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SEASONAL PATTERNS OF CIRCULATING β-ENDORPHIN, ADRENOCORTICOTROPIC HORMONE AND CORTISOL LEVELS IN PREGNANT AND BARREN MARES

E. FAZIO, P. MEDICA & A. FERLAZZO

Department of Morphology, Biochemistry, Physiology and Animal Productions − Unit of Veterinary Physiology, Faculty of Veterinary Medicine, University of Messina, Messina, Italy

Summary

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The study was carried out to investigate the changes of circulating β-endorphin, adrenocorticotropic hormone (ACTH) and cortisol concentrations in 18 Thoroughbred mares (n=12 pregnant and n=6 barren), aged 5-18 years, over a 12–month period. Blood samples were collected monthly from April to March. In pregnant mares there were no significant changes in β-endorphin and ACTH levels (P>0.05), but higher cortisol levels at the 1st (P<0.05) and at the 8th (P<0.01) and lower at the 11th gestation month (P<0.01) were observed, as compared to barren mares. Pregnant mares showed higher β-endorphin levels in August (P<0.05), lower ACTH levels in July (P<0.05) and higher cortisol levels in May (P<0.05), as compared to the previous month. Barren mares showed lower cortisol levels in April (P<0.05) and in November (P<0.01) and higher levels in March (P<0.001), as compared to pregnant mares and in the 1st post-partum month values. Barren mares exhibited higher β-endorphin levels in September (P<0.05) and lower in November (P<0.01) and December (P<0.01), vs the previous month. One-way RM-ANOVA showed a seasonal effect for β-endorphin (P<0.001), ACTH (P<0.001) and cortisol (P<0.001) changes in pregnant mares, and for β-endorphin (P<0.0001) and cortisol (P<0.0001) changes in barren mares.

Key words: adrenocorticotropic hormone, β-endorphin, cortisol, mare, pregnancy, seasonality

INTRODUCTION

The endogenous opioid peptide system and the pituitary-adrenal axis are involved during physical and psychological challenges in domestic animals, through the release of β-endorphin, adrenocorticotropic (ACTH) and cortisol hormones. The seasonal reproductive pattern in mares is the result of a circannual endogenous rhythm and recent discoveries regarding the neuroendocrine control of seasonal reproduction have allowed the manipulation of the reproductive activity in mares (Nagy *et al.*, 2000; Aurich & Aurich, 2006).

Opioid peptides regulate seasonal reproductive activity and the oestrous cycle in mares (Aurich *et al.*, 1994; 2000; Alexander *et al.*, 2000) and exert a tonic inhibition on luteinizing hormone (LH) secretion by influencing the hypothalamic secretion of gonadotropin-releasing hormone (GnRH). Inhibition is higher during the anovulatory season, and the appearance of the ovulatory cycle during the non-breeding season is related to decreased opioid inhibition of the hypothalamic-pituitary axis (Turner et *al.*, 1995; Davison *et al.*, 1998) and the rise in LH provoked by naloxone is only evident during the luteal phase (Behrens *et al.*, 1993).

ACTH exerts a negative influence on the implantation of the zygote and also inhibits the development of placenta in mares (Gill *et al.*, 1985). Maternal ACTH administration appears to accelerate foetal maturation and delivery in pony mares (Ousey *et al.*, 2000). Intrafoetal ACTH injections induced both precocious maturation of the equine foetus and a significant reduction in length of gestation (Ousey *et al.*, 1998), as well as an increase in maternal plasma progestagen concentrations (Rossdale *et al.*, 1992). The foetal adrenal gland has been proposed as the key regulator in the timing of parturition in horses (Challis *et al.*, 1993; Cudd & Wood, 1995; Cudd *et al.*, 1995; Card & Manning, 2000), although a negative correlation between plasma ACTH and time to delivery was observed (Silver & Fowden, 1994).

Glucocorticoids have been shown to influence reproductive activity in many mammalian species and stressful events may contribute to low reproductive efficiency due to glucocorticoid-mediated inhibition of hormone secretion (Alexander & Irvine, 1998; Kalantaridou *et al.*, 2004; Breen *et al.*, 2005). During the ovulatory cycle, circulating cortisol concentrations were higher during mid- to late-dioestrus than during oestrus, with the lowest concentrations occurring two days before ovulation. The frequency of follicular checks influenced a significant faecal cortisol metabolites secretion 16 h before ovulation (Berghold *et al.*, 2007). In pregnant mares, a diurnal rhythm in cortisol level was observed only up to the fifth month of pregnancy and in the first week of lactation (Flisińska-Bojanowska *et al.*, 1991; 1992), however a masking effect of pregnancy on the circadian rhythm of cortisol in the second half of pregnancy was reported (Flisińska-Bojanowska *et al.*, 1989).

In this study, our objective was to examine whether physiological circulating β-endorphin, ACTH and cortisol level changed in pregnant mares over 11 months (monthly sampling throughout a year), by simultaneously taking into account the seasonal pattern of these alterations over 12 months, from April to March.

MATERIALS AND METHODS

Experimental animals

The study was carried out from April to March and included 18 clinically healthy Thoroughbred mares (n=12 pregnant and n=6 barren), aged 5−18 years.

The experiment was performed near Catania, Sicily (37.31 °N; 15.4 °E, 150 m above sea level). The breeding season of mares in Sicily occurs from February to July. All mares became pregnant at the same time of the year and the pregnancy was nearly of the same duration in all animals studied. The stage of pregnancy by the time of blood sampling was the same in all mares studied. The data were normalized in relation to medical history and confirmed according to the time of parturition that occurred 7 to 15 days after the final blood sampling.

Mares were normally kept on green pasture from February to July and they were also individually fed twice a day (at

7.00 AM and at 5.00 PM) with 2 kg of commercial feed and 2 kg of oats, and had free access to water. From August to January mares were individually fed twice a day (at 11.00 AM and at 4.00 PM) with 2 kg of commercial feed, 2 kg of oats and 4 kg of hay, and had free access to water. Mares were placed in 20.9 m^2 individual box stalls with interindividual visual contact.

Blood sampling

Blood samples were taken from the jugular vein once monthly over the 12 months. All samples were taken between 7.00 and 8.00 AM to minimize the effect of the circadian rhythm on hormone measurements, in quiet conditions by the same operator. Blood samples were collected in evacuated tubes (Venoject, Terumo®; Belgium) and were transferred into a propylene tube containing 100 μL of EDTA solution and aprotinin (500 kIU/mL of blood, ICN Biomedicals Inc., Aurora, Ohio). Blood samples were centrifuged at 1500 g for 15 min and plasma and serum were stored in polystyrene tubes respectively at −80°C and −20°C for later hormone assays.

Parameters and methods

Blood samples were analysed for β-endorphin, adrenocorticotropic hormone (ACTH) and cortisol. Concentrations of β-endorphin were determined in duplicate utilizing a commercial RIA kit (Peninsula Lab., Inc., Belmont, CA, USA) for human β-endorphin, with 100% cross-reactivity with equines (McCarthy *et al.*, 1993; Mehl *et al.*, 2000). The assay had a detection range for β-endorphin of 1−128 pg/μL with intra- and interassay coefficients of variation (CV) 8% and 19%, respectively. Plasma ACTH concentrations were analysed in duplicate using a commercial RIA kit (ELSA-ACTH, CIS-BioInternational, Gif-sur-Yvette, France) suitable for equines (Ferlazzo *et al.*, 1998), with a detection range for ACTH of 0−440 pmol/L, intra- and interassay CVs of 7% and 15%, respectively. Serum cortisol concentrations were analysed in duplicate using a commercial immunoenzymatic kit supplied by RADIM (Pomezia, Roma, Italy). The assay had a detection range for of 0−1380 pmol/L cortisol with intra- and interassay CVs of 6.2% and 6.9%, respectively.

Statistical analysis

Data are presented as means ± standard deviation (S.D.). Statistical analysis was carried out using one way repeated measures analysis of variance (1-way RM-ANOVA). Significant differences over previous month were established using a *post hoc* multiple comparison test (Bonferroni). Significant differences among mares of different ages were established using Student's unpaired t-test. The level of significance was set at $P<0.05$. All calculations were performed using the PRISM package (GraphPad Software Inc., San Diego, CA).

RESULTS

In pregnant mares (Table 1) circulating β-endorphin concentrations averaged 28.10 and 90.02 pg/mL respectively at the 1st and the 6th gestation month, ACTH concentrations averaged 3.48 pmol/L (4th month) and 5.84 pmol/L (8th month). Circulating cortisol levels showed higher values at the 1st ($P<0.05$) and at the 8th ($P<0.01$), and lower values at the 11th ($P<0.01$) gestation month, as compared to barren mares; cortisol averaged 173.76 and 113.54 nmol/L respectively at the 2nd and the 6th gestation month.

Table 1. Changes of β-endorphin, ACTH and cortisol levels (Mean ± S.D.) in pregnant (n=12) and barren (n=6) mares

	Pregnant mares		
	β-endorphin (pg/mL)	ACTH (pmol/L)	cortisol (nmol/L)
Months of pregnancy			
1	28.10 ± 10.14	4.20 ± 2.25	145.29 ± 68.06*
2	28.60 ± 13.81	4.07 ± 1.55	173.76 ± 55.02
3	35.20 ± 14.19	4.16 ± 1.65	165.18 ± 62.60
4	60.20 ± 49.22	3.48 ± 1.91	133.78 ± 51.70
5	75.01 ± 45.07	4.42 ± 1.74	119.81 ± 30.46
6	90.02 ± 45.25	4.34 ± 1.87	113.54 ± 41.36
7	75.89 ± 44.21	4.63 ± 1.36	135.61 ± 61.54
8	56.66 ± 39.77	5.84 ± 1.69	148.18 ± 48.33**
9	28.46 ± 11.26	4.24 ± 1.44	117.25 ± 33.93
10	41.46 ± 23.42	4.46 ± 1.68	148.40 ± 52.03
11	50.50 ± 29.32	4.26 ± 1.45	119.69 ± 22.30**
1 post partum	70.90 ± 25.54	2.81 ± 0.68	129.49 ± 68.54
Months of sampling			
April	26.70 ± 10.73	3.83 ± 1.02	117.00 ± 43.36
May	27.90 ± 11.82	4.15 ± 2.18	177.02 ± 65.75°
June	34.70 ± 14.72	4.95 ± 1.60	171.73 ± 65.14
July	35.50 ± 16.55	2.84 ± 1.72°	151.31 ± 51.65
August	73.11 ± 47.28°	4.61 ± 1.70	126.58 ± 32.33
September	97.12 ± 48.43	3.69 ± 1.17	111.68 ± 33.71
October	89.89 ± 41.57	4.72 ± 1.57	103.31 ± 39.00
November	63.56 ± 38.37	5.51 ± 1.81	168.19 ± 58.43
December	28.00 ± 12.20	4.98 ± 1.87	116.58 ± 32.23
January	32.12 ± 16.29	3.95 ± 1.17	141.08 ± 51.26
February	43.50 ± 23.01	4.97 ± 1.54	137.49 ± 30.94
March	56.70 ± 23.65	3.18 ± 0.99	97.79 ± 35.27
	Barren mares		
Months of sampling			
April	26.83 ± 8.28	3.56 ± 1.07	73.52 ± 43.79*
May	25.50 ± 11.04	3.77 ± 1.30	199.95 ± 63.63
June	28.00 ± 8.67	4.22 ± 1.40	163.78 ± 57.91
July	30.50 ± 11.20	3.93 ± 1.19	108.73 ± 38.03
August	64.33 ± 56.92	4.47 ± 0.79	122.56 ± 29.89
September	124.33 ± 69.98°	3.85 ± 1.75	106.63 ± 54.82
October	129.66 ± 48.89	5.26 ± 0.76	99.70 ± 38.69
November	70.83 ± 20.49°°	4.60 ± 2.01	113.42 ± 22.15**
December	28.83 ± 10.28°°	3.44 ± 1.94	104.63 ± 42.73
January	27.60 ± 9.26	3.31 ± 1.23	140.74 ± 29.86
February	43.73 ± 13.11	4.31 ± 1.26	144.64 ± 24.88
March	49.80 ± 10.81	3.36 ± 1.02	175.85 ± 37.96***

° P<0.05; °° P<0.01: statistically significant differences vs the preceding month;
* P<0.05; ** P<0.01; *** P<0.001 statistically significant differences vs barren mares.

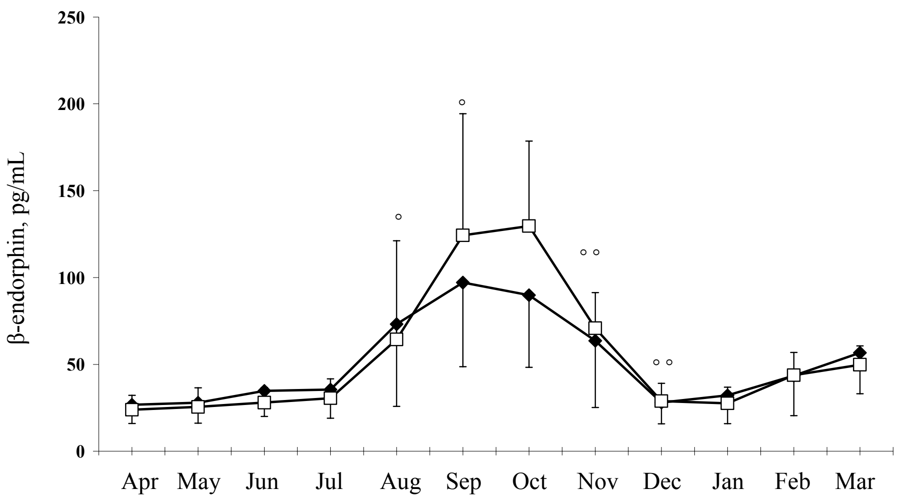

Fig. 1. Seasonal circulating β-endorphin levels in pregnant (n=12; −♦−) and barren (n=6; −□−) mares. °P<0.05; °°P<0.01 vs the previous month.

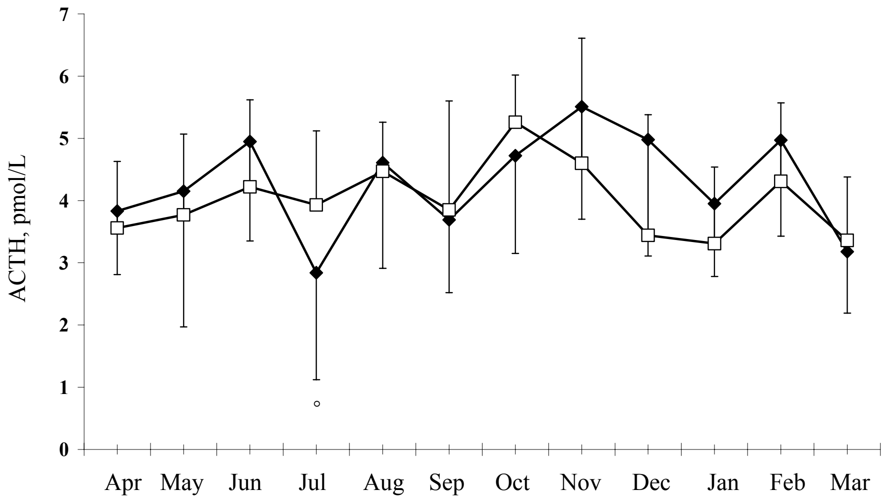

Fig. 2. Seasonal circulating ACTH levels in pregnant (n=12; −♦−) and barren (n=6; −□−) mares. °P<0.05 vs the previous month.

In barren mares (Table 1) circulating β-endorphin concentrations averaged 25.50 and 129.66 pg/mL respectively at May and October, ACTH concentrations averaged 3.31 pmol/L (January) and 4.60 pmol/L (November), and cortisol concentrations averaged 73.52 and 199.95 nmol/L respectively in April and May.

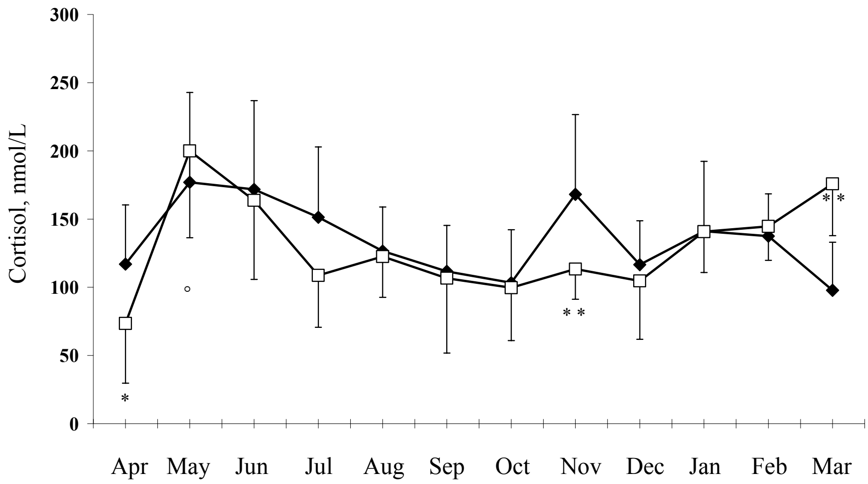

Fig. 3. Seasonal circulating cortisol levels in in pregnant (n=12; −♦−) and barren (n=6; −□−) mares. °P<0.05; vs the previous month, *P<0.05; **P<0.01 vs pregnant mares.

The seasonal patterns of studied parameters showed that circulating β-endorphin (Table 1; Fig. 1) of pregnant mares increased in August (P<0.05 vs the preceding month), and averaged 26.70 and 97.12 pg/mL in April and September respectively. One-way RM-ANOVA showed a seasonal effect on β-endorphin changes (P<0.001). In barren mares, β-endorphin increased in September (P<0.05) and decreased in both November (P<0.01) and December (P<0.01), as compared to previous two months. By April and October, its concentrations averaged 26.83 and 129.66 pg/mL respectively. One-way RM-ANOVA showed a seasonal effect on β-endorphin changes (P<0.0001). No differences (P>0.05) were observed between pregnant and barren mares for any of month.

Circulating ACTH (Table 1; Fig. 2) of pregnant mares decreased at July (P<0.05 vs June), and averaged 2.84 and 5.51 pmol/L respectively at July and November. A seasonal effect on ACTH changes (P<0.001) was shown. In barren mares no differences of circulating ACTH were observed, as compared to previous month and ACTH averaged 3.31 and 5.26 pmol/L at January and October, respectively. No effect of season (P>0.05) was shown for ACTH changes, as well as no differences (P>0.05) between ACTH concentrations of pregnant and barren mares during the same month.

Circulating cortisol (Table 1; Fig. 3) of pregnant mares increased in May (P<0.05) vs the previous month. The levels averaged 97.79 and 177.02 nmol/L respectively in March and May. One-way RM-ANOVA showed a seasonal effect on the cortisol changes (P<0.001). In barren mares circulating cortisol concentrations averaged 73.52 and 199.95 nmol/L in April and May, respectively. A seasonal effect on the cortisol changes was present (P<0.0001). The comparison of pregnant and barren animals showed lower cortisol levels in barren mares in April (P<0.05) and November (P<0.01) and higher levels in March (P<0.01) than those in pregnant mares and in the 1st post-partum month.

No differences (P>0.05) of circulating β-endorphin, ACTH and cortisol concentrations between animals of different ages were observed in both pregnant and barren mares.

DISCUSSION

The comparisons of results obtained in this study with published data reported for horses did not reveal any large discrepancies for circulating β-endorphin (McGreevy & Nicol, 1998; Mehl *et al.*, 1999; Pell & McGreevy, 1999), ACTH (Orth *et al.*, 1982; Card & Manning, 2000) and cortisol (Flisińska-Bojanowska *et al.*, 1989; Tischner & Niezgoda, 2000) levels, and any slight variation might be ascribed to differences in techniques. Some differences may also possibly be explained by influence of physiologic or geographic factors.

The results obtained exclude that some stress conditions may have determined the increase of β-endorphin levels or that stress conditions were produced by blood drawing, as it was always performed at the same time and by the same operator (Canali *et al.*, 1996; Hydbryng *et al.*, 1996).

A relationship with the variable "lighting conditions" would not be reliable as low β-endorphin levels were observed in summer as well as in winter.

The increase of β-endorphin levels in pregnant mares, observed between the 4th and the 8th gestation month respectively, could be correlated to increase of estrogens (Nett *et al.*, 1973; 1975), that activate the opioid inhibition of LH (Aurich *et al.*, 1995).

In pregnant and barren mares an increase of circulating β-endorphin levels was observed between August and November. In pregnant mares published data reported a low increase of β-endorphin levels during September-November (Turner *et al.*, 1995), while in our climatic conditions an insignificant increase of these levels was observed.

The highest β-endorphin levels of barren mares, that corresponded with the anovulatory season, may have been due to the shown tonic inhibition on LH secretion, that could represent an endogenous system of control on the reproductive activity (Beherens *et al.*, 1993; Aurich *et al.*, 1994).

No differences of ACTH levels between pregnant and barren mares showed that ACTH exhibited a similar pattern; moreover the seasonal effect observed only in pregnant mares showed that pregnancy may modify the seasonal cycle of this hormone. It is possible to consider the pregnancy as a stress condition that could modify the physiological pattern of ACTH. The higher cortisol concentration in pregnant mares, at the 1st month and the 8th gestation month and at April and November, than in barren ones, is in agreement with the higher level recorded in pregnant mares than in barren ones during spring-summer (Flisińska-Bojanowska *et al.*, 1989), while it is in disagreement with the data found in Arabian barren mares, in which the mean cortisol level was 20% higher than in pregnant mares (Gill *et al.*, 1985). In addition, our data are in agreement with the highest cortisol levels reported at the 2nd and 3rd gestation month in Thoroughbred mares (Tsumagari *et al.*, 1991). These data showed that pregnancy is an additional factor which may modify circannual cycle in the mare. On the other hand, the progressive decrease of cortisol levels from the 3rd to the 6th month in pregnant mares is partially in agreement with the data observed by Flisińska-Bojanowska *et al.* (1991) that reported a decrease of cortisol levels with the lowest concentrations from the 3rd to the 10th month. Our data are also confirm the results of other authors (Asa *et al.*, 1983; Allen, 1984) who indicated a relationship between suprarenal function and reproduction, and especially between the cortisol levels and gonadotropin secretion during oestrus in mares. The highest cortisol levels observed at the 8th gestation month could be due an increase in maternal plasma progestagen, supporting the hypothesis that this increase occurring in late gestation is the result of foetal adrenocortical activity (Rossdale *et al.*, 1992).

These results could be due to the existence of a negative correlation, specific for equines, between cortisol and oestrogens concentrations, that increase between the 3rd and the 7th–8th month in pregnant mares (Nett *et al.*, 1973; 1975). Results obtained confirm partially the acrophase observed in Arabian pregnant mares in May and June, but not in January-February, nor the acrophase observed in November-December in barren mares (Gill *et al.*, 1985). In addition, the highest cortisol levels observed in barren mares in May and February could be evoked by strong stress conditions as were the oestrus period and breeding season that occurred in our experimental conditions. However the significant seasonal variations both in pregnant and barren mares showed the existence of cortisol seasonal fluctuations, and they do not confirm the cyclicity absence observed only in barren mares (Flisińska-Bojanowska *et al.*, 1991).

The results obtained seem to lend support to the recognition of pregnancy and season in modulating the opioid peptides system and pituitary-adrenal axis in equines. In fact, significant and differentiated effects of season on β-endorphin, ACTH and cortisol changes were demonstrated. These findings indicate that seasonal effects were more efficacious in pregnant than barren mares. In fact the seasonal effects were observed for β-endorphin, ACTH and cortisol changes in pregnant mares and only for β-endorphin and cortisol changes in barren mares. We concluded that pregnancy in mares modified seasonal cycles in secretion of the hormones studied by promoting a significant seasonal effects, that barren condition abolished for ACTH changed. In addition, our data confirmed the main role of seasonal patterns that appeared to synchronize the endogenous rhythm to summer and winter.

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Correspondence:

Esterina Fazio
Department of Morphology, Biochemistry, Physiology and Animal Productions,
Unit of Veterinary Physiology
Faculty of Veterinary Medicine, 9
8168 Messina, Italy.
Phone: +39-90-3503583;
Fax: +39-90–3503975;
e-mail: esterina.fazio@tin.it