The effect of dietary protein on reproduction in the mare. IV. Serum progestagen, FSH, LH and melatonin concentrations during the anovulatory, transitional and ovulatory periods in the non-pregnant mare

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ABSTRACT

The effect of total protein intake and protein quality on the serum concentrations of certain reproductive hormones during the anovulatory, transitional and ovulatory periods were studied in 36 Anglo-Arab mares. High-quality protein stimulated FSH and LH production during the late transitional period. Serum progestagen and melatonin concentrations were unaffected by the quality of protein nutrition during the anovulatory period. Mares receiving high-quality protein exhibited a 10–14-day cyclical pattern of FSH release approximately 4–6 weeks earlier than the mares fed the lower-quality protein diet, and also ovulated 3–4 weeks earlier than the mares on the lower-quality protein diet. Progesterone concentrations during the 1st oestrous cycle after the anovulatory period were unaffected by protein quality in the diet.

Key words: equine, ovulation, protein nutrition, sex hormones, transitional period.

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INTRODUCTION

It is economically important to Thoroughbred breeders that barren and maiden mares exhibit normal, ovulatory oestrous cycles and also conceive as early as possible during the breeding season. In winter, most but not all non-pregnant mares pass through a period of anoestrus, or sexual and ovarian inactivity (anovulatory period), when neither developing follicles nor *corpora lutea* are found in the ovaries[23]. The phases that follow in the seasonal cyclical reproductive rhythm of the mare are the transitional period (early spring) and the active breeding phase (ovulatory period: late spring and summer). These changes are controlled by the reproductive hormones, which in turn are influenced by environmental factors, particularly daylight length, temperature and nutrition[4].

Studies involving ovariectomised mares indicated a definite seasonal pattern in the secretion of LH and FSH, where low baseline LH and FSH concentrations similar to those found in mid-dioestrus were found during the winter months, in contrast to higher LH and FSH concentrations during summer[6]. As these mares were ovariectomised, it can be assumed that external factors, particularly the photoperiod, played an important regulatory role in the absence of ovarian hormones. Furthermore, LH concentrations are lower during the 1st ovulation of the active breeding season compared to subsequent ovulations, which implies that LH secretion is affected by season[4,17]. The concentration of LH was also found to be higher during the penultimate ovulation than during the last ovulation during the ovulatory season[6]. A slight increase in the plasma LH concentration found 15–18 days after the last ovulation of the active breeding season indicates that failure of ovulation at the onset of the anovulatory season is due to a lack of an adequate ovulatory LH surge and final growth of a preovulatory follicle[19].

No correlation was found between FSH concentrations and the number of large follicles from the time of the penultimate ovulation until 31 days after the last ovulation[19]. The decreased LH and FSH concentrations during the non-breeding period indicate that the hypothalamus-hypophysis axis is relatively inactive during the anovulatory period but that this activity increases during the transitional period[4,6,16,19].

The exteroreceptive stimulus of daylight length results in direct stimulation of the nervous system and pineal gland and consequently the secretion of melatonin[14]. Melatonin may have a suppressive effect on the release of GnRH[14]. There is also an inverse correlation between daylight length and melatonin secretion[11]. This means that an increase in daylight length decreases the total secretion of melatonin, which in turn decreases the suppressive effect on GnRH release[7]. Although it is accepted that daylight length plays an important role on the secretion of GnRH, it is not the only factor involved, as nutrition, ambient temperature and other forms of stress also affect ovarian activity[21].

It is not known whether the diet, specifically a deficiency of essential amino-acids, affects the production of GnRH or the protein hormones, LH and FSH, in the horse. The available essential amino-acids will probably be utilised initially for essential body functions and not for production of gonadotrophic hormones. The purpose of this study was to investigate the influence of the dietary protein intake on the concentration of certain reproductive hormones during the anovulatory and transitional periods in the mare and their effect on the onset of the breeding season (ovulatory period).

MATERIALS AND METHODS

Thirty-six barren Anglo-Arab and Thoroughbred mares were allotted evenly according to age and body mass to 4 nutritional groups in which the feed varied in the essential amino-acids and total protein content. The rations were made up as follows:

Group 1: tef hay (5 kg), cubes (2 kg) (crude protein 10.8 %, threonine 0.30 %, methionine 0.11 %, iso-leucine 0.38 %, leucine 0.71 %, lysine 0.55 %, arginine 0.62 %).

Group 2: lucerne hay (5 kg), cubes (2 kg) (crude protein 14.0 %, threonine 0.5 %, methionine 0.10 %, iso-leucine 0.62 %,

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leucine 1.04 %, lysine 0.76 %, arginine 0.62 %).

Group 3: tef hay (5 kg), cubes (2 kg) fishmeal (0.2 kg) (crude protein 12.06 %, threonine 0.35 %, methionine 0.15 %, isoleucine 0.43 %, leucine 0.68 %, lysine 0.65 %, arginine 0.59 %).

Group 4: lucerne hay (5 kg), cubes (2 kg) fishmeal (0.2 kg) (crude protein 15.15 %, threonine 0.53 %, methionine 0.14 %, isoleucine 0.66 %, leucine 1.00 %, lysine 0.86 %, arginine 0.94 %).

The management, feeding procedures, teasing programme and rectal and ultrasonagraphic examinations of the ovaries of the mares, the analytical methods used in the chemical analyses of the rations and the daily crude protein and amino-acid intake of the mares in the different groups remained the same as described previously[23].

Blood samples were collected between 07:00 and 08:00 by venipuncture using 10 mℓ sterile evacuated blood collection tubes without anti-coagulant (Vac-U-Test, Radem Medical), centrifuged for 15 min at 3000 rpm and the serum removed and stored in sterile 10 mℓ glass tubes at 20 °C.

For the purpose of this study a distinction was made between 3 periods, as follows:

Period 1: anovulatory period (1 July – 28 August). Only 1 serum sample per week was collected from each of the 36 mares during this period for the determination of progestagen, FSH, LH and melatonin levels.

Period 2: transitional period (29 August – 31 September). During this period, 1 serum sample was collected from each of the 36 mares every 3rd day for the determination of progestagen, LH and FSH levels until the mare showed signs of oestrus accompanied by the development of a Graafian follicle.

Period 3: ovulatory period (1 October – 31 December). Blood samples were taken every 3rd day during this time until the onset of oestrus. During oestrus, blood samples were taken daily and thereafter every 3rd day until the commencement of the next oestrus period. These samples were analysed for progestagen, LH and FSH.

Hormone assays

Total unconjugated serum progestagen

Serum samples were analysed for total unconjugated progestagens. The radioimmunoassay procedure was principally as described by Youssefnejadian *et al.*[26] using an antibody generated in sheep according to the method of Odell *et al.*[16] against 11 α-hydroxyprogesterone hemisuccinate BSA[5] as modified by Faure[3]. In addition, the antibody shows significant cross-reactivity with α-pregnane-3,20 dione, and to a much lesser extent with other pregnane derivatives of progesterone that are known to occur in the pregnant mare[10]. Cross-reactivity with major adrenocortical C18 and C19 steroids is for all practical purposes non-existent[21].

Follicle-stimulating hormone (FSH)

FSH concentrations were determined using the Amerlex-M FSH (Code DM 3070/3071) kit (Amersham, England). This antibody was used based on the results of Alexander *et al.*[1].

Luteinising hormone (LH)

LH concentrations were determined using the method described by Niswender *et al.*[15] as modified by Visser[24].

Melatonin

The method described by Visser[24] was used for the determination of melatonin concentrations in serum.

Statistical analysis

Analysis of variance was conducted using the LSML 76 computer programme[9]. Means and standard deviations are given when analyses of variance were not used.

RESULTS

Mean serum progestagen, LH and melatonin concentrations of the mares in the 4 dietary groups during the anovulatory and transitional periods (1 July – 28 August) are presented in Table 1. No statistical differences were found in the serum progestagen, LH and melatonin concentrations between the 4 nutritional groups, and therefore only the mean values of the pooled progestagen, LH and melatonin results are given. When the first blood samples were collected on 3 July, 5 mares showed progestagen concentrations above 6 ng/mℓ. Although some mares showed signs of oestrus during the anovulatory period, no further ovulations were detected. Serum LH concentrations varied between 0.31 and 0.83 ng/mℓ in all mares during July and August (anovulatory and transitional periods), which were regarded as low baseline values.

Table 1: **Mean pooled serum progestagen** (ng/mℓ), **LH** (ng/mℓ) **and melatonin** (pg/mℓ) **concentrations of mares in 4 nutritional groups during the anovulatory period** (1 July – 28 August).

Date	Progestagen (ng/mℓ)	LH (ng/mℓ)	Melatonin (pg/mℓ)
3 July	3.7	0.58	14.1
10 July	1.3	0.43	15.1
17 July	1.7	0.43	11.3
24 July	1.5	0.34	11.1
31 July	1.6	0.44	9.2
7 Aug	1.5	0.40	7.2
14 Aug	1.1	0.50	4.5
21 Aug	1.4	0.38	4.6
28 Aug	1.0	0.46	5.2
SEM[a]	0.37	0.10	3.27
SSD[b]	1.09	0.30	9.6
	$p < 0.05$		$p < 0.05$

[a]SEM = standard error of the mean.
[b]SSD = smallest significant difference.

The mean FSH concentrations of the mares in each nutritional group during the anovulatory and transitional periods are given in Fig. 1. During the first 6 weeks of the trial the mean FSH concentrations of all 4 groups remained at baseline concentrations, with no differences recorded between groups. In the last 3 weeks of the anovulatory and transitional periods, FSH concentrations of mares in Groups 1–4 increased from 22 to 26 ng/mℓ, 27.8 to 60.2 ng/mℓ, 40.2 to 50.0 ng/mℓ and 33.2 to 82.4 ng/mℓ respectively (Fig. 1).

The FSH concentrations in Group 1 did not change during the experimental period. In Group 2, FSH concentrations increased ($p \leq 0.05$) with time. In Groups 3 and 4 the FSH concentrations did not differ significantly between each sampling but increased ($p \leq 0.05$) over time.

From 28 August serum progestagen concentrations in each mare were determined every 3rd day until the 1st ovulation. These concentrations remained at low baseline values (<1.5 ng/mℓ), with no indication of luteal activity, during the early transitional period. Serum LH

concentrations were also determined for these samples. In all cases, the LH concentrations were at low baseline values (<0.5 ng/mℓ) and no increases were found during the early transitional period. The first increases in serum LH concentrations were associated with the 1st ovulatory oestrous periods. The cumulative percentages of mares that ovulated during the transitional period in each dietary group are shown in Table 2.

The mean serum LH concentrations of mares in the 4 dietary groups during the 1st and 2nd ovulations after the anovulatory period are shown from Days –4 to +3 of the respective ovulations in Fig. 2.

The FSH concentrations of 18 randomly selected mares were determined during the transitional and ovulatory periods of September and October (Table 3; $n = 18$). Mares receiving fishmeal developed a cyclical pattern with approximate intervals of 10–14 d in their circulating FSH concentrations during the transitional period (Fig. 3B). The results of 2 mares per group (A: Groups 1 and 2, low-quality protein; B: Groups 3 and 4, high-quality protein) are given in Fig 3A,B. The number of days between FSH peaks are shown in Table 3.

The mares were covered during the 1st oestrous period in which ovulation occurred. The duration of the 1st oestrous cycles during the ovulatory period are shown in Table 4. No significant differences between nutritional groups were found. Oestrous cycle duration varied considerably (17–35 d) between mares. Four series (A, B, C, D) of mares ($n = 22$) were identified according to the duration of their oestrous cycles. The duration of the oestrous cycles in Series A mares was 18–20 d, Series B mares 21–24 d, Series C mares 25–29 d and Series D mares 30–36 d. Serum progestagen profiles of these mares are shown in Fig. 4. Serum progestagen concentrations were <1 ng/mℓ during the 24 h after ovulation in all 4 series. From Days 2–6 there was a rapid increase in progestagen concentrations, which remained high (>6 ng/mℓ) until Days 11–15. Depending on the length of the oestrous cycle, low progestagen concentrations were reached between Days 16–18. Mares that had a cycle length of 30–36 d had a prolonged luteal phase of approximately 20 d.

DISCUSSION

During the anovulatory period (July and August), no luteal activity was detected, as demonstrated by the baseline serum progestagen concentrations recorded in all mares in the 4 nutritional groups. During this period LH concentra-

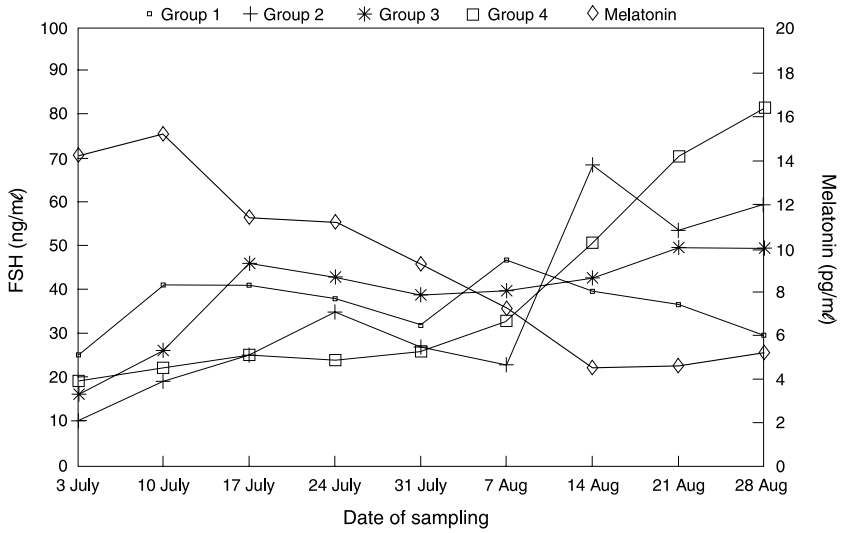

Fig. 1: **Mean serum FSH** (ng/mℓ) **and the mean melatonin** (pg/mℓ) **concentrations of mares in 4 nutritional groups during the anovulatory and transitional periods.**

Table 2: **The cumulative percentage of mares in each nutritional group that ovulated during the transitional period.**

Weeks		Cumulative percentage of mares that ovulated			
		Groups			
		1	2	3	4
August	4		10		
September	1		32		
	2		42	12	12
	3		42	12	12
	4	25	42	38	22
October	1	25	55	75	42
	2	25	55	88	88
	3	42	62	88	88
	4	57	62	100	88
November	1	85	90	100	100
	2	85	100	100	100

Table 3: **Peak FSH** (ng/mℓ) **concentrations and the number of days between peaks of individual mares in different groups during the transitional and ovulatory periods.**

Group	Mare No.	Days between peaks	FSH (ng/ml)	
			Peak 1	Peak 2
1	3	15	68	70
	5	56		
	6	71		
	9	40		
2	11	12	24	23
	13	140		
	14	9	60	46
	18	50		
3	19	24	68	83
	20	9	74	86
	23	9	92	103
	24	80		
	27	8	75	86
4	30	10	50	60
	33	35		
	34	12	76	65
	35	12	123	150
	36	19	118	156

tions were also low. Baseline concentrations were found in all 4 dietary groups and no increases were detected, which is in agreement with the findings of Freedman *et al.*[4]. As expected, no differences in serum LH concentrations were recorded between the nutritional groups during this period, confirming that serum LH concentrations only increase during the transitional period (August – September) and then again markedly just before ovulation[18]. Since these changes in LH concentrations have also been observed in ovariectomised mares by Garcia *et al.*[6], it can be assumed that the seasonal differences in baseline LH secretion are linked to the external factors of daylight length and nutrition[6].

In this trial, substantial differences were recorded between groups when the plasma LH concentrations reached their 1st peak values during the transitional period (September – October). By mid-October, only 42 % of mares in Group 1 had reached LH peak values, compared with 62 % in Group 2 and 88 % each in Groups 3 and 4 (Table 2). It is therefore evident that a direct correlation exists between the quality of protein in the diets of the 4 groups and the timing of peak LH values during the transitional period. Whether the quality of the protein in the ration has a direct effect on the secretion and release of LH or whether it is indirectly mediated through increased secretion of FSH followed by stimulation of follicular growth and oestrogen production, is not clear at this stage.

During the first 6 weeks of the trial (anovulatory and transitional periods), no differences in the mean FSH concentrations between groups were found (Table 1). The significant increase in FSH concentrations in Groups 2, 3 and 4 during the last 3 weeks of the anovulatory period indicate that dietary factors, particularly protein quality, have a direct effect on the production of FSH, which in turn results in an increase in ovarian activity and more follicles that develop to the LH-dependent stage[23]. Although serum FSH concentrations increased steadily in mares in Groups 2, 3 and 4 during the late anovulatory period, no cyclical pattern of FSH release was observed at this stage, possibly because blood samples were only collected once a week. No such increase was seen in Group 1 mares.

FSH concentrations increased steadily during the transitional period to baseline values found in cyclical mares and remained constant at these elevated concentrations. These FSH concentrations then increased above baseline values during the oestrous period and

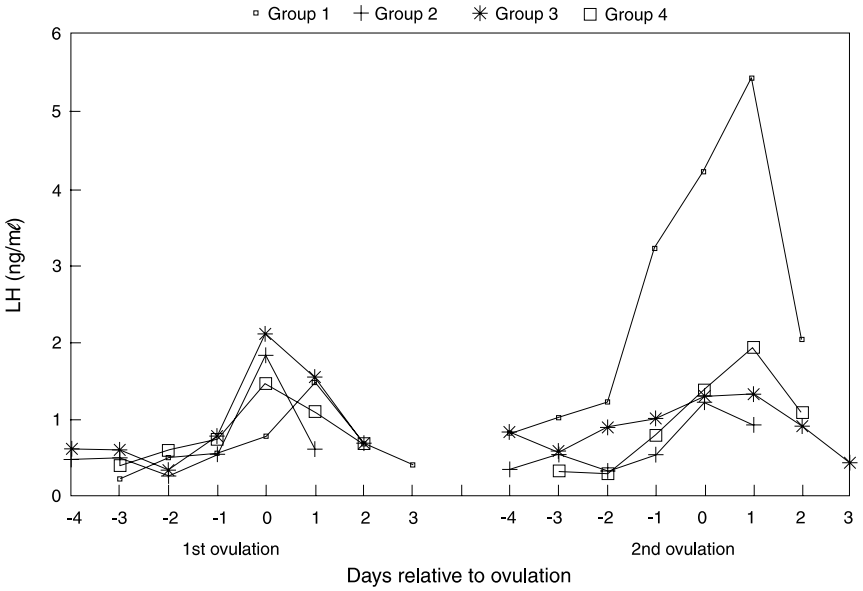

Fig. 2: **Mean serum LH** (ng/mℓ) **concentrations of mares in 4 nutritional groups during the 1st and 2nd ovulations after the anovulatory period.**

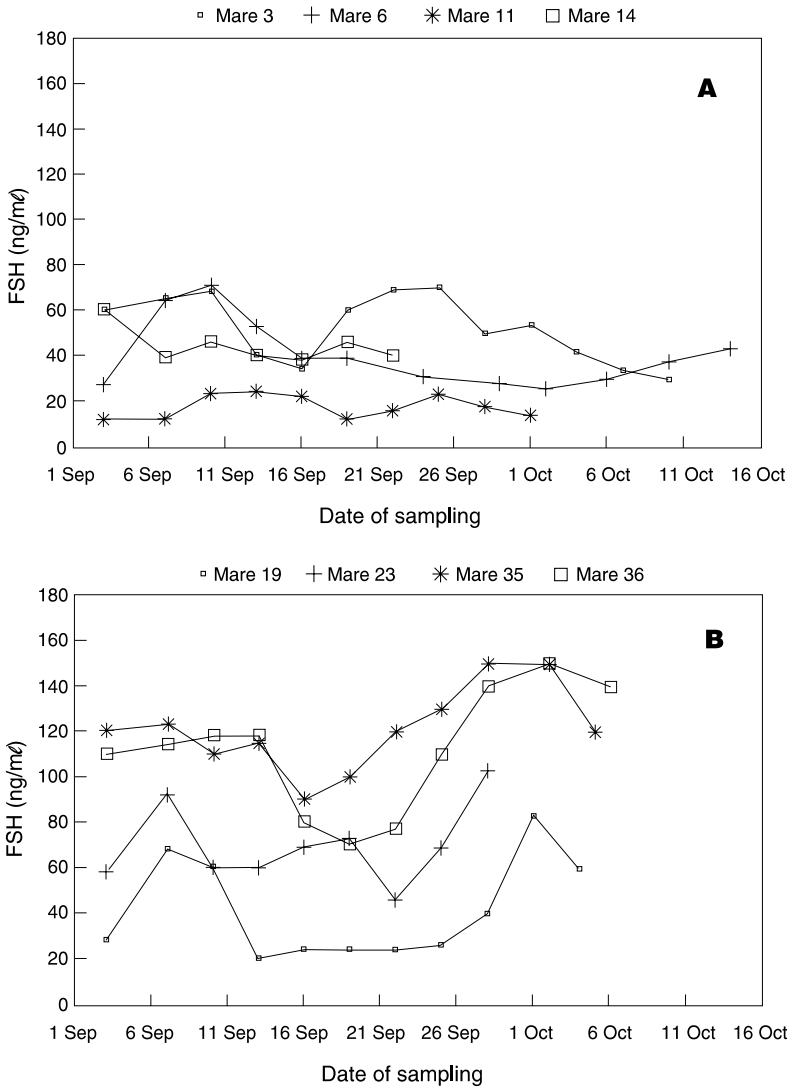

Fig. 3: **Mean serum FSH** (ng/mℓ) **concentrations of individual mares that received no fishmeal** (A) **and those that received fishmeal supplementation** (B) **during the late transitional and ovulatory periods.**

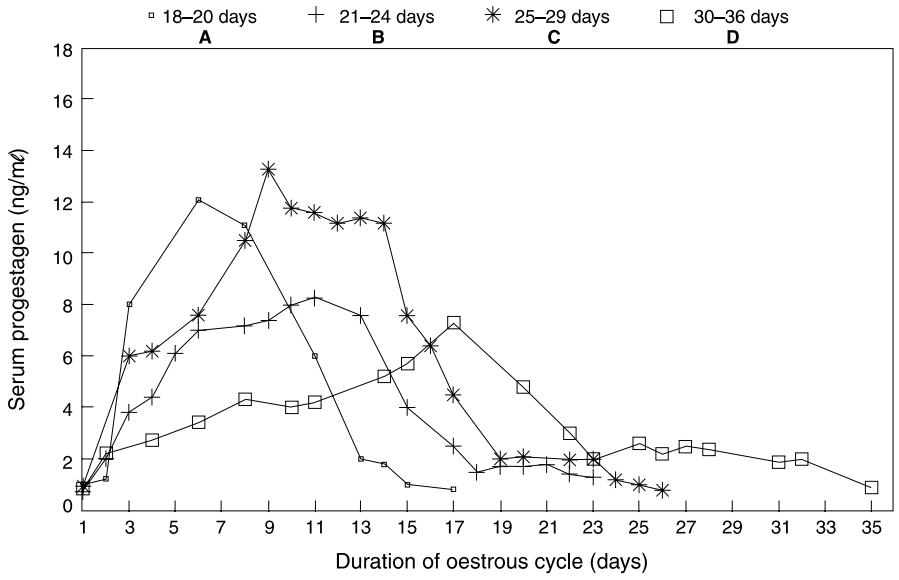

Fig. 4: **Serum progestagen concentrations** (ng/mℓ) **of mares with oestrous cycle durations of 18–20 days** (A)**, 21–24 days** (B)**, 25–29 days** (C) **and 30–36 days** (D) **after their 1st ovulations after the anovulatory and transitional periods** (Day 0 = day of ovulation).

Table 4: **Duration of the 1st oestrous cycle in days during the ovulatory period**.

Group	Number of mares	Duration of oestrous cycle	
		Range	Mean
1	2	17–23	20
2	6	22–35	25.3
3	7	19–31	24
4	7	19–31	24.2

reached a peak before ovulation. A 2nd peak occurred with maximum values recorded from Days 8–12 of the oestrous cycle before levels decreased to baseline, in agreement with the cyclical pattern of FSH release of 10–12 d intervals described by Evans *et al.*[2]. The mares in Groups 3 and 4 (fishmeal-supplemented) showed this cyclical pattern of FSH release earlier in the transitional period (Fig. 3B) compared with mares in Groups 1 and 2 that did not receive fishmeal supplementation. The results also clearly show that cyclical release of FSH does not occur before the 1st ovulation (Fig. 3A). It appears that the source of roughage (tef hay or lucerne hay) does not play a significant role in the cyclical release of FSH.

The ovarian activity of the mares in this study has been described in an earlier paper in this series[23]. From the current results it is clear that mares on a lower plane of protein nutrition, in spite of the fact that the total daily protein intake complied with the National Research Council (NRC), Washington DC (1989), recommendations, showed lower FSH concentrations during the transitional period and also took longer to manifest the 10–12 d cyclical FSH release pattern, which delayed the 1st ovulation in the breeding season[23].

Owing to the large variation in serum progestagen concentrations and oestrous cycle duration between individual mares, it was not possible to detect any nutrition-related effect on progesterone production. Consequently the results of the 21 mares were pooled. Four groups were identified according to the time from the 1st to the 2nd ovulation: Series A (18–20 d, 9 % of mares), Series B (21–24 d, 50 % of mares), Series C (25–29 d, 27 % of mares) and Series D (30–36 d, 14 % of mares). The patterns of the serum progestagen profiles (Fig. 4) are very similar for mares in Series A, B and C, which varied from 18–29 d and included 86 % of the mares. Serum progestagen concentrations increased within 24 h post-ovulation, reaching maximum values on Day 6 (Series A), Days 10–11 (Series B and C) and only on Day 17 in Series D. Although the basic patterns of Series A, B and C were similar, differences occurred when the concentrations declined (Series A, Day 9; Series B, Days 11–12; Series C, Day 15; Series D, Day 18). In most mares, serum progestagen concentrations reached baseline values 3–4 d before the next ovulation. These results show that most of these mares (86 %) had normal functional *corpora lutea* that remained active for 12–15 d, which is in agreement with the literature[8,14,22].

The practical implications are that the progestagen secretion patterns found in the mares in Series A (oestrous cycle duration of 18–20 d) might result in early embryonic death due to early luteal regression, as concentrations decreased to baseline levels by Day 15. In the case of the mares in Series D (oestrous cycle duration of 30–36 d), the initial progestagen concentration might be too low to maintain early pregnancy, because it only increased gradually to 8 ng/mℓ by Day 17 and then decreased to 4 ng/mℓ by Day 20. This is in agreement with the findings of Yuthasastrakosal *et al.*[27] that in sheep, *corpora lutea* formed after the inactive breeding period remained active for a shorter period compared with subsequent ovulations. In addition, the observation has been made in both cattle and sheep that the 1st *corpora lutea* formed after parturition also have a shorter lifespan[12,13,25].

For ease of comparison, the interactions between extrinsic (environmental) and intrinsic (ovarian) factors in regulation of FSH and LH concentrations during the anovulatory, transitional and ovulatory (breeding) periods obtained in these experiments and previous studies cited, are shown diagrammatically in Fig. 5.

1. The interaction between extrinsic (environmental) factors, of which the photoperiod is the most important, and its effect on the central nervous system (pineal gland) and consequently the hypothalamic and hypophysial control of FSH and LH production and release, and 2. Intrinsic (ovarian) factors, which include the effect of steroid hormones produced by the ovaries on the hypothalamus and hypophysis in the cyclical regulation of the normal oestrous cycle during the ovulatory period.

During the anovulatory period, low baseline serum FSH and LH concentrations were maintained in ovariectomised mares[6]. However, the concentrations of these hormones increased as daylight length (photoperiod) increased during the transitional period, and as no ovarian steroids were secreted in these ovariectomised mares, only the extrinsic factors (photoperiod) could be responsible for this increase[6]. High, non-fluctuating basal

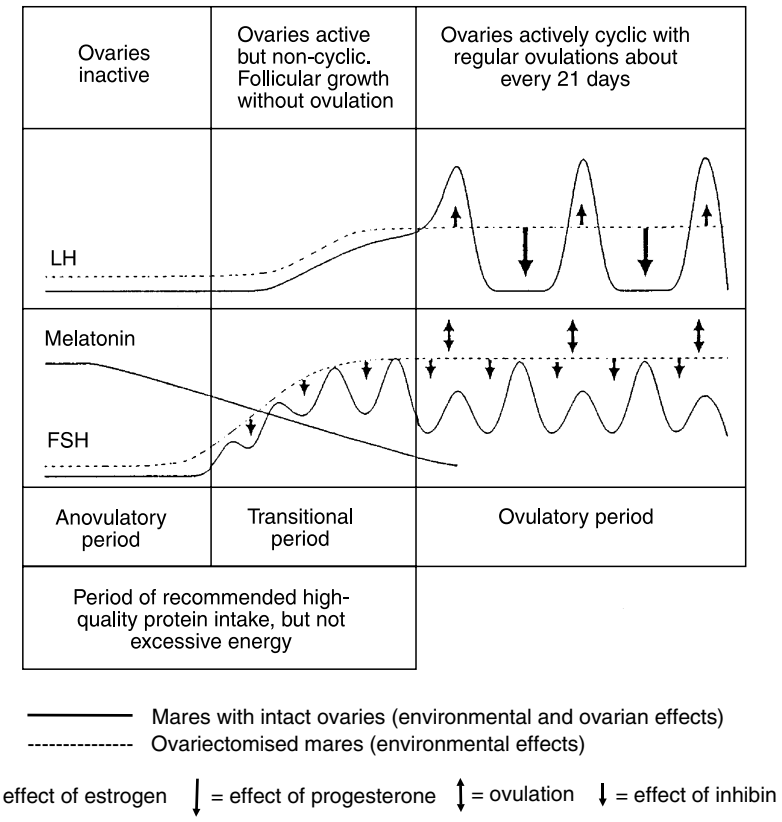

Fig. 5: **Schematic representation of the interaction between the extrinsic** (environmental) **and intrinsic** (ovarian) **factors in the regulation of FSH and LH concentrations during the anovulatory, transitional and ovulatory** (breeding) **periods.**

concentrations of FSH and LH are maintained in these mares during the ovulatory season in the absence of the intrinsic factors (oestrogen, progesterone and inhibin) that are produced in the ovaries. In mares with intact ovaries, the FSH and LH concentrations also remained low, at the same level as those of ovariectomised mares during the anovulatory period. This indicates that the pituitary-ovarian axis is inactive during this period. More importantly, plasma melatonin concentrations are high during the anovulatory period with its short photoperiod, and consequently inhibit the production and release of FSH and LH.

As the concentration of melatonin decreases during the transitional period in ovariectomised mares and mares with intact ovaries, plasma levels of FSH increase, followed by an increase in LH levels. Photoperiod can therefore be considered to be the main extrinsic factor in the regulation of FSH and LH secretion through its effect on melatonin secretion. However, this study reveals that nutrition, another extrinsic factor, plays an important role in the duration of the anovulatory and transitional periods, since the supplementation of high-quality protein in the diet stimulates release of FSH and LH 3–4 weeks earlier in the transitional period. As the concentration of FSH increases, follicular growth is stimulated and consequently secretion of inhibin and oestrogens commences. However, the concentration of the oestrogens produced by these follicles during the early transitional stage is insufficient to stimulate a LH peak, which is necessary for the ovulatory process. Inhibin, also secreted in the walls of the large follicles, suppresses the release of FSH, which results in atresia of the large follicles and also suppresses the growth of new follicles. As the atretic follicles cease producing inhibin, release of FSH occurs and new follicles develop. When these follicles reach the Graafian stage, sufficient oestrogen is produced to stimulate LH release and consequently the first LH peak, which is followed by ovulation. The intrinsic (ovarian) factors, progesterone, oestrogen and inhibin, thus play important roles in the control and functioning of the normal cyclical pattern of the oestrous cycle.

CONCLUSIONS

It is concluded that supplementation of high-quality protein in the diets of mares in Groups 3 and 4 increased FSH concentrations during the later part of the transitional period. These mares therefore exhibited a 10–12 d cyclical FSH release pattern 3–4 weeks before the mares in Groups 1 and 2 that did not receive the high-quality protein. Mares in Groups 3 and 4 ovulated on average approximately 3 weeks earlier than those in Groups 1 and 2. These higher FSH concentrations indicate that follicular development was stimulated to the extent that the *theca interna* layer became active, secreting oestrogen earlier in the transitional period. In addition, the higher oestrogen concentrations are considered to be partly responsible for the earlier ovulation due to stimulation of LH release, resultant peak LH concentrations and ovulation of the LH-dependent Graafian follicle (Fig. 5).

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