



Interrelationship between reproductive hormones and acute phase proteins during estrous cycle and pregnancy in Spanish purebred broodmares

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ABSTRACT

In some species, female steroid hormones modify the profile of acute phase proteins (APPs) during the estrous cycle and pregnancy, according to the ovulation, embryonic implantation and placental development; however, nowadays there's no experimental evidence for equine species. Objectives of this study were: to compare the serum amyloid A (SAA), haptoglobin (Hp) and C-reactive protein (CRP) concentrations between cyclic and pregnant mares, and to analyze the influence of estradiol-17 β (E₂) during estrous cycle or estrone sulfate (E₁) during pregnancy, and progesterone (P₄) on these proteins to assess their potential role to identify the cyclicality or pregnancy in Spanish mares. Blood samples were taken from 20 Purebred Spanish mares on the day of ovulation (day 0), on days +5 and +16 post-ovulation, and then, monthly during the whole pregnancy. SAA, Hp and CRP did not change between day 0, +5 and +16 post-ovulation days. P₄ concentrations were significantly higher on day +16 than on days +5 and 0; and E₂ concentrations were significantly higher on day 0 than day +5. On the other hand, pregnancy was characterized by a progressive increase in the Hp, variable modifications of E₁ and P₄ concentrations, without changes in SAA and CRP. The absence of significant differences in the APPs between days 0, +5 and +16, suggested that these proteins cannot be used as biomarkers of diagnosis of heat or pregnancy in Spanish mares, at least early, since the Hp later increases during the gestation. Nevertheless, it is possible to use them for comparative purposes with other equine breeds, as supervisor instrument of health status in breeding females as diagnostic tools to monitor pregnancy's development and/or subclinical reproductive inflammations, that could lead to the early embryonic death.

1. Introduction

The knowledge of organic interactions and metabolic modifications during the estrous cycle and pregnancy has a special relevance in order to assess the physiological course of reproductive activity and ensure the fertility in mares [Canisso et al., 2014, Coutinho da Silva, Canisso, MacPherson, Johnson & Divers, 2013]. The acute-phase reaction (APR) is a nonspecific response of the immune system, primarily started in response to inflammatory cytokines. The acute phase proteins (APPs) are related with the regulation of the immune response, inflammation, protection against infection and in the recovery of damaged tissues (Cray et al., 2009). Serum amyloid A (SAA) is a major sensitive APP, but not specific, and it is highly considered a relevant marker of inflammation in horses (Jacobsen & Andersen, 2007). The ability to produce a

vast amount of SAA with larger amplitude (>100-fold) represents the response to an inflammatory stimulus (Jacobsen, Vinther, Kjelgaard-Hansen & Nielsen, 2019). Low or undetectable SAA levels (<0.5–20 mg/L) in healthy horses facilitate the interpretation of mildly elevated concentrations; the quick response time and the short half-life of SAA cause its blood increase after an inflammatory stimulus, and a close parallel decrease with successful treatment and resolution of disease (Jacobsen & Andersen, 2007; Jacobsen et al., 2019; Satué, Calvo & Gardón, 2013). Haptoglobin (Hp) is an important APP for the equine species, although it is used to monitor and diagnose the inflammatory processes in horses, Hp has a late response and a relevant variation in healthy animals. Hp concentrations in healthy horses (0.2–1 g/L) increase only 1–10 times during inflammation. Although C-reactive protein (CRP) also responds to inflammation, it is considered a minor APP in

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the horse (Jacobsen & Andersen, 2007).

In mares, the profile of APPs has been analyzed in various pathological conditions such as endometritis (Canisso et al., 2014; Christoffersen et al., 2012; Coutinho da Silva et al., 2013; Sikora et al., 2015; Wolf, Maslchitzky, Gregory, Jobim & Mattos, 2012), placentitis (Coutinho da Silva et al., 2013), early embryonic mortality (Krakowski et al., 2011) and foal heat (Krakowski et al., 2017, 2020). Therefore, the assessment of APP levels is useful as an additional tool for clinical reproductive performance.

In many species, including women, bitches and heifers, steroid hormones like estrogens and progesterone (P_4) modulate the cytokine synthesis during the reproductive cycle, establishing a close relationship between reproductive hormones and APPs' profile. Indeed, plasma Hp rises in heifers during estrous, preceding the estrogens' dynamic, and the lesser extents to P_4 ; in fact, from the ovulation time, a simultaneous increase in E_2 and Hp (Krakowski & Zdzisinska, 2007) is observed. However, the endogenous release of E_2 during the follicular phase induces lower levels of C-reactive protein (CRP) in women (Gaskins et al., 2012), according to the anti-inflammatory properties of estrogens, represented by a reduction of the cytokines' expression and of molecules' adhesion (Straub, 2007). On the contrary, P_4 levels during the luteal phase are associated with an increased CRP (Gaskins et al., 2012; Wander, Brindle & O'Connor, 2008), settling down a positive relationship between this protein and P_4 .

In women, Gatzka et al. (Gatzka et al., 2002) reported levels of Hp 39% lower in pregnant compared with nonpregnant subjects, suggesting that this fall is related to the effect of haemodilution and increased estrogen's concentrations during pregnancy. On the other hand, in pregnant bitches no correlations between this protein and hormonal profile have been established (Ulutas, Musal, Kiral & Bildik, 2009).

However, the CRP response to pregnancy does not seem consistent among different publications. In the bitch, Kuribayashi et al. (Kuribayashi et al., 2003) showed an increase of CRP from 30 to 45 post-ovulation days, more evident during the second half of gestation (Eckersall et al., 1993), when the gradual estrogens' increase and haemodilution occurred (Valtonen et al., 2009), but also during the embryonal implantation and placental development (Kuribayashi et al., 2003). Although Eckersall et al. (Eckersall et al., 1993), Vannucci et al. (Vannucci, Mirandola & Oliveira, 2002) and Kuribayashi et al. (Kuribayashi et al., 2003) suggested that APPs could be used as a source of diagnosis of pregnancy in the bitch, Ulutas et al. (Ulutas et al., 2009) were not agree. In mares, it is unknown whether reproductive hormones can alter the APPs' profile, the magnitude of the inflammatory response to pregnancy, and thus whether these proteins can be used for estrous or pregnancy diagnosis. Therefore, the objectives of this study were 1) to analyze the levels of SAA, Hp and CRP in cyclic and pregnant Spanish Purebred mares, and 2) to evaluate the interrelationship between hormonal profile during estrous (E_2 and P_4) and gestation periods (E_1 and P_4) and SAA, Hp and CRP concentrations.

2. Materials and methods

2.1. Animals

All methods and procedures used in this study were in compliance with the guidelines of the Spanish law (RD 37/2014) that regulated the protection of animals used for scientific purposes. The Animal Ethics Committee for the Care and Use of Animals of the CEU-Cardenal Herrera University (Spain) concluded that the proposed study did not need ethical approval, as it did not qualified as an animal experiment under Spanish law.

Twenty cyclic non-pregnant mares, aged between 4 and 15 years, were used in this study. The inclusion criteria of mares were as following: 1) reproductive history: the presence of normal cyclicity during the previous breeding seasons, absence of reproductive pathologies, as endometritis, pyometra or other processes related to the loss of

fertility or physiological deliveries of viable foals; 2) absence of inflammatory and infectious processes or hospitalized case occurred a month before the start of blood samples and treated with antibiotics or anti-inflammatory and 3) to be wormed and vaccinated. All animals were subjected to the same treatments of feeding and management. The amounts of concentrate were 2–3 kg twice daily, fiber needs were covered with 2–3 kg of alfa-alfa hay and wheat straw. The water supply was ad libitum.

2.2. Reproductive control of mares

Non-pregnant mares were tested when they showed signs of estrous. Transrectal and transvaginal palpations and vaginoscopic examination of vulva, vaginal vestibule, vagina and cervix were carried out to ensure the physiological integrity of the reproductive barrier against external contaminants; hence, the predisposition to suffer of post mating induced endometritis (PMIE) is increased in mares with prepartum injuries, alterations of perineal and vulvar conformation. Transrectal ultrasonography, using a 5 MHz probe (Sonosite 180 Plus) was made to determine the enlargement of the preovulatory follicle, the presence of uterine edema and to predict the ovulation time.

When follicular diameter reached equal to or greater than 35 mm, the mares were intramuscularly treated with 1500 IU of hCG (Chorulon, Intervet). The insemination was carried out 30 h after this hormonal treatment using the cooled semen.

The semen samples came from stallions of the Spanish State Stallion Deposit, located in Zaragoza (Spain), using a standardized protocol in the preparation of the samples. The protocols for sending and using the seminal doses were the same in all cases, for each mare. The insemination volume (60 mL), with a sperm concentration of 300 million sperm / dose, was deposited in the uterine body. Once artificial insemination was performed, the ovulation was verified by ultrasound examination at 48 h, ruling out the presence of uterine inflammatory fluids at days +5 after the ovulation.

Most of the mares were bred once, only 2 of them needed twice to become pregnant.

2.3. Blood samples

In non-pregnant mares the samples were obtained when preovulatory follicle reached the maximum diameter (day 0) and at +5 and +16 post ovulation days, detected by ultrasound evaluation. Once the pregnancy was confirmed, the blood exaction follow-up monthly continued until delivery. All mares became pregnant in late February, March and early April. Mean pregnancy length was of 330.1 ± 10.1 days (range: 290–348 days). The last blood samples were taken 7 to 15 days before parturition. After foaling, all 20 mares were in the lactation period and had foals on their sides.

Blood collections were always performed by jugular venipuncture between 8:00 and 11:00 A.M., using 20 mL disposable syringes, with luer cone (Becton Dickinson Discardit® II) attached to 40 mm. 18–20 G needles (Sterican®, Braun Melsungen AG). A total of 20 mL was collected and each blood sample was added to glass tubes with clot activators and PS granules to collect serum (Tapval®). Samples were protected from light during collection, refrigerated at 4 °C for transport, and then were centrifuged at 3500 rpm for 10 min (P Selecta® Centrifuge); the serum obtained was stored at –20 °C until analyzed.

2.4. Determination of serum amyloid type a (SAA), haptoglobin (hb), C-reactive protein (CRP), 17 β -estradiol- (E_2), estrone sulfate (E_1) and progesterone (P_4) concentrations

The determinations of SAA (mg/dL) were performed with an automatedimmunturbidimetric method adapted to a Cobas Mira Plus, using a test kit LZ SAA, Eiken Chemical Co. (Eiken SAA TIA). The Hp concentrations (mg/L) were analyzed using a Phase Haptoglobin

commercial kit (Tridelata Development Limited, Ireland) and ELISA plate reader. Concentrations of CRP (g/dL) were determined using an automated immunoturbidimetric method adapted to a Cobas Mira Plus with a CRP Randox kit.

Plasma concentrations of E2 (ng/mL) were determined by a competitive enzyme-linked immunosorbent assay (E2 Sensitive, Demeditec ELISA DE4399) validated specifically in the equine species. The limit of detection of E2 was 1.4 ng/mL. The percentage of recovery was of 98.72%. The intra- and inter-assay coefficients of variation (CVs) at low and high concentrations were 7.87% and 5.52%, and 8.78% and 6.78%, respectively. Plasma E1 was also measured by RIA (DSL-5400, Diagnostic Systems Laboratories, Texas, USA). The sensitivity of the assay was 0.03 nmol/L, the intra- and inter-assay CVs were 3.1 and 7.9%, respectively. Plasma concentrations of P4 (ng/mL) were determined using a solid-phase I-125 radioimmunoassay (RIA) (Coat-a-Count Progesterone, Diagnostic Products Co., Los Angeles, LA, USA). The minimal assay sensitivity of P4 was 0.1 ng/mL. The inter- and intraassay CVs were 16.1% and 4.3% at 3.5 ng/mL, 7.3% and 8.5% at 22.5 ng/mL, and 23.3% and 6.4% at 54.8 nmol/L.

2.5. Statistical analyses

Descriptive statistics including mean, maximum and minimum values and standard deviation (SD) of all parameters were obtained. The normality and homoscedasticity of the data were verified using the Kolmogorov-Smirnov and Levene test. Logarithmic transformations of the data were performed to achieve homogeneity of variance. A one-way analysis of variance (ANOVA) was performed to compare the protein fractions (SAA, Hp and CRP) and the corresponding hormones (E2, E1 and P4) among day 0, +5 and +16 post ovulation days as well as between each month of all the 11 months of gestation, and comparisons of means were realized by the Tukey HSD test. The interrelationships between proteins and hormones were examined by linear regression analysis and the correlation was expressed by Pearson's correlation coefficients. A level of significance was considered when $P < 0.05$. Analyses and plots were performed with Statistica 12.0 software (StatSoft, Tulsa).

3. Results

Table 1 shows the descriptive statistics of SAA, Hp, CRP, E2 and P4 concentrations at day 0 and at +5 and +16 post ovulation days, in cyclic mares, and no significant differences of APPs were observed. However, compared to day 0, E2 concentrations were lower ($P < 0.05$), while P4

Table 1

Mean \pm SD of serum amyloid type A (SAA), haptoglobin (Hp), C-reactive protein (CRP), 17- β estradiol (E₂) and progesterone (P₄) in 20 cyclic Spanish purebred mares. Note that E₂ was not measured on day +16, instead, E₁ began to be determined.

Parameters	Post ovulation Days	Ranges	Mean \pm SD
SAA (mg/dL)	0	0.00 - 10.1	7.04
	+5	0.00 - 7.79	2.66 \pm 5.13
	+16	0.00 - 3.54	0.71 \pm 1.58
Hp (g/L)	0	1.70 - 5.52	3.26 \pm 1.12
	+5	1.66 - 11.3	3.97 \pm 2.17
	+16	0.00 - 5.00	1.84 \pm 1.27
CRP (g/dL)	0	3.20 - 21.6	11.1 \pm 6.75
	+5	0.60 - 22.6	10.7 \pm 6.32
	+16	0.30 - 5.00	3.14 \pm 1.95
E ₂ (pg/mL)	0	21.2 - 59.1	37.3 \pm 8.27
	+5	22.2 - 49.0	33.8 \pm 7.08 ^a
P ₄ (ng/mL)	0	22.2 - 49.0	0.43 \pm 0.18
	+5	0.28 - 2.56	1.60 \pm 0.71
	+16	1.10 - 22.5	7.04 \pm 6.56 ^{ab}

Superscripts indicate significant differences vs Day 0: $a = P < 0.05$; vs Day + 5: $b = P < 0.05$.

concentrations were higher ($P < 0.05$) in day +16 than day +5 and day 0.

Table 2 shows the descriptive statistics of SAA and CRP concentrations in pregnant mares, and no significant differences between them were observed during the different months of pregnancy.

Hp concentrations showed a biphasic trend, with a decrease from the 1st to the 3rd month of pregnancy, and a progressive increase from the 4th to 10th month (Fig. 1). Compared to the 3rd month, Hp concentrations increased significantly in the 7th, 8th, 9th and 10th month of gestation ($P < 0.05$). The maximum Hp values were reached in the 9th month compared to the 2nd, 3rd and 4th month, and in the 10th month compared to the 1th and 2nd month ($P < 0.05$).

Serum E1 concentrations increased significantly from the 2nd to the 6th month ($P < 0.05$), and significantly decreased from the 7th month, until the end of gestation ($P < 0.05$) (Fig. 2). The maximum E1 values were reached in the 5th month compared to 9th month, in the 6th month compared to the first and the last trimester, and in the 7th month than the first trimester and the 10th month of pregnancy ($P < 0.05$).

The maximum P4 values were reached in the 2nd and 3rd month compared to the 6th, 7th, 9th and 10th month, and in the 4th month than to the 6th and 7th month ($P < 0.05$) (Fig. 3).

A significant positive correlation between Hp and P4 ($r = 0.49$; $P < 0.05$) in cyclic mares was found (Fig. 4), and no other correlations were observed in pregnant mares (Table 3).

4. Discussion

In cyclic Spanish purebred mares, the concentrations of SAA, Hp and CRP did not change between day 0 and day 5, according to the results observed for CRP in women (Capobianco et al., 2010), bitches (Ulutas et al., 2009; Vannucchi et al., 2002) and cows (Samimi, Aghamiri, Babaei & Heidarabadypor, 2020). Nevertheless, controversial data showed an increase of CRP and SAA concentrations during the ovarian cycle in women (Wander et al., 2008; Kristensen et al., 2009; Clancy, Baerwald & Pierson, 2013) and heifer (Krakowski & Zdzisinska, 2007), indicating the influence of gonadotropins on the protein's profile, and in patients undergoing controlled ovarian hyperstimulation, especially after human chorionic gonadotropin (hCG) administration (Kosaka et al., 2002; Orvieto et al., 2004). However, despite the involvement of cells and inflammatory mediators on follicular rupture, the ovulation does not seem to affect the systemic inflammatory response (Wander et al., 2008), as could be probably occurred in the Spanish Purebred mare. On the contrary, lower CRP concentrations due to the endogenous E₂ release during the follicular phase were observed (Gaskins et al., 2012), suggesting that estrogens regulate the anti-inflammatory effects (Chakrabarti, Lekontseva & Davidge, 2008), with a reduction of the cytokines expression and molecular adhesion (Straub, 2007). Another point is that, the Hp concentration during estrous phase was significantly higher compared to diestrous and proestrous in non-synchronized estrous cycle in cows (Samimi et al., 2020), according to its higher

Table 2

Mean \pm SD of serum amyloid type A (SAA) and C-reactive protein (CRP) in 20 pregnant Spanish purebred mares from 1 to 11 months.

Months	SAA (mg/dL)	Mean \pm SD	CRP (g/dL)	Mean \pm SD
	Ranges		Ranges	
1	0.00 - 3.54	0.71 \pm 1.58	0.30 - 5.00	3.14 \pm 1.95
2	0.00 - 3.30	0.93 \pm 1.59	0.00 - 14.2	7.06 \pm 5.99
3	0.00 - 3.58	1.01 \pm 1.73	0.60 - 36.5	12.2 \pm 13.8
4	0.00 - 4.73	2.60 \pm 2.09	0.00 - 34.4	11.2 \pm 11.8
5	3.35 - 9.90	4.95 \pm 2.49	1.00 - 30.5	11.2 \pm 11.1
6	0.00 - 3.82	1.41 \pm 1.94	2.10 - 28.4	13.1 \pm 9.70
7	0.00 - 7.20	2.03 \pm 2.82	0.00 - 14.5	6.60 \pm 4.99
8	0.00 - 3.82	3.84 \pm 2.33	0.00 - 15.2	5.05 \pm 7.00
9	0.00 - 3.82	2.05 \pm 1.94	0.00 - 17.4	6.87 \pm 6.65
10	0.00 - 4.12	3.01 \pm 1.38	0.00 - 18.7	7.43 \pm 7.28
11	0.00 - 0.01	0.10 \pm 0.01	0.00 - 14.9	3.38 \pm 6.47

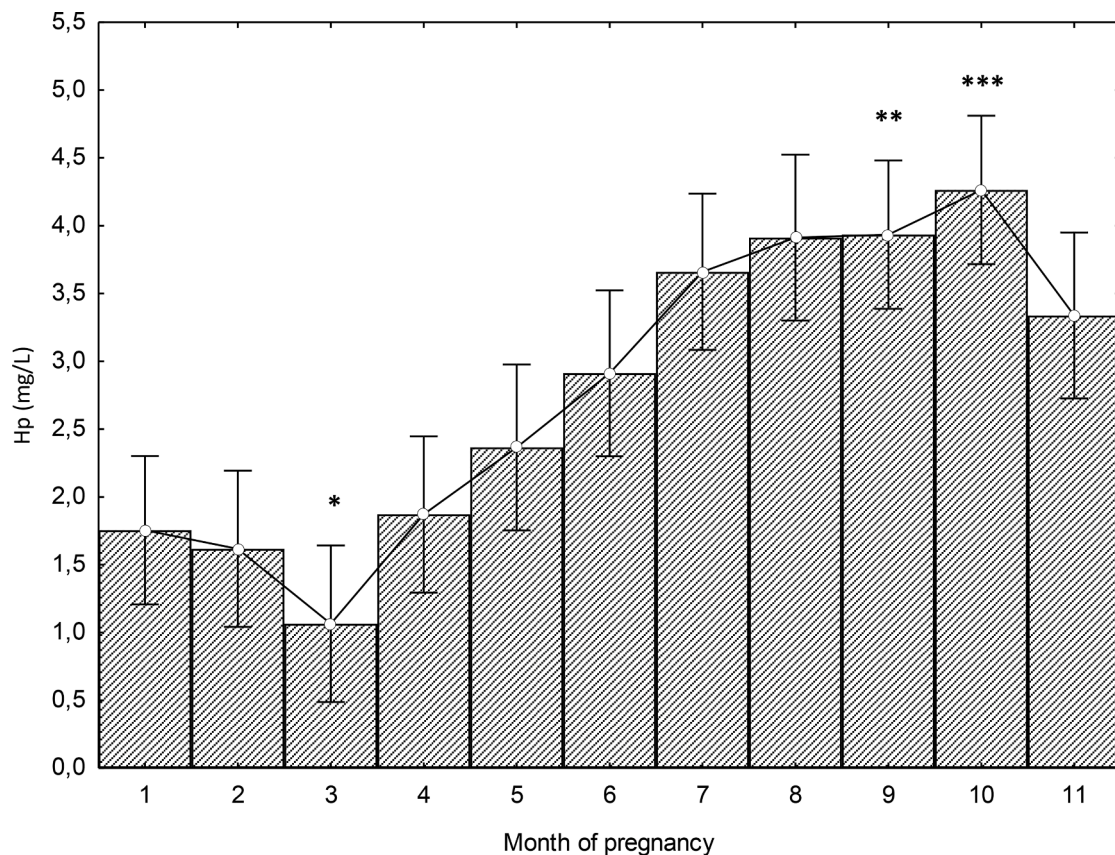


Fig. 1. Concentrations (mean \pm SD) of haptoglobin (Hp) in 20 pregnant Spanish purebred mares. Symbols indicate significant differences: *vs 7, 8, 9, 10 month of pregnancy ($P < 0.05$), ** vs 2, 4 month of pregnancy ($P < 0.05$), *** vs 1, 2 month of pregnancy ($P < 0.05$).

production from the reproductive tract around the time of ovulation. What is more, behavioral stress during the ovulation, as the increased oxidative activity and released free radicals across estrous, could be other reasons for the Hp increases (Sciorsci, Galgano, Mutinati & Rizzo, 2020).

During luteal phase, the endogenous release of P_4 substantially induces an increase of CRP (Gaskins et al., 2012; Wander et al., 2008), promoting both the neutrophil chemotaxis and an increased production of some inflammatory mediators, as interleukin-6 (IL-6). In Holstein heifers short term progesterone-releasing intravaginal device treatment increased Hp concentrations, due to local vaginal inflammation (Kuru, Merhan, Kaya, Oral & Kukurt, 2015). Nevertheless, the relation established between Hp and P_4 was limited in Spanish purebred mares (Satué et al., 2018).

Unlike what happens in bitches (Ulutas et al., 2009; Vannucchi et al., 2002), the determination of different APPs does not support sampling or clinical decision making at specific time points of estrous cycle in Spanish Purebred mares.

In nonpregnant and pregnant Spanish Purebred mares the profile of SAA did not change, confirming the results obtained in bitches (Ulutas et al., 2009) and sheep (Albay et al., 2014). However, previous studies carried out in Thoroughbred and Quarter Horse mares by Nunokawa et al. (Nunokawa et al., 1993) and Satoh et al. (Satoh, Fujinaga, Okumura & Hagio, 1995) showed the existence of variations in SAA during the last four months of gestation, with the higher values than those shown in Spanish purebred mares. SAA levels are generally elevated close the parturition, due to tissue damage, induced by the passage of the fetus through the birth's canal and after parturition, with a rapid and short-term increase recorded in mares (Coutinho da Silva et al., 2013). According to our results the SAA cannot be used as a source of diagnosis of pregnancy in Spanish Purebred mares because it was not able to

discriminate between pregnant and non-pregnant mares.

Moreover, the results observed in women and goat considerably differed by those obtained in mares. Indeed, in the goat SAA concentration significantly increased in the 2nd month and remained elevated until the end of pregnancy, reaching the peak concentration at kidding (Czopowicz et al., 2017). The presence of SAA in trophoblast at the first trimester (Kovacevic et al., 2006) and in placental cells at the third trimester (Sandri et al., 2014), suggests that this protein may exhibit an important function during the progression of pregnancy, and its presence may be related to the modulation of IL-1 β , IL-6 and IL-8 (Bowen et al., 2002; Osman et al., 2003) and metalloproteases (Xu, Alfaidy & Challis, 2002), which trigger the onset of labor and delivery.

Then, maternal SAA and CRP levels in normal pregnancy could differ from nonpregnant levels, due to increased hormonal values, and adipose tissue, and/or secondary to modifications of the inflammatory response (Sacks, Studena, Sargent & Redman, 1998).

Hp concentrations in pregnant mares were significantly higher than those obtained on day 0 and day 5, reaching the highest values from the 7th to the 10th month of gestation, confirming partially data obtained in women and bitches. In fact, in pregnant bitches, the Hp increase occurs from the third week after the LH surge, according to the embryo implantation and the placental formation, and remains high until the delivery; for this reason, the Hp is considered a source of pregnancy diagnosis, with values higher than 112.42 mg/dL, as indicative of pregnancy in this species (Vannucchi et al., 2002).

In women, the Hp showed a biphasic pattern, with two peaks of release during the first and third trimester, with a decrease at 24 weeks of gestation (Haram, Augensen & Elsayed, 1983; Larsson, Palm, Hansson, Basu & Axelsson, 2008), hypothesizing its placental synthesis (Berkova et al., 2001), according to the angiogenic role of the placenta and fetal development (Herrier, Krusche, Müller-Schöttle & Beier,

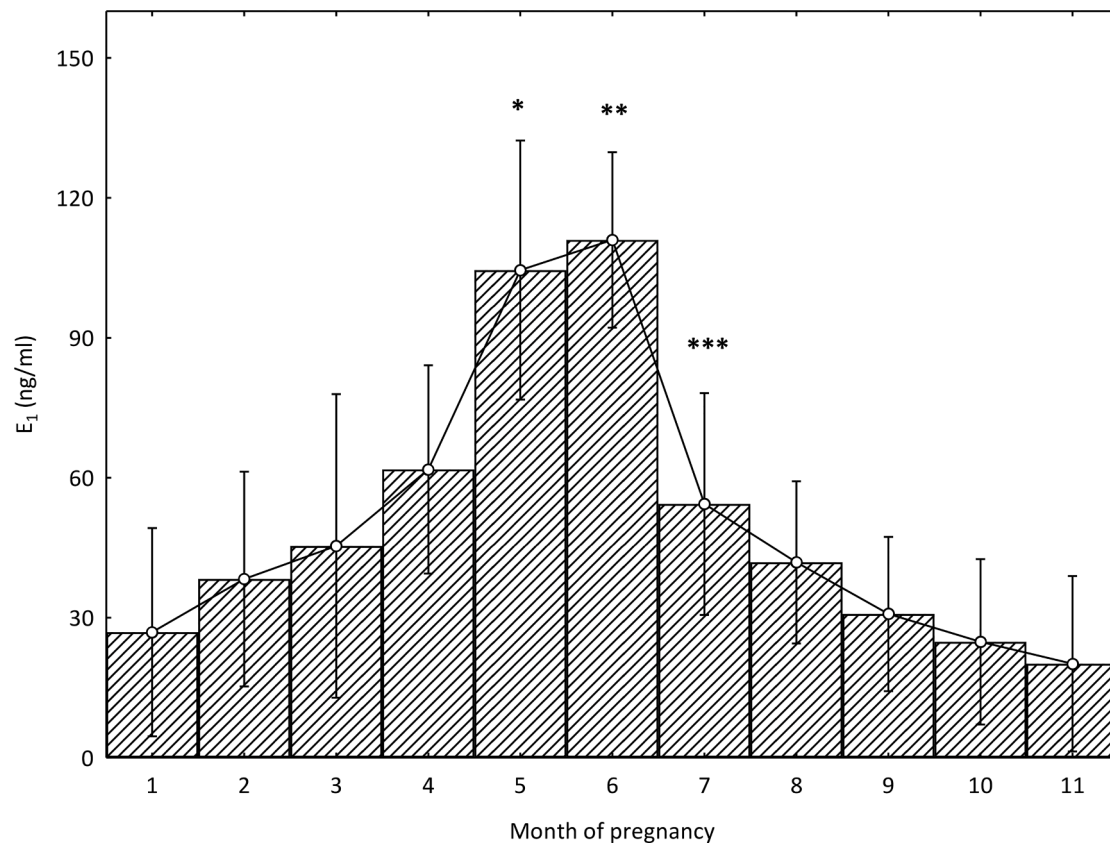


Fig. 2. Concentrations of oestrone sulfate (mean \pm SD) in 20 pregnant Spanish purebred mares. Symbols indicate significant differences: *vs 9 month of pregnancy ($P < 0.05$), ** vs 1, 2, 3, 9, 10, 11 month of pregnancy ($P < 0.05$), *** vs 1, 2, 3, 10 month of pregnancy: ($P < 0.05$).

2004).

On the contrary, Gatzka et al. (Gatzka et al., 2002) reported a decreased Hp concentration along pregnancy in women, as effects of hemodilution and of estrogen, as observed by experimental application of estrogen derivatives (Bicho, Pereira da Silva, Matos, Silva & Bicho, 2009). Other studies showed no difference on Hp between pregnant or nonpregnant and its value was stable throughout the pregnancy in sows (Sorrells, Eicher, Harris, Pajor & Richert, 2007), bitches (Ulutas et al., 2009; Vannucchi et al., 2002), goats (Czopowicz et al., 2017), and at term of pregnancy in healthy mares, with normal foals, compared to mares with placentitis (Canisso et al., 2014).

The absence of significant differences in the APP between day 0, +5 and +16 post ovulation days, suggested that these proteins cannot be used as biomarkers of diagnosis of heat or pregnancy in the mare, at least early; thereby, the Hp increases during the late gestation, as previously documented in bitches (Vannucchi et al., 2002), differing considerably by a close relationship between estrogens and Hp recorded in mares during the last four months of pregnancy (Taira et al., 1992).

The absence of changes in CRP levels in this study contrasted with the dynamics shown in Thoroughbred mares, in which this protein decreased during the last 4 months of gestation, reaching minimum values two months before foaling (Yamashita, Fujinaga, Okumura, Takiguchi & Mizuno, 1991). By contrast, in bitches CRP increased significantly during pregnancy; indeed, by day 16 after the LH surge, endometrial implantations' sites were established, with a release of cytokines, resulting in hepatic synthesis of APPs (Gruys, Toussaint, Niewold & Koopmans, 2005). Hence, it has been suggested that CRP, as well as Hp, could be used as a source diagnosis of pregnancy in bitches (Kuribayashi et al., 2003; Eckersall et al., 1993; Vannucchi et al., 2002) although this suggestion was not supported by Ulutas et al. (Ulutas et al., 2009) in this same species. In women there was a lack of consistency regarding the results of the CRP during pregnancy, since 52.2% of the

studies revealed fluctuations, 30% progressive reductions, while the remaining 17.4% manifested a progressive increase (Belo et al., 2005). It is hypothesized that the complexity of stimuli triggers the synthesis of numerous proinflammatory cytokines, according to different animals' species, promoting these discrepancies (Sacks, Seyani, Lavery & Trew, 2004).

Although the origin of the absence of CRP changes is unknown, it is well known that endometrial cups are present at day 35–40 of gestation in the mare. These structures exhibit the ability to modulate immunity, since the chorionic junctions suppress lymphocyte proliferation and the expression of various cytokines (Flaminio & Antczak, 2005). Larsson et al. (Larsson et al., 2008) showed no significant increased APP's trend during pregnancy in women, until week 38, coinciding with the time of delivery. This increase is due to the activation of macrophages during the birth trauma and related release of prostaglandins (PGF 2α) associated with the synthesis of IL-1 and IL-6, and the progressive increase in estrogens (Heikkinen et al., 2003; Belo et al., 2005). It is well known that certain pathological processes such as, preeclampsia, chorioamnionitis, tocolysis failure, premature rupture of fetal membranes, intrauterine growth restriction and alterations in the neonate were associated with substantial increases of this protein in women (Larsson et al., 2008).

Our observations may have a practical application, since an increase of SAA, Hp or CRP concentrations during periovulatory period or pregnancy could be facilitate an early diagnosis of some insidious diseases, such as endometritis (Canisso et al., 2014; Tuppits, Orro, Einarsson, Kask & Kavak, 2014), early embryonic mortality (Krakowski et al., 2011) and placentitis (Coutinho da Silva et al., 2013; Canisso et al., 2014).

5. Conclusion

SAA and CRP resulted no able to discriminate between nonpregnant

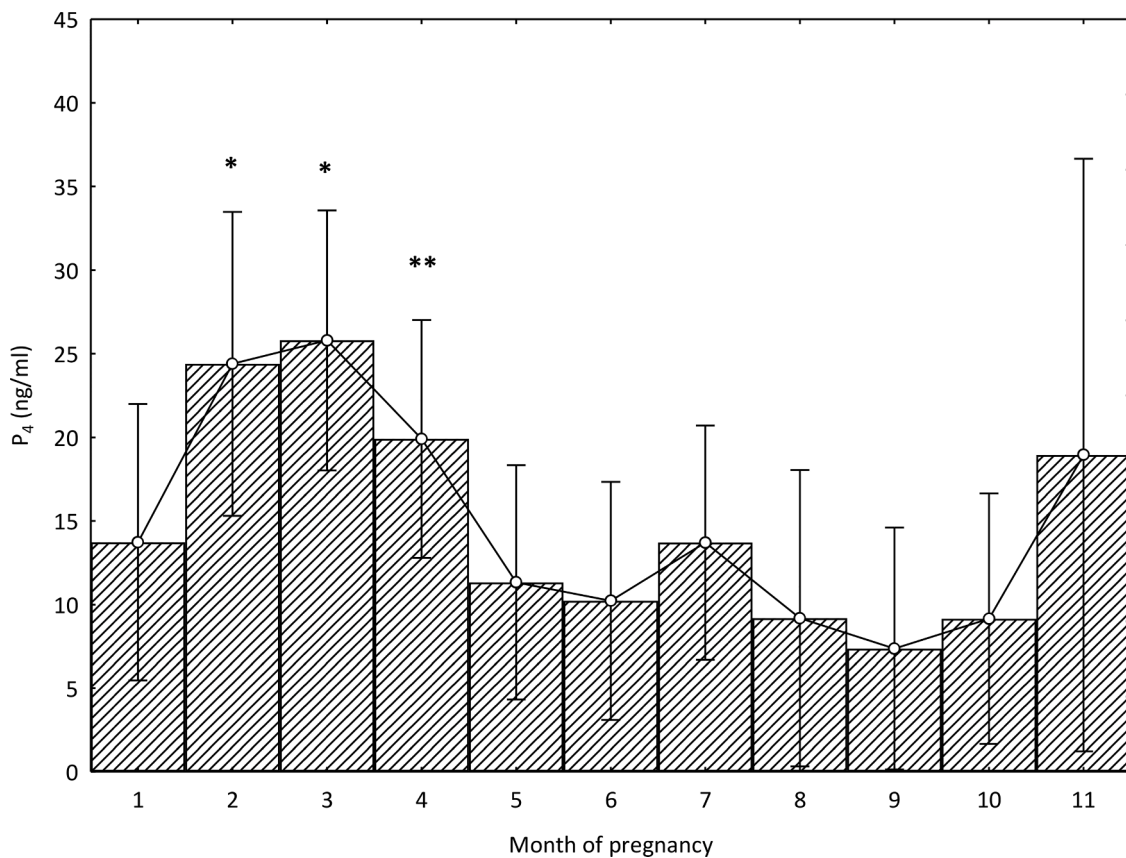


Fig. 3. Concentrations (mean ± SD) of progesterone (P₄) in 20 pregnant Spanish purebred mares. Symbols indicate significant differences: *vs 6, 7, 9, 10 month of pregnancy ($P < 0.05$), ** vs 6, 7 month of pregnancy ($P < 0.05$).

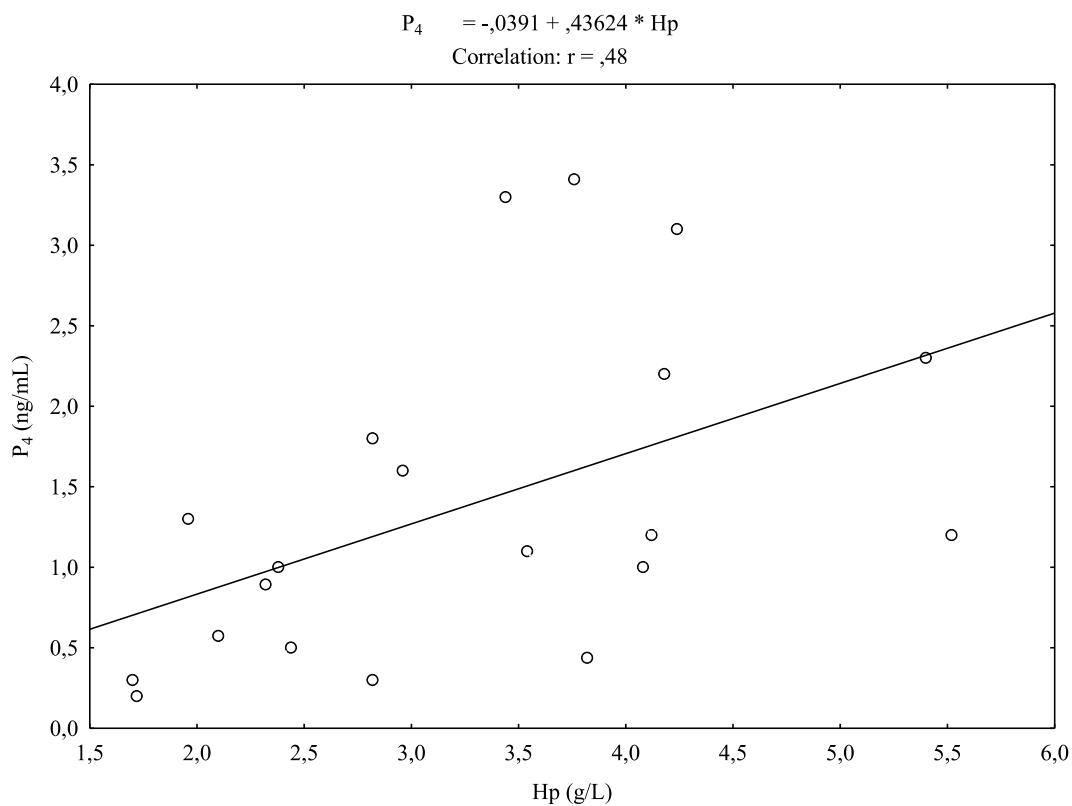


Fig. 4. Correlation between haptoglobin (Hp) and progesterone (P₄) concentrations in cyclic mares ($P < 0.05$).

Table 3
Correlations' coefficients among serum amyloid type A (SAA), C-reactive protein (CRP) and haptoglobin (Hp) in 20 cyclic and pregnant Spanish purebred mares.

	Estrous		Pregnancy	
	P ₄	E ₂	P ₄	E ₁
SAA	$r = 0.02$	$r = -0.36$	$r = -0.07$	$r = 0.33$
CRP	$r = 0.16$	$r = 0.13$	$r = 0.20$	$r = 0.06$
Hp	$r = 0.49^*$	$r = -0.11$	$r = -0.21$	$r = 0.06$

*($P < 0.05$).

and pregnant mares, and therefore cannot be used as a source of estrous or pregnancy diagnosis in Spanish Purebred mares. The absence of temporal relationship and the low correlations among Hp, E₁ and P₄ profiles, suggest that the mare shows a limited increase of the APR during pregnancy, independent of the hormonal dynamics. The evaluation of APP in cyclic and pregnant Spanish Purebred mares will be used for comparative purposes among different equine breeds, adding a new scientific segment related to the physiological range of these proteins, to monitor their health status or the pathological conditions.

Declaration of Competing Interest

None of the authors of the present article has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

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