

# Evaluation of the effect of two training regimes on the resumption of the ovarian activity by analysis of faecal 20 -hydroxyprogesterone in trotter mares

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Onset of cyclic ovarian activity in mares, after seasonal anoestrus is characterized by the appearance of the first heat and the beginning of seasonal breeding activity. The aim of this work was to evaluate the effect of different training regimes on the resumption of ovarian activity by analysis of 20 -hydroxyprogesterone in faeces. Sixteen trotter mares, housed in single boxes, were allocated into two groups: in group A, mares (n = 8) were trained outside for one hour each day while in group B (n = 8), the mares were remained inside their boxes all day. Faecal and plasma samples were collected every Monday and Friday morning from the 16th of February until the 13th of May and stored at -20 °C until used for hormone assay. Plasma levels of progesterone and faecal level of 20 -hydroxyprogesterone were measured by radioimmunoassay. In group A, the onset of cyclic activity occurred one month earlier than in group B (P < .001). Moreover, during the reproductive season, the resumption of the cyclic activity was more gradual in group A than in group B, in which cyclic activity was enhanced between the 15th and 30th of April. In conclusion, 20 -hydroxyprogesterone concentrations in faeces can be used to evaluate cyclic activity in mares. Resumption of cyclic ovarian activity was positively affected by regular physical work.

**Keywords.** Mare, cyclic activity, 20 -hydroxyprogesterone, faeces, training.

**Évaluation de l'effet de deux régimes d'entraînement sur l'activité ovarienne de la jument de trot par l'analyse de la 20 -hydroxyprogestérone fécale.** Le retour de l'activité ovarienne chez la jument, après un anoestrus saisonnier, s'apprécie essentiellement par la détection des chaleurs. Après la mise au point d'une méthode d'analyse de la 20 -hydroxyprogestérone dans les fèces, l'objectif de ce travail a été d'évaluer l'incidence de l'entraînement physique sur la reprise de l'activité sexuelle de la jument de trot. Hébergées en box individuel, 16 juments de trot ont été réparties en deux groupes homogènes : le groupe A (n = 8) dont les animaux étaient soumis à un entraînement quotidien d'une heure et le groupe B (n = 8) dont les animaux restaient en permanence dans leur box. Des échantillons sanguins et de matières fécales ont été prélevés chaque lundi et vendredi, entre le 16 février et le 13 mai. Les taux plasmatiques de progestérone et les taux fécaux en 20 -hydroxyprogestérone ont été déterminés par dosage radioimmunologique. Dans le groupe A, la reprise de l'activité ovarienne survient un mois plus tôt que pour le groupe B. Par ailleurs, cette reprise de l'activité sexuelle est progressive dans le groupe A alors qu'elle apparaît brusquement dans le groupe B (entre le 15 et le 30 avril). En conclusion, la validation du dosage de la 20 -hydroxyprogestérone permettra l'emploi de la technique développée pour évaluer l'activité ovarienne au sein des deux groupes de juments. Ces observations montrent que l'activité physique favorise la reprise de l'activité sexuelle.

**Mots-clés.** Jument, cyclicité, 20 -hydroxyprogestérone, matières fécales, entraînement.

## INTRODUCTION

The identification of the first heat and the confirmation of pregnancy are two essential aspects for the correct

management of reproduction efficiency in a stud farm, especially if the horses are kept for breeding race horses. Indeed, the race registers use the first of January (in the northern hemisphere) as the official

birthday for all the colts born during the year even though the calendar difference is six months. Consequently, the younger horse will be disadvantaged in competitions as it will always have reached an earlier stage of development.

Horses differ from the other domestic animal species because they often have a lower reproductive efficiency (Ginther, 1992). The causes of this probably lie in the inability to determine the onset of seasonal oestrus activity in the mare, combined with a high risk of reabsorption of the embryo in the first sixty days of pregnancy.

The analysis of plasma progesterone levels allows an accurate evaluation of luteinic activity (Seren *et al.*, 1974) and therefore, the start of the cyclic activity in spring, the exact duration of the ovarian cycle and the possible incidence of precocious abortion in animals diagnosed as pregnant. A limit to this approach is represented by the fact that this type of analysis requires blood samples that could be responsible of a stress. The ability to obtain similar information from a more easily obtained sample would make this approach a much simpler task. It is well known that plasma progesterone is liver metabolized in 20-hydroxyprogesterone and 20 $\alpha$ -hydroxyprogesterone before being partly eliminated in the urine and the faeces. In 1992, Schwarzenberger *et al.* proposed to use 20 $\alpha$ -hydroxyprogesterone concentrations in faeces to evaluate luteinic activity.

The aim of this work was to evaluate the effect of different training routines on the resumption of ovarian activity by the determination of the 20 $\alpha$ -hydroxyprogesterone concentration in the faeces.

## MATERIALS AND METHODS

### Animals

Sixteen trotter mares (8 to 16 years old) were fed with grass hay twice a day and with commercial concentrates to cover the horses requirements defined in the NRC (NRC, 1989). Water was supplied *ad libitum*. The litter was renewed daily. All animals were housed in single boxes and kept in identical conditions.

### Experimental design

Mares were allocated into two groups: in group A, mares (n = 8) were trained outside for one hour each day; in group B (n = 8), the mares were remained inside their boxes the whole day. Samples of faeces and blood were collected every Monday and Friday morning from 16<sup>th</sup> February until 13<sup>th</sup> of May. Blood samples (10 ml) were taken from the jugular vein into EDTA tubes, immediately refrigerated at 4 °C and

centrifuged at 2,000 g for 10 min. The plasma was separated and stored at -20 °C until used for hormone assay. The faeces were immediately stored at -20 °C.

### Hormone determinations

Progesterone plasma levels were determined by a radioimmunoassay procedure adapted from the method described for bovine species by Seren *et al.* (1974). Briefly, 1,000 cpm of progesterone 1,2,6,7- $H^3$  dissolved in phosphate buffer was added to 10 ml screw-cap culture tubes which had been previously rinsed with petroleum ether. Plasma (400  $\mu$ l) added with 600  $\mu$ l of bidistilled water were extracted twice in the screw-cap culture tubes with 10 ml of petroleum ether by mixing for 30 min. The screw-cap culture tubes were centrifuged at 2,300 g for 15 min and the combined supernatants were dried down in a glass vial in a water bath (38 °C) under a stream of nitrogen. Thereafter, 1 ml of phosphate buffer (0.1% gelatin) was added to the vial and mixed for 1 h. An aliquot of the solution (500  $\mu$ l) was used for recovery and correction of procedure losses. Another two aliquots (duplicate) (200  $\mu$ l) were supplied with phosphate buffer (0.1% gelatin) containing the antibody (diluted at 1:3,000) and 100  $\mu$ l of phosphate buffer (0.1% gelatin) containing progesterone 1,2,6,7- $H^3$  (10,000 cpm). After an incubation at 4 °C overnight, 1 ml of dextran-coated charcoal solution was added to the tubes in an ice bath, incubated 15 min and then centrifuged at 3,000 g for 7 min. The supernatants were decanted into scintillation vials and counted. The intra- and interassay coefficients of variation were 8% and 13%, respectively.

The radioimmunoassay for 20 $\alpha$ -hydroxyprogesterone in faeces was performed by a method adapted from the procedure described by Schwarzenberger *et al.* (1992). Briefly, 500  $\mu$ l bidistilled water and 4 ml methyl alcohol were added to 500 mg faeces and the solution was energetically shaken on a vortex for 30 min. Three ml of petroleum ether (95%) were added to remove possible apolar lipids, which may interfere in the analysis, and the whole mixture was shaken for 10 s. After centrifugation (1,500 g for 15 minutes), the sample was incubated at -20 °C for 30 min. During this time, separation of the methanolic and the ether phases occurs. Fifty  $\mu$ l were withdrawn from the methanolic phase and were diluted at a ratio of 1:5 with methyl alcohol. The radioimmunoassay was carried out in glass tubes according to the following protocol. Fifty  $\mu$ l of diluted extract were dried, dissolved in 100  $\mu$ l phosphate buffer (0.05M EDTA, pH 7.5) containing 0.1% bovine serum albumin and then, shaken for 5 min. NET-236-[1,2- $^3H(N)$ ]-20 $\alpha$ -hydroxyprogesterone was added at the rate of 60 pg per tube. Rabbit antiserum raised against

20 $\alpha$ -hydroxyprogesterone-3-CMO-BSA was produced in our laboratory and used at a working dilution of 1:20,000 (100  $\mu$ l per tube). The tubes were shaken for one minute and incubated for 30 min at 37 °C. A charcoal-dextran solution (charcoal 0.25% and dextran 0.02% in phosphate buffer) (250  $\mu$ l per tube) was added. After an incubation of 15 min at 4 °C, the tubes were centrifuged for 7 min at 3,000 g. Finally, 500  $\mu$ l of the supernatant were measured using a scintillation counter.

### Statistical analysis

Data are always presented in terms of means  $\pm$  standard error. The results of the parallelism and recovery tests were subjected to linear regression. The significance of the difference between animals and animal parameters were assessed using Student's *t* tests.

## RESULTS

### Validation of the 20 $\alpha$ -hydroxyprogesterone method in faeces

The parallelism test was carried out by reducing the concentration of the sample by 25% at each level of concentration. The linear regression analysis gave the following equation with a correlation coefficient of 0.993:

$$y = 1.14x - 26.3$$

where *x* = the theoretical concentration in the tube and  
*y* = the observed concentration in the tube.

The recovery test was realised by adding various amounts of 20 $\alpha$ -hydroxyprogesterone (25, 50, 75, 150 and 250 pg of hormone per tube) to the faecal samples. In this case, the linear regression gave the following equation with a correlation coefficient of 0.999:

$$y = 0.82x + 0.85$$

where *x* = the theoretical concentration in the tube and  
*y* = the observed concentration in the tube.

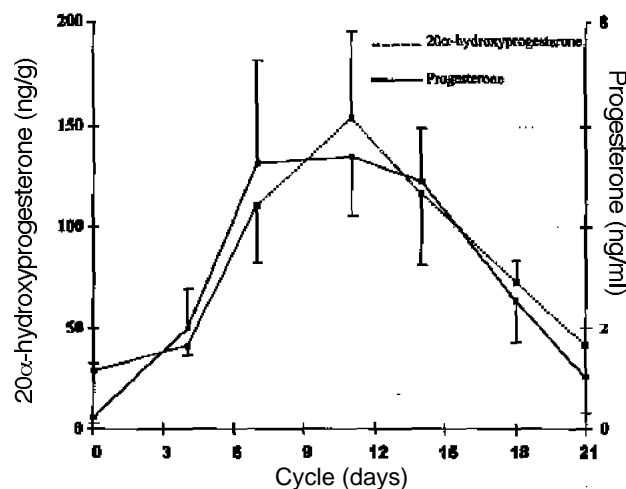
The sensibility of the analyses, in terms of the interpolated dose as a response to zero concentration, minus the statistical error (Programme Riastar, Canberra-Packard), was  $1.76 \pm 0.26$  pg/tube.

### Evolution of plasma progesterone and 20 $\alpha$ -hydroxyprogesterone in faeces during cyclic oestrus.

The analyses of the 20 $\alpha$ -hydroxyprogesterone in the mare's faeces were compared with the plasma

progesterone in four mares (**Figure 1**). The faecal concentrations of the 20 $\alpha$ -hydroxyprogesterone varied from  $25 \pm 2.7$  ng/g during the oestrus (days 0-4 of the cycle) to  $152 \pm 41.6$  ng/g during the dioestrus, while the plasma progesterone varied from  $0.5 \pm 0.1$  ng/ml in oestrus up to a maximum of  $5.5 \pm 1.1$  ng/ml. Correlation between progesterone and 20 $\alpha$ -hydroxyprogesterone during the oestrus cycle was highly significant ( $r = 0.89$ ;  $P < 0.01$ ).

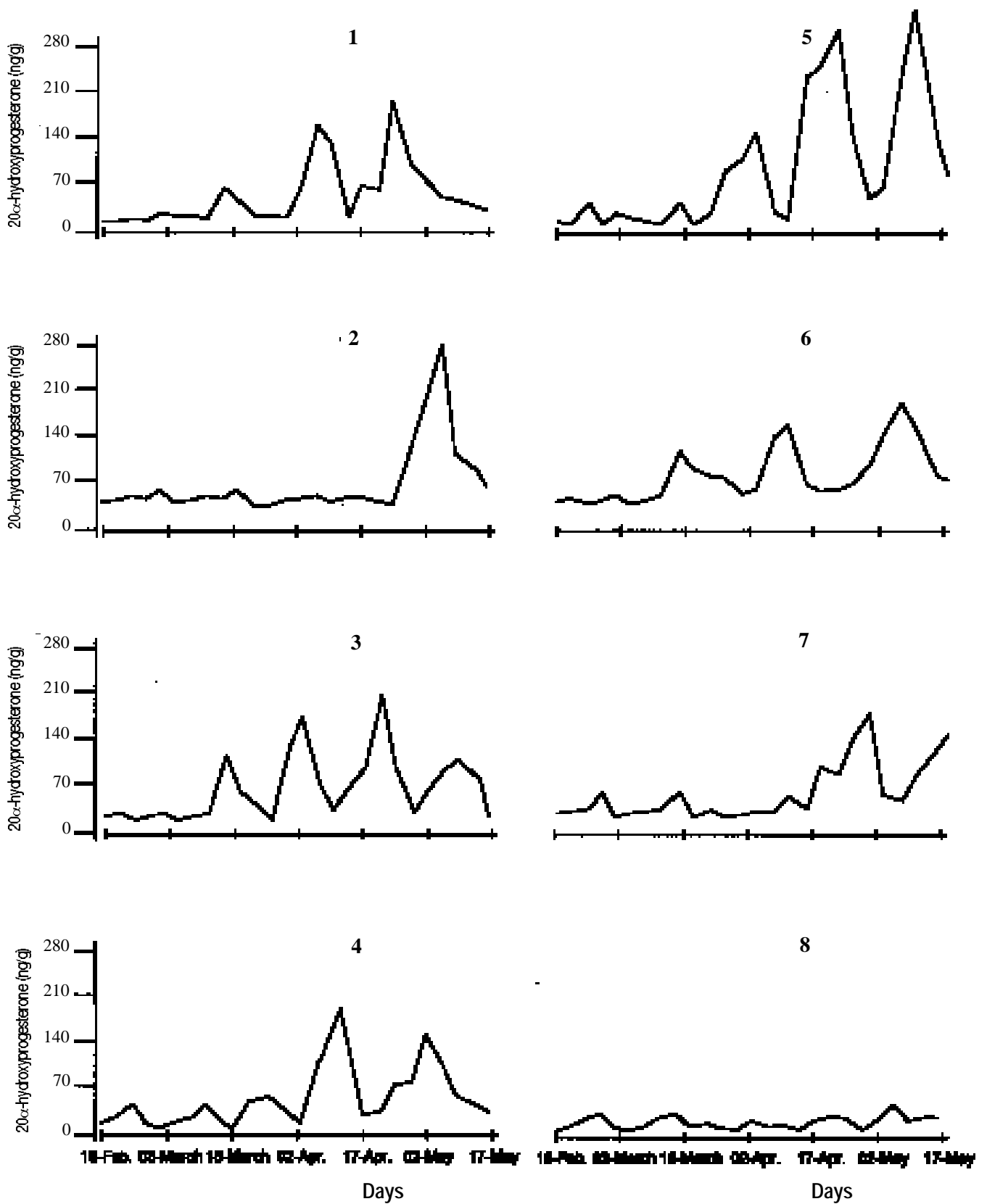
The variation of the faecal progesterone metabolite during the oestrus cycle has been represented in **figures 2** and **3**. During the dioestral phase, the quantity of 20 $\alpha$ -hydroxyprogesterone measured in the faeces progressively increased and reached maximum values towards the 12th-14th day of the cycle (mean value  $212 \pm 46$  ng/g for cyclic mares of group A and B), and then decreased quickly to reach the basal level in the following oestrus.



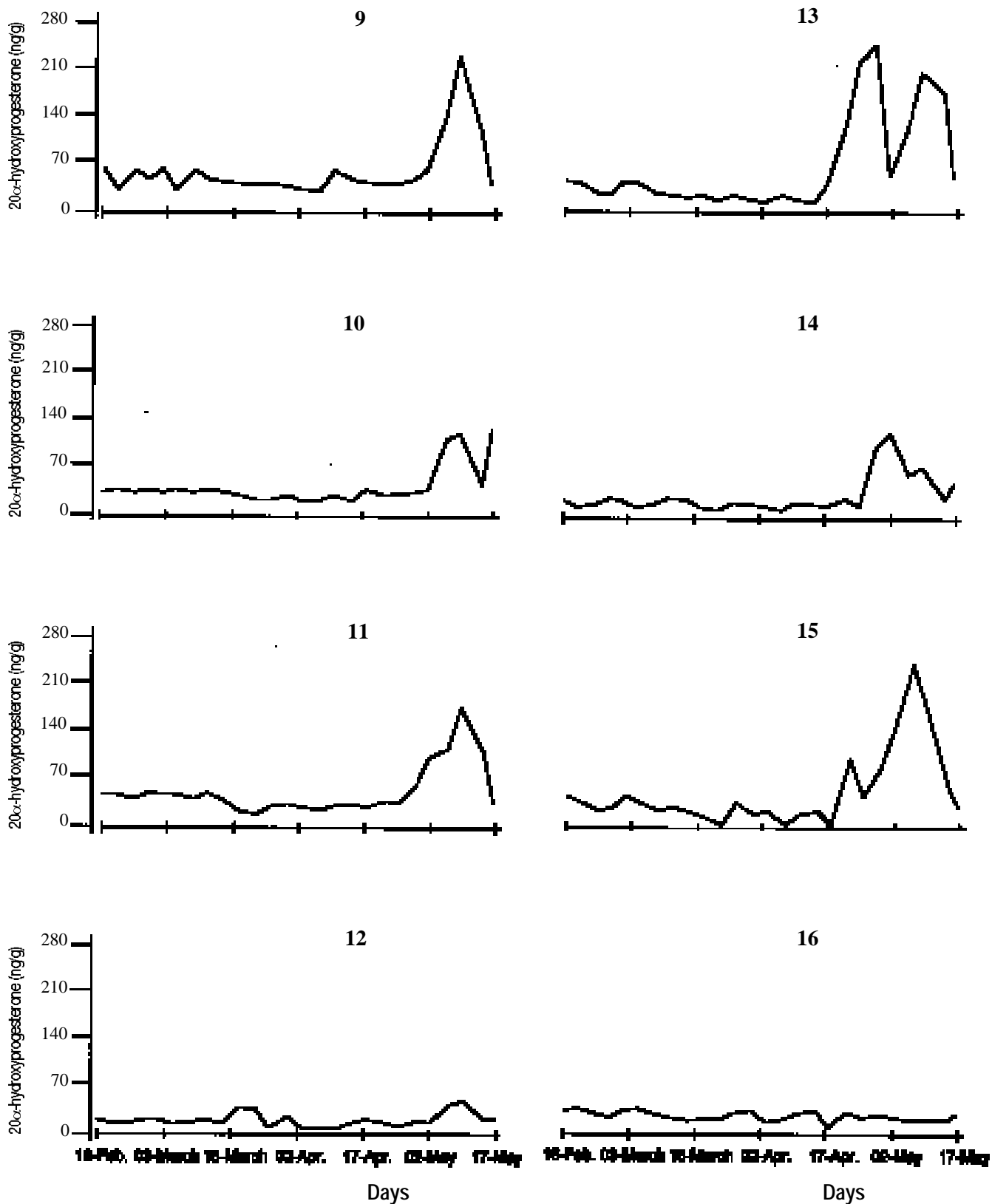
**Figure 1.** Comparison between plasma progesterone and faecal 20 $\alpha$ -hydroxyprogesterone concentrations — *Comparaison entre les taux de progestérone plasmatique et de 20 $\alpha$ -hydroxyprogestérone fécale.*

### Effect of training on faecal 20 $\alpha$ -hydroxyprogesterone concentrations

The analysis of the faecal 20 $\alpha$ -hydroxyprogesterone was used to evaluate the ovarian activity in the mares with or without training. In group with training (group A), the beginning of the reproductive season occurred during the first few days of March and 50% of the mares came out of the acyclic seasonal phase in the first ten days of April. (**Figure 4**). The percentage of acyclic mares progressively decreased to 12.5% at the end of April.

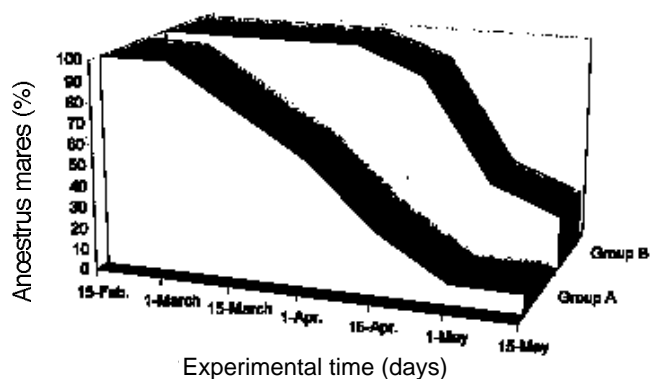


**Figure 2.** 20  $\alpha$ -hydroxyprogesterone profiles in faeces of mares submitted to training (group A) —  
*Profils en 20  $\alpha$ -hydroxyprogestérone fécale dans le groupe de juments soumises à l'entraînement (groupe A).*



**Figure 3.** 20  $\alpha$ -hydroxyprogesterone profiles in faeces of mares non-submitted to training (group B) —  
*Profils en 20  $\alpha$ -hydroxyprogestérone fécale dans le groupe de juments non soumises à l'entraînement (groupe B).*

In group B (without training), the beginning of the reproductive season started after a considerably delay (in the early days of April) and only 50% of the animals were cycling at the end of the month. The percentage of the acyclic mares in this group also diminished progressively, but there were still about 25% of non-cycling animals around the middle of May.



**Figure 4.** Comparison between breeding mares in group A (with training) and group B (without training) — *Comparaison entre les juments du groupe A (avec entraînement) et du groupe B (sans entraînement).*

## DISCUSSION AND CONCLUSION

The high correlation coefficients obtained in the parallelism and the recovery tests ( $r = 0.993$  and  $r = 0.999$ , respectively) demonstrated that the assay of 20 -hydroxyprogesterone in faeces from mares is a reliable and valid assay. This process allowed very accurate profiles of the different concentrations of the metabolite during the oestrus cycle of the mare to be obtained, as reported by Schwarzenberger *et al.* (1992).

The evolution of the 20 -hydroxyprogesterone concentrations in the faeces in cycling mares was similar to the one observed for the plasma progesterone, although out of phase by two or three days. Schwarzenberger *et al.* (1991) regard this delay as the time necessary for the metabolite to pass into the faeces.

The comparison between the variations of the progesterone metabolite in the faeces with the plasma progesterone levels confirmed that it is possible to evaluate the oestrus cycle in the mare by studying the faecal levels of the 20 -hydroxyprogesterone, which represents an extremely precise and objective source of the ovarian functional character, as demonstrated by the trends presented in **figure 1**. The results are in agreement with those of Gunther *et al.* (1980), Ginther (1992) and Schwarzenberger *et al.* (1992). This faeces

approach has two main advantages: the sample of faeces is easy to obtain and to store and the non-invasiveness of the technique allows measurements to be made on animals without disturbing them in any way.

The analyses performed compared two groups of mares. It immediately separated the cycling mares from the acyclic animals. It is evident that in the training group (group A) the resumption of the cyclic activity occurred a month earlier than in group B. In addition, during the reproductive season in breeding mares in group A, the release from anoestrus was gradual in comparison with group B, in which there was a surge of cycling activity between the 15<sup>th</sup> and 30<sup>th</sup> April. Environmental (latitude, temperature and feeding) and physiological conditions were identical. Except light, the only difference was the daily physical activity. It can be observed that in the group of mares subjected to daily physical activity the resumption of the cyclic activity was about one month earlier in comparison with the group which was kept continuously in the stables.

In conclusion and in agreement with Ginther (1992), the physical work to which the animals were subjected appears to positively affect the resumption of the cyclic ovarian activity in mares.

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