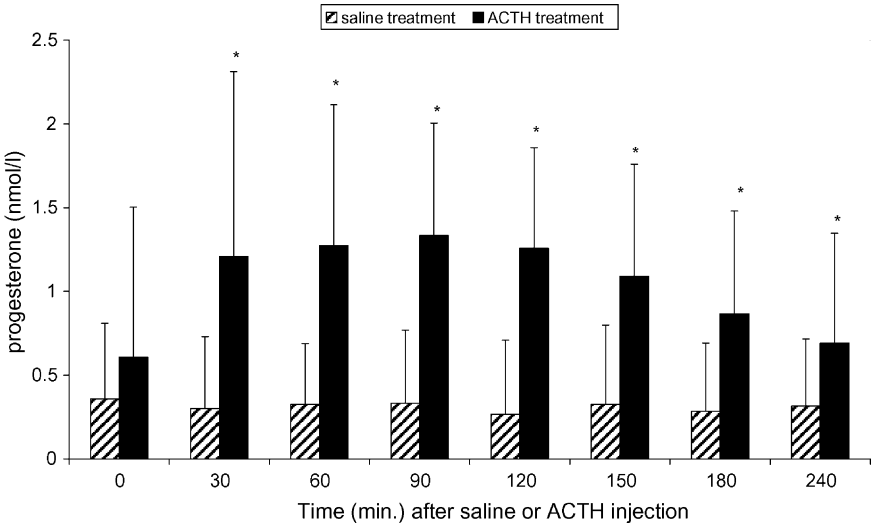

Fig. 3.4. Progesterone values (mean ± S.D.) on the saline- and ACTH treatment days (all mares). *Significant at $P < 0.05$.

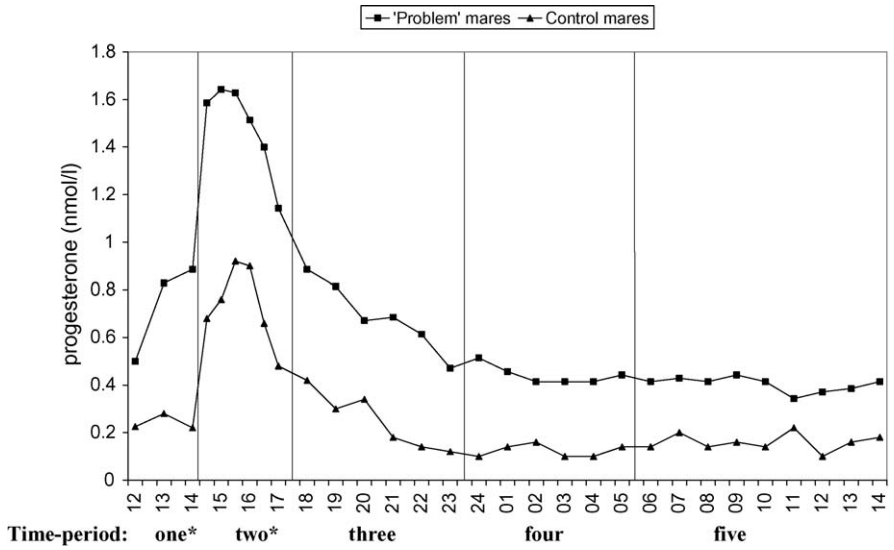

Fig. 3.5. Mean progesterone values on the ACTH treatment day for control and 'problem' mares. ACTH treatment given at the beginning of time period two. *Significant at $P < 0.05$.

most likely sampled too early, with still some progesterone from a corpus luteum present in the plasma.

3.4.3. Androstenedione

A significant effect of time period ($P < 0.001$) was found on the saline treatment day (Fig. 3.1). The mean (±S.D.) concentrations of androstenedione during the first 4 h after the ACTH injection in both groups of mares are shown in Fig. 3.6. The ACTH treatment resulted in a significant

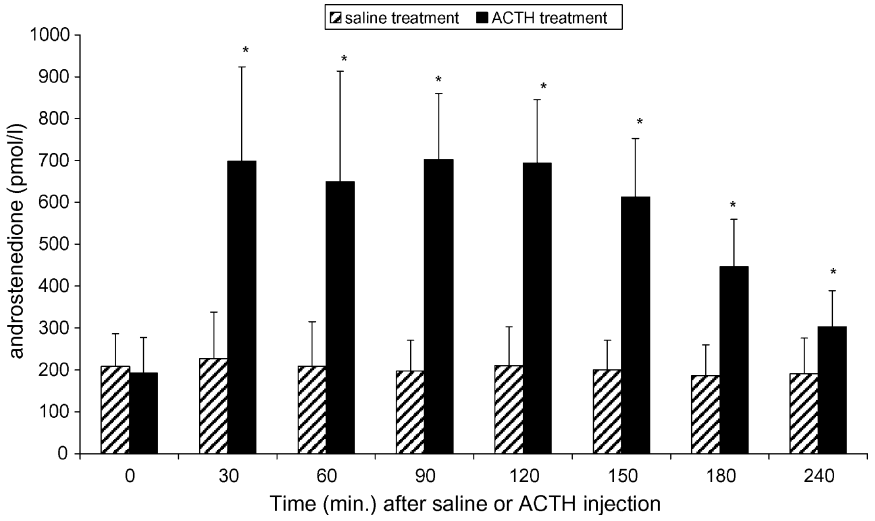

Fig. 3.6. Androstenedione values (mean ± S.D.) on the saline- and ACTH treatment days (all mares). *Significant at $P < 0.05$.

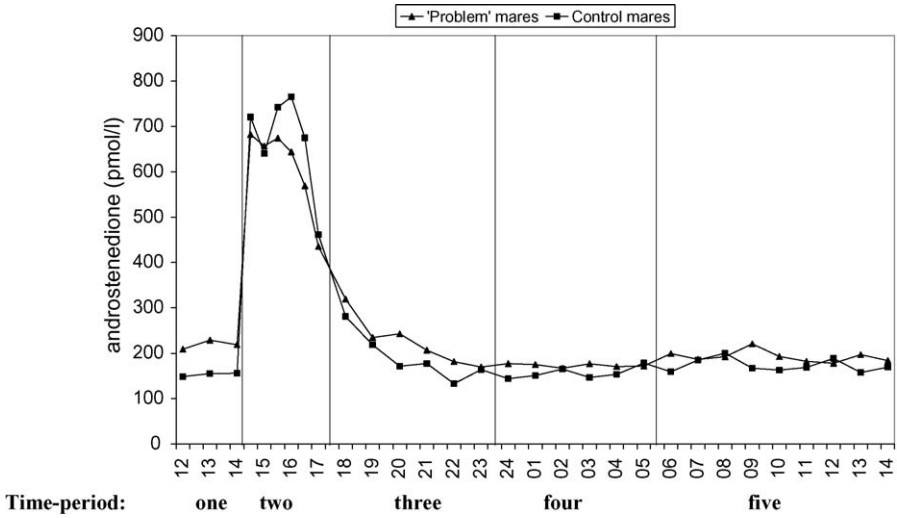

Fig. 3.7. Mean androstenedione values on the ACTH treatment day for control and 'problem' mares. ACTH treatment given at the beginning of time period two.

increase in plasma androstenedione concentrations in all mares ('problem' and control) with levels significantly higher, as compared with the saline treatment day, from 30 min (698 ± 225 pmol/l) to 4 h (303 ± 86 pmol/l) after the injection ($P < 0.05$). Highest average androstenedione level was reached 90 min after the ACTH injection (703 ± 158 pmol/l). Individual peak values ranged from 444 to 1119 pmol/l, representing an increase from pre-injection values in the range of 157–884 pmol/l. No significant effect of mare group (control versus 'problem' mare) was found in androstenedione response to ACTH (difference between treatment days; $P = 1.0$) (Fig. 3.7).

3.4.4. Testosterone

The mean (±S.D.) concentrations of testosterone during the first 4 h after the ACTH injection in both groups of mares are shown in Fig. 3.8. The ACTH injection resulted in a significant increase in plasma testosterone concentrations in all mares ('problem' and control) from 30 min (149 ± 50 pmol/l) to 5 h (103 ± 36 pmol/l) after the injection ($P < 0.05$). Highest average testosterone level was reached 120 min after the ACTH injection (196 ± 63 pmol/l). Individual peak values ranged from 140 to 320 pmol/l, representing an increase from pre-injection values in the range of 90–254 pmol/l. No significant effect of mare group was found in testosterone response to ACTH (difference between treatment days; $P = 0.7$) (Fig. 3.9).

4. Discussion

The present study showed that the mares, selected from a questionnaire as having estrous behavioral problems, was a very heterogeneous group in behavior, showing from intense signs to normal to weak estrus. This may indicate that some owners misinterpret their mare's behavioral signs. In some cases, behavioral problems may be due to different diseases or infections, but, in the present study, such causes were ruled out by the thorough clinical examination. In addition, the mares had normal HPA function as confirmed by the dexamethasone suppression test. Environmental and handling factors may also have an effect on the behavior of some mares.

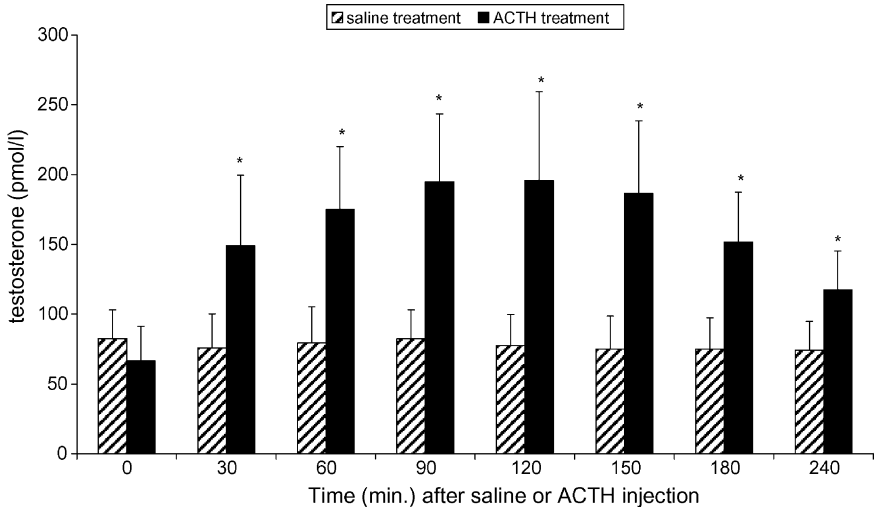

Fig. 3.8. Testosterone values (mean ± S.D.) on the saline- and ACTH treatment days (all mares). *Significant at $P < 0.05$.

For example, five out of the seven 'problem' mares were said to display disturbed behavior most frequently in association with the mare being exercised and/or at competition (see Table 2.1). At the department, mares were handled according to a set routine but were not ridden or driven.

In the present study, after the ACTH treatment, plasma levels of cortisol, progesterone, androstenedione and testosterone increased in all mares and within the same time-frame (30 min to 4 or 5 h post-injection), thereby strongly indicating an adrenal source for the significantly increased levels of all hormones. The mares in the present study were intact. Therefore, the hormonal concentrations measured during the saline treatment (control day) were likely to be of

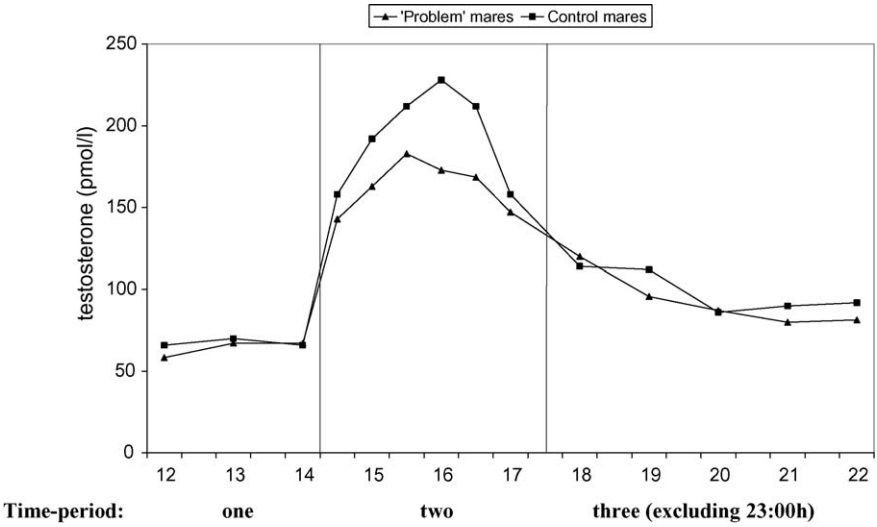

Fig. 3.9. Mean testosterone values on the ACTH treatment day for control and 'problem' mares. ACTH treatment given at the beginning of time period two.

both adrenal and ovarian origin. For example, mares have been shown to have a pre-ovulatory rise in androstenedione (Noden et al., 1975; Munro et al., 1979) and follicular fluid of mares in estrus contains androstenedione, epitestosterone and progesterone (Younglai, 1971). In women, 25–30% of circulating levels of 17-hydroxyprogesterone, androstenedione and testosterone were of ovarian origin, with the remaining being produced by the adrenal gland (Piltonen et al., 2002). The adrenal source in the increase in hormone level as a result of ACTH treatment was confirmed when some of the same mares were ovariectomized and again treated with ACTH (Hedberg et al., 2006, accompanying paper).

The increase in progesterone concentration in the ACTH cycle is not in agreement with the study of Watson and Hinrichs (1989) who found no statistically significant change in progesterone level when ovariectomized mares were injected with a similar dose of ACTH. However, adrenal progesterone production has been described in various other mammals, such as ovariectomized rats (Piva et al., 1973), intact and ovariectomized deer (Plotka et al., 1983; Jopson et al., 1990), ovariectomized sheep (Van Lier et al., 1998) and ovariectomized Zebu cows (Bolaños et al., 1997). Progesterone is a precursor to cortisol (Nelson, 1980) and it has been suggested that both cortisol and its precursors are simultaneously released when the adrenal gland is stimulated (Van Lier et al., 1998). In the present study, intact mares were used, which could be one reason for the discrepancy between our results and that of Watson and Hinrichs (1989). However, this explanation was ruled out after ovariectomy (Hedberg et al., 2006, accompanying paper).

All of the mares in the present study showed a significant increase in androstenedione and testosterone after the ACTH treatment, with about 3.5 times higher concentration of androstenedione being released compared to testosterone. This agrees with Silberzahn et al. (1984), who found that incubation of cortical tissue from mare adrenals with ^{3}H-pregnenolone yielded 33 times more ^{3}H-androstenedione than ^{3}H-testosterone, i.e. a higher androstenedione than testosterone level produced by the mare adrenal gland. The increased levels of androgens in response to ACTH varied markedly between individuals, with some mares showing a much greater increase than others. However, no significant difference between controls and 'problem' mares in either androgen was found, thus not supporting the theory of abnormal androgen synthesis in the adrenal gland as a cause of disturbed behavior. In addition, there was no direct effect of the adrenal hormone increase on estrous behavior in either mare group.

Androstenedione is in itself a weak androgen, but can be converted peripherally to testosterone by the enzyme 17β-hydroxysteroid dehydrogenase (17β-HSD), which was, e.g. found in several tissues of the rhesus monkey (Martel et al., 1994). Therefore, it cannot be excluded that parts of the increased testosterone values observed in the present study after the ACTH injection may have been due to peripheral tissue conversion from androstenedione. However, considering that both hormones increased at a similar time after the ACTH treatment (90–120 min post-injection), a direct adrenal source of testosterone is more likely. 17β-HSD activity was also shown to be present in the adrenal gland of rhesus monkey (Martel et al., 1994). In the adrenal cortex, it is the enzyme distribution as well as the enzyme characteristics that determine the steroid hormones to be produced. Also, species differences exist [Hornsby and Aldern, 1984; Conley and Bird, 1997 (review)]. To our knowledge, the distribution of enzymes in the equine adrenal gland has not been studied.

Although basal cortisol levels did not differ between mare groups, 'problem' mares showed a significantly lower increase in cortisol level during the first 3 h after the ACTH injection, indicating a difference in adreno-cortical reactivity between the 'problem' mares and controls. In cattle, chronic stress has been shown to alter adreno-cortical reactivity, with changes in response to ACTH treatment, despite similar basic adrenal activity (Ladewig and Smidt, 1989). However, type of

chronic stressor used and species studied influences whether the stress results in an enhanced or depressed adreno-cortical reactivity (Friend et al., 1979; Ladewig and Smidt, 1989; Von Borell and Ladewig, 1989; Klemcke, 1994). In pigs it has been shown that the adreno-cortical reactivity had also an individual characteristic, regardless of influences from the environment (Von Borell and Ladewig, 1989). The effect of chronic social stress in the horse has been studied by Alexander et al. (1996) who proposed that there is an ACTH inhibitory factor, resulting in a hypo-responsiveness to corticotrophin-releasing hormone (CRH) in chronically stressed horses. Despite being unresponsive to CRH, acute stress, simulated by hypoglycaemia, induced both ACTH and cortisol responses in the stressed horses. In the present study, it is tempting to speculate that the 'problem' mares experienced a greater endogenous stress level during the estrous cycle compared with control mares, resulting in a changed adreno-cortical reactivity with a blunted cortisol response to exogenous ACTH. This may indicate that mares with estrous-related behavioral problems are more susceptible to stress and therefore a stress-free environment may be of benefit for such mares. The fact that many of the 'problem' mares failed to show aberrant behavior at our department and, also, that clients have frequently commented that a change of environment recommended by us has improved their mare's behavior, seems to support such a notion. However, individual variation and breed differences may have confounded the present results, since it was not possible to match the mare groups for age or breed. In horses, social rank may affect cortisol levels, with cortisol levels being inversely related to rank (Alexander et al., 1996).

After the ACTH treatment in the present study, the progesterone levels in 'problem' mares increased more, but cortisol levels increased less, as compared to controls. Therefore, another possible explanation for the differences in cortisol response to ACTH as found in the present study may be an aberrant synthesis of adrenal hormones, which may cause a deficient cortisol response. For example, in humans, a defect in the gene encoding the 21-hydroxylase enzyme in the adrenal gland leads to deficient cortisol production and an elevation in 17α-hydroxyprogesterone (17 OH-progesterone), 17 α-hydroxypregnenolone (17 OH-pregnenolone), DHEA, androstenedione and testosterone (Glatt et al., 2005). ACTH stimulation tests are in some cases used to discern such defects in adreno-cortical function, where high levels of 17 OH-progesterone are diagnostic (Carlson et al., 1999). The progesterone levels reached in the present study were relatively low and therefore it would have been of interest to measure also the progesterone metabolite, 17 OH-progesterone. This analysis was not possible in the present study. The biological effect of the significantly greater progesterone response in the 'problem' mares is not clear. The maximum levels of progesterone observed in the present study were in all mares considerably lower than normal luteal phase values (12–66 nmol/l) (Ginther, 1992).

Although easily disrupted by, e.g. a novel environment (Irvine and Alexander, 1994), cortisol in the horse is known to exhibit a diurnal rhythm, with peak levels in the morning and low levels in the afternoon/evening (Hoffis et al., 1970; James et al., 1970; Larsson et al., 1979; Irvine and Alexander, 1994). This agrees with the present study, where peak cortisol levels occurred between 07:00 and 10:00 h and trough levels between 18:00 and 23:00 h. In the present study, there was also a significant effect of time period in the saline cycle for progesterone and androstenedione (testosterone was only measured for 14 h). There are conflicting reports as to the existence of diurnal rhythms in other adrenal hormones. In postmenopausal women, no statistically significant diurnal rhythm in androstenedione and testosterone was discovered (Lønning et al., 1989), whereas in another study of women (age 16–40 years) a rhythm in 17 OH-progesterone, androstenedione, 11-hydroxyandrostenedione, testosterone, DHEA and its sulfate was observed (Mikulecky et al., 1995). A diurnal rhythm in progesterone has been demonstrated in children over two years of age (Gröschl et al., 2003). In the present study, the levels of androstenedione and progesterone

changed over the 26-h sampling period. A subtle trend of increasing levels of androstenedione and decreasing levels of progesterone was seen, but not indicating a 24-h rhythm. The changes in these hormone concentrations observed may therefore partly have been due to changed production by the ovary. This is supported by data from ovariectomized mares (see Hedberg et al., 2006, accompanying paper).

For progesterone, there was a significant interaction between mare group and time period on the saline treatment day, indicating that the 'problem' mares differed from controls. However, because progesterone values on the saline treatment day were low (< 2 nmol/l), these differences may be without biological significance, and also, it cannot be excluded that the differences were partly due to intra-assay variation.

5. Conclusion

In response to ACTH, increased levels of cortisol, progesterone, androstenedione and testosterone were found in intact mares. An adrenal source of these hormones was most likely. A substantial amount of androgens, especially androstenedione, was released upon ACTH stimulation, but the amount of progesterone was low. A difference in adrenal response between 'problem' mares, as judged by owners to have disturbed estrous behavior, and control mares was found.

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