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HORMONAL PARAMETERS IN FOAL HAIR: FROM BIRTH TO POST WEANING.

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ABSTRACT

The aim of this study was to investigate the trend of two steroids, cortisol and progesterone (P4) in hair matrix from birth up to post weaning in foals, in order to obtain information about the last period of gestation and peripartum using a non-invasive sampling that provides information on the long term. In order to assess the relationship between mare and foal, we studied the trend of the same hormones in hair samples collected from the mothers of examined foals.

Little is known about hormonal profile and changes that occur in the foetus and newborn. Hair revealed to be a very effective matrix for the study of this delicate period.

Advantages in the use of tegumentary derivatives include: non invasive collection of samples, stability of the capillary structure that incorporates blood-derived hormones or xenobiotics in the growth process, the fact that they are not affected by short-term changes such as circadian rhythm or acute stress and storage at room temperature without any problem.

In contrast to blood, saliva, urine and feces, hair steroids concentrations provide a retrospective picture of the period preceding the time of sampling.

In the equine foetus, hair begins to grow at 270 days of gestation so that cortisol and progesterone concentrations measured in hair samples collected at birth reflect hormones accumulated in the last third of pregnancy, but not those accumulated in the last 15 days of gestation.

The section of hair located beneath the scalp of horse, representing a lag time of 1-2 weeks because the growth of the hair is 1 cm per month, is not collected because hair is always cut or shaved close to the skin and not plucked.

Therefore, hair cortisol concentrations at 30 days of age reflects cortisol accumulated between 15 days before foaling and 15 days of age.

Incorporation of lipophilic substances within hair matrix is still not fully elucidated. Bibliographic sources agree that it occurs mainly through a mechanism of passive diffusion from blood capillaries to the cells of the hair follicle, which then generate the stem.

Therefore, polar bond are formed between some structures of the hair (primarily keratin and melanin) and lipophilic substances contained in the blood.

This study was conducted on 11 Italian trotter foals (6 males and 5 females) and mares, all coming from the same herd in order to standardize environmental conditions and management.

Foalings were included in the study when the criteria defining normal parturition were satisfied: delivery in recumbency; allantochorion rupture and unassisted foetal delivery; dorsal anterior presentation of foetus; second stage of delivery (from allantochorion rupture to foetus expulsion) no longer than 20 min; spontaneous rupture of the umbilical cord; placental expulsion time no longer than 120 min, and normal gross appearance of the placenta. Newborn foals criteria for inclusion were: normal maturity and normal viability, as assessed by Apgar score >7 measured within 5 min after birth, and time to stand up (TSU) and time to first suck (TFS). At term, only multiparous (more than one foaling) mares were allowed to foal spontaneously with no obstetric intervention,

Mares and foals, that remained in herd for the entire period of the study, were daily checked to control health conditions and normal neonatal and pediatric development.

There were no clinically detectable diseases.

Hair samples, collected by electric raser and always by the same operator, were performed in correspondence of left scapular region. After the first sample, only regrowth hair was taken. Samplings, carried out at distance of 30 days, were 8 in foals, starting from birth, and 9 in mares, in which the first sample was collected 30 days before the expected date of delivery. Weaning occurred in all foals at the age of 6 months, at the seventh sampling.

Hair samples were stored in paper envelopes in a dry place at room temperature. They were washed thoroughly with isopropanol to remove contaminations deposited on the outer surface, extracted and finally analyzed by RIA.

Cortisol in foals showed a significant decreasing trend in the first three samples, from 47.64 ± 18.59 pg / mg of birth to 15.84 ± 6.94 pg / mg on samples collected at 1 month of age and to 8.39 ± 4.39 pg / mg of the 2nd month, probably due to the interruption of foetal placental

connection and progressive adaptation to extrauterine life. From the 3rd month, cortisol concentrations remained constant at levels of 4.9 ± 0.65 pg / mg until after weaning.

In mares there were no significant differences in the different samples, because cortisol concentrations remained at levels of 3 ± 1.7 pg / mg. Differences in cortisol concentrations compared to foals trend in peripartum period are probably due to the central role that the utero placental and foetal tissues play in the production of this steroid.

As for progesterone, in foals a significant fall of the concentrations between the first and second sampling (from 469.68 ± 240.6 pg / mg to 184.65 ± 117.58 pg / mg) was noted but, unlike cortisol, in the third sampling concentrations remained high (111.78 ± 123.27 pg / mg). In this sampling, carried on foals still prepubertal, P4 cannot be produced by adrenals in high stress conditions, because the cortisol in the same sample undergoes a significant fall.

We can therefore hypothesize that the source of this steroid is food, since all mares start cycling immediately after delivery and transfer large amounts of progesterone to foals through the milk (up to 10 ng / ml). In the first month of life, in fact, foal, which is constantly with mother, attaches from 60 to 70 times during the day, assuming 160-220 grams of milk for each feeding, for a total of about 12000 g of milk per day; these amounts then decrease with time. Foal in development phase gradually changes from an exclusive milk diet to one more and more similar to that of the mother, managing subjectively the amount of milk to swallow. This could be the cause of the high individual variability observed in the third sampling. In correspondence of the third month of foal life, P4 decreases again significantly (35.96 ± 21.01 pg / mg) and then does not present any further significant change. In mares, that haven't a milk diet, after a significant and physiological increase of P4 in the hair between the first and the second sampling (from 85.87 ± 39.17 pg / mg to 211.94 ± 151.18 pg / mg recorded at birth), the concentrations of the steroid remained stable at levels of 42.19 ± 5.7 pg / mg.

The biological significance of this hormone after birth needs to be explored, in order to clarify how the relationship between mother and fetus doesn't stop with delivery, but continues through the milk.

Milk is also configured not only as a bringer of nutrients and energy but assumes the characteristic of a nutraceutical. Progesterone is an important hormone with multiple functions

including the protective and anabolic ones, characteristics critical for an organism like the foal that in the first month of life doubles its weight.

In conclusion, hair seems to be an excellent matrix for studying the peripartum period both in foal and in mare. Results obtained in the hair, thanks to non-invasiveness and storage in the long term of this matrix, is innovative respect to previous studies carried out on plasma samples. In fact hair permits to study P4 transfer through breast milk, reflecting the nutraceutical functions of this food. It was possible to study foal cortisol and P4 concentrations in the last period of gestation with samples collected at birth, without risk for animals health. Progesterone results confirm that the relationship between mother and foetus does not stop with the delivery.

Finally, weaning, although traumatic for the animal, is not reflected in an increase in hair cortisol.

INTRODUCTION

Progesterone (4-pregnen-3,20-dione or P4) and cortisol are steroids extremely important in equine gestation, parturition and foal life.

During pregnancy P4 plays an important role in maintaining uterine quiescence. It reduces uterine contractility by hyperpolarizing myometrium and by reducing the number of gap junctions and receptors for uterotonic hormones, such as prostaglandin (PG)F 2alpha and oxytocin, on the myometrium (Lye et al., 1998; Challis et al., 2000; Fowden et al., 2008).

During the first 150 days of equine pregnancy, P4 is detectable at high concentrations in maternal plasma and is produced by the primary and secondary corpora lutea (Pashen, 1984). Thereafter plasma P4 levels fall during the last third of gestation (Holtan et al., 1991), and quiescence of uterus is maintained by P4 and metabolites of pregnenolone (P5), collectively known as progestagens (Silver & Fowden, 1994; Chavatte et al., 1997; Ousey, 2004). Their concentrations rise during the last 20 – 30 days of gestation and fall precipitously only in the last 24 – 48 h before delivery. At birth total maternal progestagen concentrations are reduced by 80% compared to pregnancy levels (Fowden et al., 2008) and continue to fall during the 6 h after delivery (Haluska et al., 1987) (Figure 1).

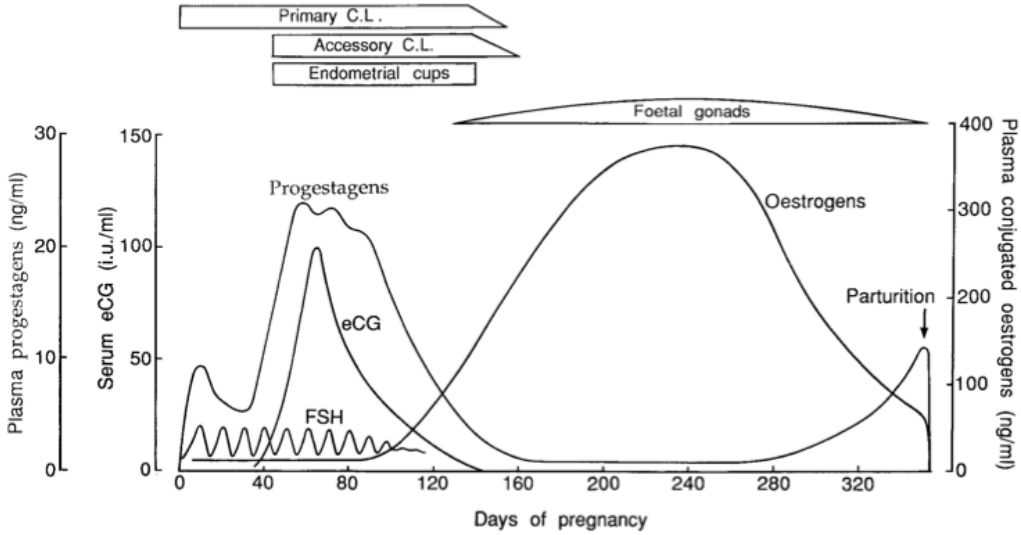


Figure 1: Progestagens trend during mares gestation (Allen WR, 2001).

The source of progestagens for pregnancy maintenance is foeto-placental unit, that converts pregnenolone (P5), derived from foetal circulation, into others progestagens (Fowden et al., 2008) (Figure 2). P5 is converted into P4 in the placenta (Hamon et al., 1991; Han et al., 1995). Most (70%) of P4 produced in late gestation is returned to foetus reaching levels >10 ng/ml (Holtan et al., 1991), the remaining 30% is excreted into the maternal (uterine) circulation along with other progestagens(Ousey, 2006).

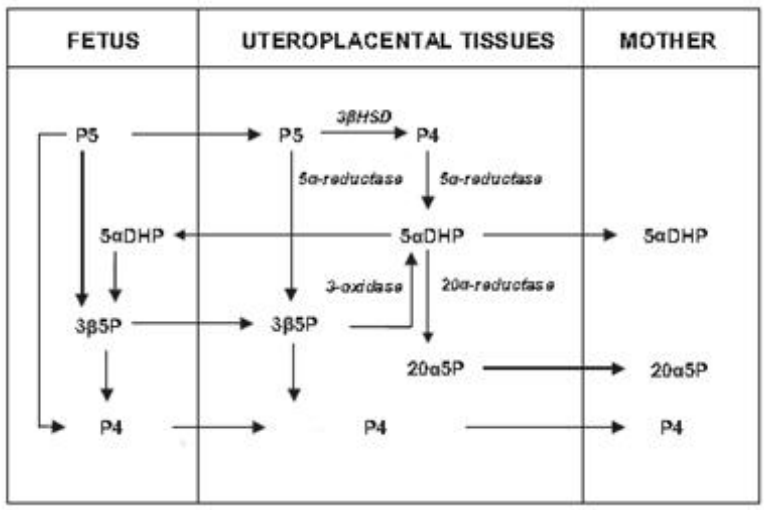


Figure 2: Progestagen production by the foetal and uteroplacental tissues of the horse during late gestation. P5, pregneolone; P4 , progesterone; 5 _ DHP, 5 _ -pregnane-3, 20-dione; 20 _ 5P, 20 _ -hydroxy-5 _ -pregnan-3-one; 3 _ 5P, 3 _ -hydroxy-5 _ -pregnan-20-one (Adapted from Fowden et al, 2008).

As regards cortisol, it isn't produced by foetus for most part of pregnancy. Foetus is also protected for most part of gestation from excessive passage of maternal glucocorticoids through placenta (Nathanielsz et al, 1975). In fact, the enzyme 11β-hydroxysteroid dehydrogenase-2 (11β-HSD2) converts cortisol to the relatively inactive cortisone. The foal in uterus is, therefore, largely protected from excessive exposure to cortisol, may be because this event might be deleterious with respect to premature birth and/or growth-retarding effect (Rossdale et al., 1997). Premature birth with precocious adrenal cortical maturation is usually accompanied by skeletal or other organ defects which result in medium or long-term deficits. Placental activity of 11β-HSD2, that is present from an early stage of gestation (Chavatte et al., 1996), falls during late pregnancy allowing the passage of maternal cortisol. In addition, at the end of gestation foetal adrenals switch from producing P5 to cortisol (Fowden et al., 2008). This would explain both the precipitous fall in maternal progestagen concentrations and the very narrow window of prepartum cortisol surge.

Foetal cortisol levels remain low until 4 – 5 days before term and rise exponentially only in the final 24 – 36 h before birth. The increment in foetal cortisol is, therefore, confined to the last 1 – 2 % of gestation and it is essential for foetal maturation, (Silver, 1990; Silver & Fowden, 1994 ; Fowden et al., 2008 ; Ousey, 2004) (Table 1).

AGE	BASAL LEVELS (ng/ml)	AFTER ACTH (ng/ml)	% INCREMENT
<300 days gestation	6.4±0.7	No response	0
>310 days gestation	11.4±2.4	+6.1±.5	54
0-60 min post partum	80	+48	60
10-29 h post partum	41.2±4.9	+85.8±10.1	208
31-48 h post partum	21.2±3.3	+46.5±5.7	220

Table 1: plasma cortisol concentrations (mean±s.e.) in foetal and newborn foals before and after ACTH stimulus (Rossdale et al, 1997).

Cortisol rise at the end of gestation leads to multi-organ final maturation process (Table 2) and triggers the process of parturition.

TISSUE	CORTISOL EFFECTS
Liver	Deposition of glycogen Induction of glucose-6-phosphatase for gluconeogenesis Induction of β adrenergic receptors Decreased production of cortisol binding globulin
Thyroid	Increase in T3, possibly via induction of hepatic T4 deiodinase
Lung	Indirect evidence for a role in lung maturation
Gut	Indirect evidence for a role in maturation of the gastrointestinal tract
Adrenal	Increased sensitivity to corticotrophin hormone Induction of 17α-deydroxylase for cortisol production
Bone marrow	Increase in leucocytes, particularly neutrophilis
Cardiovascular	Increase in blood pressure and plasma angiotensin converting enzyme

Table 2: maturational effect of the pre-partum cortisol surge on various tissues in equine foetus (Ousey, 2006).

Plasma cortisol and P4 levels at birth are used as indicators of foals maturity and health. Mature foals show cortisol concentrations higher than premature (Silver et al., 1991) and there is a marked difference in plasma progestagens concentrations between normal and premature foals (Houghton et al, 1991). Normal foals show a marked decrease in P4 levels in the first 24 hours after delivery, reaching concentrations so low to be difficult to be determined (Holtan et al, 1991). Conversely in premature foals P4 increases markedly in the 30-50 hours following birth, reaching levels of 50 ng/ml, and then falls to levels of 0.6 ng/ml.

In post partum period weaning could be a possible cause of HPA axis activation and cortisol production, as animals are separated from mothers and could be forced to a change of feeding.

Correct weaning in foal could take into account the individual capacity of foal, its psychological development and its ability to feeding milk-free. Several studies revealed that weaning practiced gradually over time did not result in any negative effect on the development of the young animals, preventing onset of stressful conditions and the incomplete development of digestive system (Kelly & Coutts, 2000; Magistrelli et al., 2013; Poore et al, 2014).

In this period foals plasma P4 concentrations are very low (<0.09 ng/ml; Nogueira et al., 1997) until fillies reach puberty at the age of 10-12 months for spring-born foals and 7-8 months for autumn-born foals, exhibiting progesterone concentrations >2 ng/ml (Brown-Douglas et al., 2004).

Cortisol and P4 levels in post partum period are routinely determined from blood, saliva, urine, faeces and milk samples (Raeside & Liphap, 1975; Capezzuto et al., 2008; Panzani et al., 2009; Nagel at al., 2012;). Whilst these are well-established methods, it is important to note that they reflect acutely circulating steroids levels (plasma or saliva) or integrated steroid secretion over the examined sampling period, usually not exceeding 24 h (urine). In addition, considerable problem when aiming to derive information on long-term cortisol secretion from these measures is the high variability of HPA axis activity depending on circadian rhythmicity (Haritou et al., 2008; Irvine & Alexdander, 1994), acute stress (Kirschbaum et al., 2003), or exercising (Strzelec et al., 2011).

In recent years there has been widespread the use of hair matrix. Steroid measurement in hair samples, used in several species (Koren et al., 2002; Villain et al., 2004; Davenport et al., 2006; Sauvè et al., 2007), provides a “retrospective picture” of previous hormone accumulation and incorporation from plasma over a period of time (Kirschbaum et al., 2009; Gow et al., 2010; Russel et al., 2012). Because it is not invasive, hair concentration measurements represent an interesting alternative for studies focused on the antenatal period because it

avoids blood collection from foetuses. A single measurement (i.e., from hair collected at birth) provides data from a wide but datable antenatal time frame as demonstrated in human by Yamada et al. (2007), and in horse by Comin et al. (2012) and Montillo et al. (2014).

Hair analysis has emerged as a beneficial tool in forensic toxicology, environmental toxicology and in many research laboratories.

The interest in measuring substances, and in particular steroids, in hair started in 1960s to test abuse of steroids in athletes. In fact hair may provide a long-term memory that documents chronic substance abuse (Gaillard et al., 2000; Bévalot et al., 2000).

Benefits of hair analysis are numerous. The ability to detect past lipophilic substances exposure is a unique feature of this matrix, as it provides researches with a “window in the past” (Gow et al., 2010). Secondly, hair sampling is non-invasive and painless compared to traditional blood sample collection. The procedure is quite simple and samples can be taken by a non-professional. Once taken, samples can be stored at room temperature and sent through the mail.

Hair is a complex tissues whose structure and biology are only partially understood. Hair is an epidermal outgrown from a hair follicle. A bulb at the base of the follicle contains matrix cells which give rise to layers of the hair shaft including cuticle, cortex and medulla (Montagna & Van Scott, 1958). Cuticle is the most outermost layer of hair, and the cortex is located between the cuticle and the innermost region, the medulla (Montagna & Van Scott, 1958; Harkey MR, 1993) (Figure 3).

Cortex forms the bulk of the hair shaft and is located immediately beneath the cuticle (Harkey, 1993). Medulla is the innermost region of the hair and consists of scattered cells and hollow space (Harkey, 1993; Ward & Lundgren, 1954). It is small in relation to the cortex and may be continuous, discontinuous or absent in adult hair.

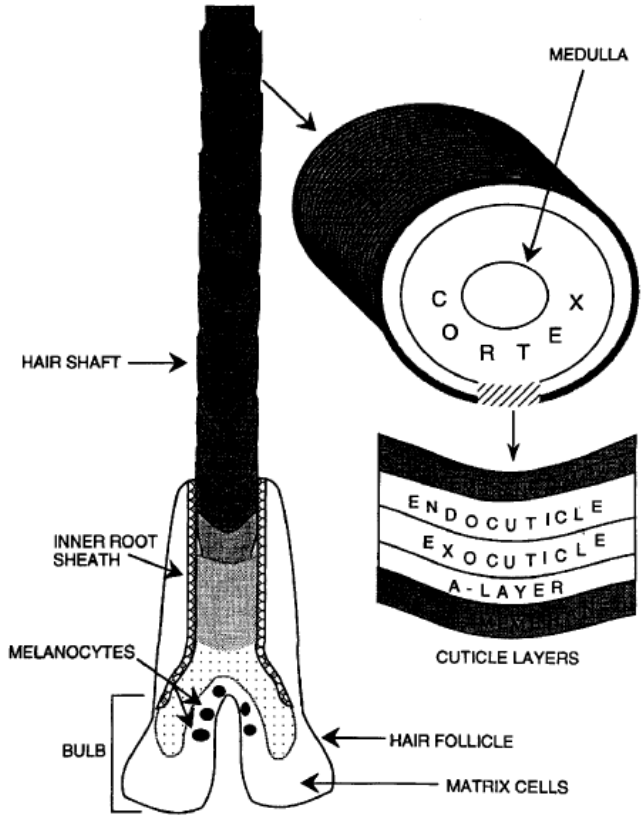


Figure 3: Anatomy of hair follicle and shaft (Klinz P, 1996).

Formation of these layers, so the entire hair growth process, isn't a continuous process, but it follows a cyclical process in which hair follicle goes through three stages:

- Anagen: growth phase
- Catagen: transition phase
- Telogen: resting phase

Matrix cells at the base of the hair follicle undergo morphological and structural changes as they move upward during grown to form different layers of the hair shaft during the anagen

phase. Proteins are synthesized within cells which ultimately determine the durability and strength of the hair shaft which emerges from the follicle. Matrix cells also may acquire pigment as they differentiate to form individual layers of hair and pigment present in hair cells determines the color of the hair shaft.

In this matrix there are several potential binding sites for lipophilic substances. Keratin and melanin contain many polar groups, and evidence strongly suggests that they can serve as attachment points for binding drugs, chemicals and metals (Kintz, 1996). Accordingly, differences between hair types in the structure, organization and concentration of melanin and keratin may affect the degree of binding. Lipids in hair may also serve as possible binding sites. However, the exact binding sites in hair have not been determined.

The mechanism of incorporation of substances in hair has not been clearly defined so far and there are several potential mechanisms that are generally accepted in the scientific community:

- Entrance via blood capillaries during formation. This is considered the primary mechanism of incorporation (Cone, 1996).
- Entrance through sebum and sweat secretion into the hair follicle after formation or outside of the hair shaft as it emerges from the scal. (Henderson, 1993; Cone, 1996).
- Externally substances can ,deposit onto hair from environment via smoke, pollution or physical contact, chemicals, etc. (Henderson, 1993; Cone, 1996). For example, lot of creams and ointments contain steroids that can bound the external surface of hair (Thomson, 2008; Thomson et al., 2010).

AIM OF THE STUDY

The aim of this study was to investigate the trend of two steroids, cortisol and progesterone (P4) in the hair matrix from birth up to post weaning in foals in order to obtain information about the last period of gestation and peripartum, using a non-invasive sampling that provides information on the long term. In order to assess the relationship between mother and foal, we studied the trends of the same hormones in hair samples carried on the mothers of foals examined.

MATERIALS AND METHODS

Although hair sampling is a non-invasive and painless procedure, the study was carried out in accordance with the EC Directive 86/609/EEC for animal experiments.

Animals

This study was conducted in a single standardbred farm (Allevamenti Toniatti s.a.s., Via Falcomer, 16, S. Michele al Tagliamento, 30028 Venezia, Italy). The study was performed on 11 Italian trotter foals (5 females and 6 males), all born in spring 2013, and their mothers.

Only at term and multiparous mares were included in the study. They delivered spontaneously with no obstetric intervention; however, all foalings occurred under staff surveillance and parturition and neonatal parameters were recorded. Foalings were included in this study when the normal parturition criteria (Rossdale & Ricketts, 1980; Whitwell & Jeffcott, 1975; Veronesi et al., 2005) were satisfied; these criteria were defined as delivery in recumbency, allantochorion rupture and unassisted fetal delivery, dorsal anterior fetal presentation, a second stage of delivery (from allantochorion rupture to fetal expulsion) no longer than 20 minutes, spontaneous rupture of the umbilical cord, placental expulsion time no longer than 120 minutes, and normal gross appearance of the placenta. The criteria for including the newborn foals were: normal maturity according to data reported by Rossdale et al (1984), normal viability as assessed by an Apgar score >7 (7.27±0.46) measured within 5 minutes of birth, normal time to stand up (TSU 89.36 ± 29.34 minutes) and normal time to first suckle (TFS 132.9 ± 38.06 minutes) (Koterba, 1990). Data concerning pregnancy length (340.36 ± 11.26 days, mean ± SD), age (9.63 ± 2.94 years) and parity (6.36±4.15) for each mare were

recorded, as well as the sex of foals and their birth-weight (48.54 ± 3.67 Kg) which were measured within 30 minutes of birth, before nursing. To assess successful passive immune transfer, a mandatory prerequisite for the neonatal adaptation process, IgG were determined on serum collected from each foal 24 hours after birth using the NAP ® Foal IgG test (IDEXX).

Animal number	Sex	Date of birth	Gestational lenght	Weight (Kg)	Apgar score	Time of birth	Time to stand up (minutes)	Time to first suck (minutes)
346	F	05/03/13	342	48	8	20:44	125	139
347	F	05/03/13	335	46	8	20:57	110	134
348	F	20/03/13	343	49	7	23:09	99	105
349	F	19/03/13	344	56	7	22:13	100	149
350	M	21/03/13	351	49	7	02:50	106	125
351	M	20/03/13	350	43	8	02:57	62	132
352	M	23/03/13	356	46	7	21:38	109	122
353	F	31/03/13	332	53	7	20:58	26	64
354	M	23/03/13	329	51	7	19:20	72	115
355	M	02/03/13	317	46	7	22:44	65	220
356	M	15/04/13	345	47	7	23:34	109	157

Table n 3: foals clinical data.

The mares and the foals were reared in single stalls during the first 5 days after birth (Figure 3). After that, they were brought into common paddocks, divided for sex of foals and were checked daily for health conditions and normal development (Figure 4). Animals had been grazing continually on excess pasture and received dietary supplementation with mixed alfalfa hay and fodder.

Figures 3 and 4: Delivery room. Sample area is visible on the left side and mare and newborn foal in paddock.

Foals were weaned at the age of 6 months. Animals were maintained in the same groups of foals before and after weaning process. (Figure 5).

Figure 5: weaned foals in common paddock in farm

None of the foals showed clinical disease.

Mares started to cycling immediately after birth and become pregnant at different times as showed in table 4.

MARE	DAYS
A	12
B	10
C	108
D	101
E	104
F	63
H	EMPTY
I	11
K	87
L	40
M	65

Table 4: days between parturition and mares insemination with positive diagnosis of pregnancy.

Hair sample collection

Within 30 minutes of birth, hair samples were carefully collected from the withers region, shaved close to the skin with an electric raser (Figures 6 and 7). The hair samples were successively collected from the same area to obtain only regrown hair. The hair samples were stored in paper envelope at room temperature until the end of the study.


Figures 6 and 7: collection of hair sample. Sampling is easy and can be done by single operator directly in paddock.

The mane and tail hair of horses grows 2.5 cm/month (Dunnet & Lees, 2004), but there are no information on body hair growth rate. At the time of sample collection, hair length was measured, and the growth rate was approximately 1 cm per month, which is in agreement with the hair growth observed in dairy cows (Schutzer & Holtan, 1996).

In the equine fetus, the hair begins to grow at 270 days of gestation (Roberts, 1986; Ginther, 1993), so that cortisol concentrations measured in the hair samples collected at birth reflect the cortisol accumulated in the last third of pregnancy, but not the cortisol accumulated in the last 15 days of gestation. The section of hair located beneath the scalp of horse, representing a lag time of 1-2 weeks because hair grows at the speed of 1 cm per month, was not collected because hair was always cut or shaved close to the skin and not plucked, similarly to what observed in humans by Russel (2012). Therefore, hair cortisol measurement at 30 days of age

reflects the cortisol accumulation between 15 days before foaling and 15 days of age. The same reasoning is fine for other samples.

Extraction from hair

Briefly, the hair strands were washed in 5 ml isopropanol, as suggested by Davenport et al. (2006), and 60 mg of trimmed hair were put in a glass vial and extracted with 3 ml of methanol (Macbeth et al., 2010). The vials were incubated at 37°C for 18 h. Next, liquid in the vial was evaporated to dryness at 37°C under an airstream suction hood. The remaining residue was dissolved in 0.3 ml of phosphate-buffered saline (PBS), 0.05 M, pH 7.5.

Hair cortisol assay

Cortisol concentrations were assayed by a solid-phase microtiter RIA assay as described by Peric et al (2013). The rabbit anti-cortisol antibody was obtained from Biogenesis (Poole, UK). Cross-reactivities of this antibody with other steroids are as follows: cortisol 100%, corticosterone 1.8%, progesterone, pregnegnolone and aldosterone <0.1%). Intra-assay and inter-assay coefficients of variation were 3.6% and 9.8%, respectively. The sensitivity of the assay, calculated as the interpolated dose of the response to a concentration of zero minus the statistical error, was 1.23 pg/well. The relationship between hair cortisol and standard cortisol curve determined through linear regression was linear, with a correlation coefficient of $r = 0.99$, and the model was described by the equation $y = 1.004x + 4.139$.

Hair progesterone assay

Hair progesterone levels were measured using a solid-phase microtitre RIA assay. In brief, a 96-well microtitre plate (Optiplate, Perkin-Elmer Life Science, Boston, MA, USA) was coated with goat anti-rabbit γ-globulin serum diluted 1:1000 in 0.15 mM sodium acetate buffer, pH 9, and the plate was incubated overnight at 4°C. The plate was then washed twice with RIA buffer, pH 7.4, and incubated overnight at 4°C with 200 µl of the rabbit anti-11α-OH-progesterone-hemisuccinate-BSA antibody produced in our laboratory diluted 1:8.000. Cross-reactivities of this antibody with other steroids are as follows: 11 β-OH-progesterone, 46%; 17 α-OH-progesterone, 0.4%; 20α-OH-progesterone, 0.04%; testosterone, 0.08%; cortisol, <0.01%; estradiol 17β, <0.01%; estradiol 17α, <0.01%; and estrone, <0.01%. After washing the plate with RIA buffer, standards (5–200 pg/well), a quality control extract, the test extracts and tracer (Progesterone [1,2,6,7-3H (N)]-, Perkin-Elmer Life Sciences, Boston, MA, USA) were added, and the plate was incubated overnight at 4°C. Bound hormone was separated from the free one by decanting and washing the wells in RIA buffer. After the addition of 200 µl scintillation cocktail, the plate was counted on a beta-counter (Top-Count, Perkin-Elmer Life Sciences, Boston, MA, USA). Intra-assay and inter-assay coefficients of variation were 4.06% and 11.47%, respectively. The sensitivity of the assay, calculated as the interpolated dose of the response to a concentration of zero minus the statistical error, was 0.56 pg/well. The relationship between hair cortisol and standard cortisol curve determined through linear regression was linear, with a correlation coefficient of $r = 0.99$, and the model was described by the equation $y = 0.99x + 1.158$.

STATISTICAL ANALYSIS

The statistical analysis was performed using SPSS for Windows (version 7.5.21, Inc 1989–1997). Normality of data distribution was tested using Shapiro-Wilk test. Hair cortisol and progesterone levels were analyzed using linear mixed models for repeated measures considering sampling period as repeated-measure factor. For multiple comparisons, Bonferroni adjustments were made. Model selections were based on Akaike and Schwarz's Bayesian information criteria (Wang & Goonewardene, 2004). In order to determine linear, quadratic and cubic effects of sampling time on hormone levels, the procedure proposed by Sheck and Ma (2011) was followed. With the aim to assess the possible relations between mare hormone level at delivery and hormone level of foal, hair cortisol and progesterone levels of mare were included as covariate in the mixed model previously explained. Pearson coefficients were used to determine associations between variables.

RESULTS

A total of 11 foals and 11 mares fulfilled criteria for study inclusion. The statistical analysis failed to detect differences ($p>0.05$) in hair cortisol and progesterone concentrations in relation to the clinical data considered (sex of foals, gestational length, foal weight at birth, TSU, TSU, mares age and parity). One mare has been considered outliers and its results are shown separately.

Cortisol and P4 values were not correlated.

Mares values, for both cortisol and progesterone, recorded at birth didn't show correlations with foals values at the same time:

- Correlation between P4 at delivery of mare and P4 at birth of foals: *r=0.111; P=0.761, n=10*
- Correlation between cortisol at delivery of mare and cortisol at birth of foals: *r=0.216; P=0.549, n=10.*

Hormones levels during the whole experimental period have been investigated and results for foals and mares are shown in tables below:

	Sampling time								SEM
	ST0	ST1	ST2	ST3	ST4	ST5	ST6	ST7	
Cortisol, (pg/mg)	51.23a	14.49b	8.52bc	5.58c	5.30c	3.95c	5.20c	6.01c	0.808
Progesterone, (pg/mg)	485.33a	183.27ab	115.76b	30.40b	31.54b	27.31b	39.09b	50.03b	15.286

Table 5: Estimated marginal means of hair cortisol and progesterone levels recorded in foals (ANTE(1) ante dependence CV).

[a,b,c]: Within the same row and factor with unlike letters differ significantly at P<0.05

ST: sampling time.

	Sampling time									SEM
	ST-1	ST0	ST1	ST2	ST3	ST4	ST5	ST6	ST7	
Cortisol, (pg/mg)	3.12	3.68	2.16	2.04	2.32	2.70	2.55	1.89	3.00	0.156
Progesterone, (pg/mg)	99.01a	175.46a	93.23ab	52.58ab	47.12b	35.42b	47.25b	41.97b	47.52b	4.646

Table 6: Estimated marginal means of hair cortisol and progesterone levels recorded in mares (ANTE(1) ante dependence CV).

[a,b,c]:Within the same row and factor with unlike letters differ significantly at P<0.05

ST: sampling time.

However, given that test used resulted non complete to describe perinatal hormonal trends, tests have been applied for the first 3 samples, corresponding to ST1, ST2 and ST3.

	Sampling time				SEM
	ST0	ST1	ST2	ST3	
Cortisol, (pg/mg)	51.23a	14.49b	8.52c	5.58c	1.461
Progesterone, (pg/mg)	485.33a	183.27b	115.76bc	30.40c	30.301

Table 7: Estimated marginal means of hair cortisol and progesterone level recorded in foals (ANTE(1) ante dependence CV)

ST: sampling time;

[a,b,c]: Within the same row and factor with unlike letters differ significantly at P<0.05.

Hair cortisol in foals showed a decreasing trend in the first 3 samples and remained constant in subsequent samples. Progesterone instead decreased significantly from ST0 to ST1 bur remained high in ST2, non showing statistical difference. In ST3 P4 significantly decreased and remained constant up to ST7.

	ST-1	ST0	ST1	ST2	ST3	SEM
Cortisol, (pg/mg)	3.11	3.68	2.16	2.04	2.32	0.148
Progesterone, (pg/mg)	99.01a	175.46a	93.23ab	52.58b	47.12b	7.348

Table 8: Estimated marginal means of hair cortisol and progesterone level recorded in mares (ANTE(1) ante dependence CV).

ST: sampling time;

[a,b,c]: Within the same row and factor with unlike letters differ significantly at P<0.05

In mares hair cortisol concentrations did not show significant changes, while P4 significantly decreases after birth.

Cortisol trend over time

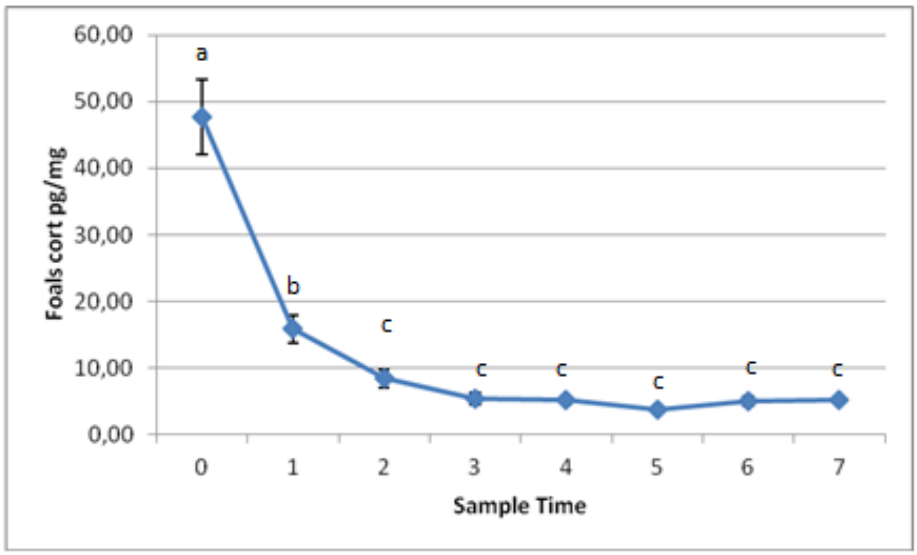


Figure 8: cortisol mean ± standard error in different sample times in 11 foals taken into account in this study.

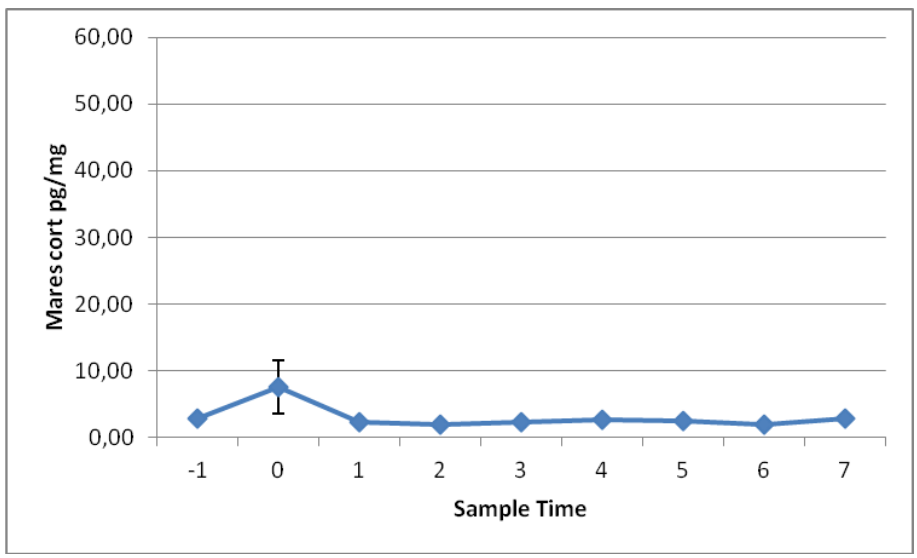


Figure 9: cortisol mean ± standard error in different sample times in 11 mares taken into account in this study.

As previously reported, hair cortisol in foals was significantly affected by sample time (P<0.05). In particular, cortisol level decreased up to three months of age and a linear (P<0.01), quadratic (P<0.01), and cubic (P<0.01) age effect was found. Conversely, from 4 to 7 months of age, an effect of time on cortisol level of foals was not observed (P>0.05). Considering the mares, the level of cortisol was not affected by time (P>0.05).

Progesterone trend over time

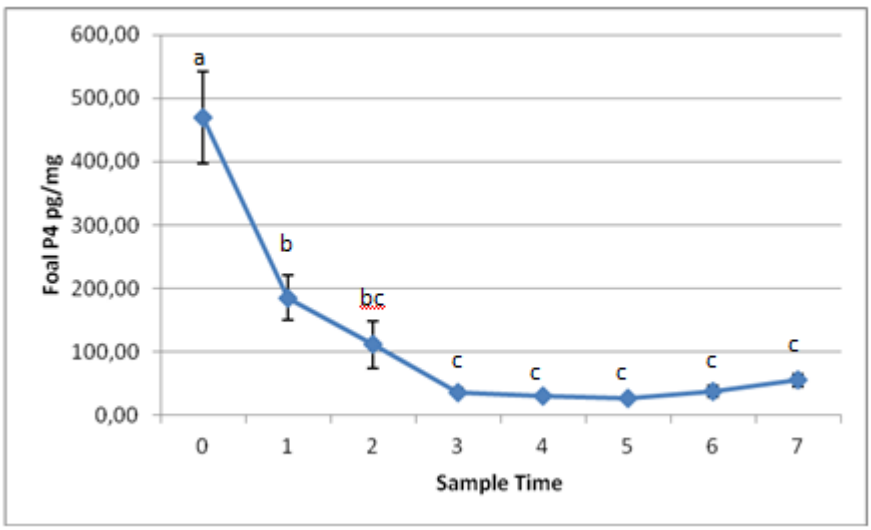


Figure 10: progesterone mean ± standard error in different sample times in 11 foals and taken into account in this study.

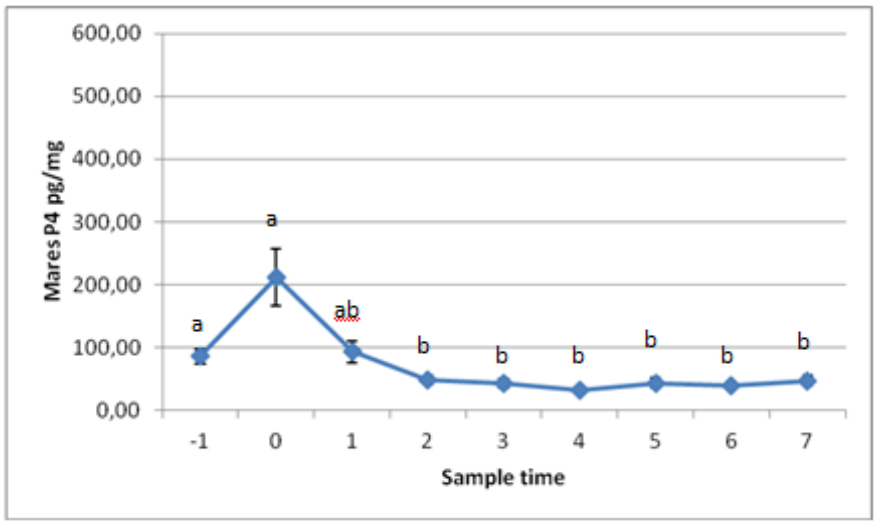


Figure11: progesterone mean ± standard error in different sample times in 11 mares taken into account in this study.

As previously reported, hair progesterone in foals and mares was significantly affected by time ($P<0.05$). In particular, progesterone level decreased up to three months of age and up to three months after delivery for foals and mares respectively. However, progesterone curves revealed a linear ($P<0.01$) and quadratic ($P=0.04$) trend for foals and, similarly, a linear ($P<0.01$) and quadratic ($P=0.03$) trend for mares. Conversely, from 4 to 7 months, an effect of time on progesterone level of foals and mares was not observed ($P>0.05$).

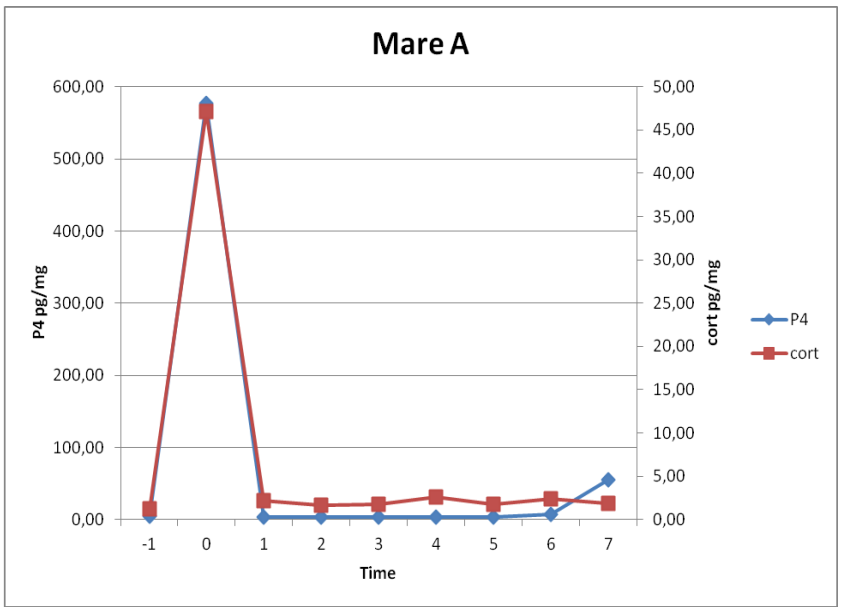
Covariate analysis

With the aim to assess the possible relations between the mare hormone level at delivery and hormone level of foal, other statistical analyses were conducted with hair cortisol and progesterone levels of mare at delivery treated as covariate in the mixed model previously considered. It is interesting to note that hair cortisol and progesterone levels of mare were not

significantly related to hair cortisol (P=0.53) and progesterone levels (P=0.95) of foals respectively.

Mare A results

One mare has been considered outlier and its results are reported in Figure 12. In particular, this animal showed high concentrations at birth sampling, both for cortisol (47.14 pg/mg) and progesterone (576.75 pg/mg). No additional clinical data were reported for this mare.



DISCUSSION

P4 and cortisol are steroids extremely important in equine gestation, parturition and foal life. Cortisol during late pregnancy is fundamental, leading to multi-organ final maturation process and triggering the process of parturition, while progesterone regulates a plethora of biologically distinct processes in a broad range of tissues. These hormones have been studied in hair matrix in foals and mares in order to obtain information about intra uterine life, peripartum and post partum period up to post weaning.

Intra uterine foal life is described by samples collected at birth (ST0). In the equine fetus, hair begins to grow at 270 days of gestation (Roberts, 1986; Ginther, 1993) so that cortisol and progesterone concentrations measured in hair samples collected at birth reflect hormones accumulated in the last third of pregnancy, but not those accumulated in the last 15 days of gestation. The analysis of steroids in the hair is retroactive because any variation in the blood is observed in the hair approximately 15-20 days later, which is the lag-time between shedding and the subsequent anagen period (Russel et al., 2012). Values found at ST0, because all the newborns are mature and viable, are representative of a normal prenatal development in the horse.

At birth both cortisol and progesterone foals levels show a wide range of values, evidencing a different inter-individual ability in hair incorporation. Incorporation of different substances is closely related to their affinity for keratin and melanin, to their lipophilicity (Shen et al., 2009), and depends on their physicochemical properties and functional groups (Shen et al., 2009). Nakahara & Hanajiri (2000) demonstrated that hydroxyl group shows a negative effect on drug incorporation into hair. So progesterone could bind hair more effectively than cortisol.

Peripartum period in foals is described by ST1. Basing on hair growth rate (Schwertl et al., 2003; Montillo et al., 2014), we could estimate that this sampling reflects steroids incorporated between 15 days before foaling and 15 days of age.

In foals between ST0 and ST1 there is a significant fall ($p<0.05$) in both hair cortisol and P4 concentrations, being about 60%. This physiologic fall in steroids concentrations, observed after birth also in plasma samples (Silver at al., 1991), is due to the interruption of foetal-maternal relation. Because hair cortisol detected at 30 days of age represents the cumulative effect of parturition and neonatal period, the significant decreasing trend suggests that preparation for birth is a stronger HPA axis stimulus in comparison to neonatal period, at least in normal, healthy foals.

In the same phase hair mares concentrations of both cortisol and P4 are about 10 times lower than those observed in foals, reflecting the central role of foeto-placental tissues in hormones production in the second half of horse pregnancy. In mothers cortisol concentrations don't show any significant difference, probably because they rise only a few days before birth (Fowden et al., 2008), while P4 decreases significantly after birth.

ST2 and ST3 describe, instead, post partum period in foals. Taking always into account hair growth rate and the fact that we collected only regrowth hair, cortisol and P4 concentrations recorded in these samples are the result of foal steroid production.

Despite in this phase animals have to cope on their own with a huge variety of stressors and have to set physiological and endocrine balances, hair cortisol in foals shows falling trend. This drop, shown also by Comin et al. (2012), describes a framework of progressive decrease in HPA axis activation, probably due a gradual adaptation of foals to extra-uterine life. So foals

compensatory mechanisms and management of breeding enable the foals to buffer environmental changes without a chronic variation in cortisol levels.

In this phase hair foal progesterone, unlike the cortisol, remains high and doesn't show statistical difference in either ST2 or ST3. In this period we can rule out ovarian P4 production, as we find high concentrations both in male and female foals. Moreover, all these animals are prebuberal, since puberty in spring-born foals starts at 10-12 months (Brown-Dounglas et al., 2004).

Progesterone could be produced by adrenals in high stress conditions, positively related to cortisol production, as reported by numerous authors (Purdy et al., 1991; Barbaccia et al., 1996; Genazzani et al., 1998; Girdler et al., 2001; Klatzkin et al., 2006; Wirth et al., 2007). However, we did not notice an HPA axis activation as in the same samples hair cortisol shows a significant fall.

Having thus excluded these endogenous sources of progesterone, we hypothesized that this source could be milk. In fact, all mares start cycling immediately after delivery and transfer to foal large amounts of progesterone through the milk (from 2 ng/ml to 20 ng/ml during estrous cycle and up to 40 ng/ml during pregnancy) (Boyd & Houpt, 1994), similarly to what observed in humans by Grosvenor et al. (1993). It is known, in fact, that steroid hormones pass the blood-milk barrier (Prandi et al, 1994), but only 10% of milk P4 reaches foals circulation, the remaining 90% is inactivated by the first-pass mechanism of the liver (Fritsche & Steinhart, 1999). Therefore, finding this high P4 levels in hair should not be surprising, as two and three months old foals can assume with milk up to 40 g of P4 per day. In fact, in these first months of life, in which foal is constantly with mother, it attaches from 60 to 70 times during the day, with times of 2-3 minutes and taking 160-220 grams of milk for each feeding (Anac Notizie 9-

11/2011-73). In the first two weeks of life foals take from 4 to 8 liters of milk per day, at 2-3 months of life they can suck up to 18 liters of milk per day (Anac Notizie 9-11/2011-73).

ST2 and ST3 also show a high variability, because foals feeding gradually changes from exclusively milk diet to one more and more similar to that of the mothers, so gradually animals reduce the amount of ingested progesterone. This change in feeding is in part subjectively managed by foals in both timing and amounts of milk and solid food ingested, but can also be affected by the will of mothers to breastfeed.

In this moment mares don't show any significant variation in either cortisol or P4 concentrations. Hair P4 levels in mothers are lower than those in foals, because P4 concentration in milk is higher than in blood plasma (Ginther et al., 1976; Gunther et al., 1980).

From ST4 both hair cortisol and P4 concentrations remain constant up to post weaning, that in our study is made in correspondence of ST6.

Correct management of foals in this delicate phase is very important, given that weaning can be a very stressful time for animals. Our results didn't show any HPA axis activation in long period, in fact foals remained with mothers up to 6 months of age and change in feeding was gradual over time, in addition animals were maintained in the same groups of foals before and after weaning process.

From the fourth to the seventh month postpartum, hair cortisol and P4 concentrations in mares don't show any significant variation and we didn't observe differences in P4 levels between pregnant and cycling mares. This is due to the fact that hair performs an integral of P4 production, that is constant during this whole period in pregnant mare and is low only for a few days in cycling ones.

In conclusion, it was possible to study foal cortisol and P4 concentrations in the last period of gestation with a sample made at birth, without risks for animals health. Hair cortisol can be useful as a tool for perinatal non-invasive retrospective studies of HPA axis activity in the horse foals.

It was possible to verify that the highest concentrations of steroids are reached at birth. The end of gestation and parturition corresponds to the maximum activation of all foals systems, in particular those fundamental for its health and vitality.

Hair cortisol and progesterone results allowed the study of some relevant environmental changes in foals, as the passage from intra to extra uterine life and the weaning period. Hair P4 also showed a possible passage of this steroid from mares to foals through the milk.

The biological significance of this hormone after birth needs to be explored, in order to clarify how the relationship between mother and fetus doesn't stop with delivery, but continues through the milk. Recent researches have indicated that many milk hormones/growth factors survive the environment of the gut of the neonate, become absorbed into the neonatal circulation and exert important functions in the neonate (Grosvenor et al. 1993). So mothers thought milk, that is configured not only as a bringer of nutrients and energy but assumes the characteristic of nutraceutical, help foals to adapt to extra uterine environment.

The study of hair P4 could gives additional information about parental care, which increases growth rates, quality, and/or survival of young, and ultimately increases inclusive fitness of parents (Lack, 1968; Trivers, 1972). In fact, verifying the transfer of P4 with hair analysis, we could study the length of relation between mother and foal.

In conclusion, hair seems to be an excellent matrix for the study of peripartum period both in foal and in mare. Results obtained in this matrix, thanks to its non-invasiveness and storage in

the long term, is innovative respect to those from previous studies carried out on plasma samples.

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