Seasonal absence of supplementary corpora lutea in pregnant mares and the relationship with pregnancy loss

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ABSTRACT

Two experiments were conducted in the Northern (UK) and Southern (Brazil) hemispheres to determine the effect of season (month of conception) on the development of supplementary CL (SCL) and the relationship with pregnancy loss. In experiment 1, 199 pregnancies were followed between Day 14 and term, to determine the number of SCL and pregnancy viability (Northern Hemisphere). From the 199 pregnancies, 178 were obtained from inseminations during the breeding season (March–September), while the rest, 21 pregnancies resulted from conceptions in the non-breeding season (October to February). Pregnancies conceived in the breeding season were more likely ($P < 0.01$) to have at least 1 SCL (75.8 %, 135/178) than pregnancies from the non-breeding season (33 %, 7/21). However, the pregnancy loss between Days 35 and 120 of pregnancy in mares with no SCL was similar (3.5 %, 2/57; $P > 0.1$) than from mares with SCL (1.4 %, 2/142). In Experiment 2 (Southern Hemisphere), three groups of recipients were used based on their ovarian activity at the time of embryo transfer: Anestrus ($n = 8$), transitional ($n = 7$) and cyclic ($n = 7$) recipient mares. While all transitional and cyclic mares developed at least 1 SCL, only 50 % of anestrous recipients (4/8) developed SCL by 120 of gestation. In conclusion, the development of SCL in pregnant mares is influenced by the time of season of conception, therefore it appears to be regulated by the photoperiod and the endogenous seasonal variation in gonadotropin concentrations. Mares with no SCL were not at increased risk of pregnancy loss.

1. Introduction

All mammals, including equids [1,2], need a source of progestagen throughout pregnancy to maintain the pregnancy state. In the mare this is initially provided by the primary *corpus luteum* (CL) or CL *verum*, which originates from the ovulatory follicle in the oestrous cycle in which conception occurred. Following ovulation, the CL develops from residual granulosa and thecal cells and begins to secrete progesterone [2]. Luteinizing hormone (LH) of pituitary origin plays the major role in ensuring that the CL develops and functions normally, aided by the action of other hormones in many species [2]. Beyond the time of maternal recognition of pregnancy, which occurs around Day 12 after ovulation and prevents the cyclic release of prostaglandin F2a (PGF2a) to prevent luteolysis, the primary CL continues to produce progesterone [3,4], which is termed the first luteal response to pregnancy [1]. From about Day 20 of pregnancy, luteal production of progesterone decreases gradually until Day 30, reaching levels of around 4 ng/mL [5].

Following the invasion of the fetal chorionic girdle cells into the endometrium, at around Day 35 to 40 of pregnancy, and the formation of equine Chorionic Gonadotropin (eCG)-secreting endometrial cups, eCG enters the mare's bloodstream and there is a luteal resurgence in progesterone production by the primary CL due to its luteotropic effect [6]. In addition, eCG stimulates oestrogen and androgen production by the CL [7,8]. This is termed the second luteal response to pregnancy [1]. The third luteal response to pregnancy, which further contributes to progesterone levels, occurs as a result of the LH-like action of eCG which causes the ovulation or luteinization of follicles in the ovary to form supplementary CL (SCL) [9–11]. The selection and growth of these

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follicles is controlled by endogenous gonadotropins [12]. However, there is considerable variation in the degree of follicular growth and, therefore, the number of SCL that develop during early pregnancy can range from none to as many as 30 [11,13,14].

Supplementary CL can form in two different ways: from ovulatory follicles (secondary CL) or from luteinization of anovulatory follicles (accessory CL) [1,15,16]. Supplementary CL are first observed in the pregnant mare from Day 39 to 45 of pregnancy [16]. All corpora lutea: primary, secondary, and accessory CL, regress on average at Day 180 of gestation (range of 140 to 210 days of gestation) [17]. However, from Day 140 of pregnancy, the ovaries are not necessary for pregnancy maintenance in the mare, as interruption of luteal production of progesterone by ovariectomy does not result in abortion [18]. In reality the timing of placental progesterone production occurs prior to Day 140 since earlier ovariectomy of pregnant mares at Day 70 of gestation resulted in the abortion of only 43 % of treated mares (6/14) [18]. Furthermore, in a study involving pregnancies obtained from anestrous recipient mares supplemented with altrenogest, the interruption of treatment at Day 120 of gestation did not cause abortion in any mare regardless of the absence of SCL [16]. Therefore, the fetal-placenta unit must start producing sufficient progestagens to maintain pregnancy sometime between Day 70 and 120 of gestation. Hence, the critical period in which eCG and the presence of SCL are believed to play a relevant role in the maintenance of pregnancy, by contributing to the progesterone output of the primary CL, is between 35 and 120 days of pregnancy. In fact, it has been suggested that mule pregnancies (mares inseminated with donkey semen) are at increased risk of pregnancy loss compared to horse pregnancies due to a decreased rate of SCL formation [19] hypothesized to occur due to the lower concentrations of eCG and progesterone typically seen in such pregnancies [20–23].

Mares are long-day seasonal breeders, with the physiological ovulatory season ranging from April to October (Northern Hemisphere) [24]. Photoperiod is believed to be the major factor influencing seasonality, with increasing daylength inhibiting the pulsatile release of melatonin from the pineal gland. This in turn stimulates the release of follicle stimulating hormone (FSH) and LH from the pituitary gland to initiate seasonal cyclicity [24]. However, some mares may cycle and conceive throughout the year, including during the non-breeding season [25]. Since FSH and LH levels, secretion pattern, and, hence, ovarian follicular activity are influenced by month and season [26–28], the development and number of SCL in pregnant mares might also be seasonal dependent, as suggested by Allen [29] and Cuervo-Arango and coworkers [15]. However, the former study was undertaken before the use of ultrasound to visualize the ovaries, and the latter one in mares which were aborted between 60 and 65 days of pregnancy.

The objectives of this study were threefold. Firstly, to compare the incidence of SCL in mares becoming pregnant during the breeding and non-breeding season. Secondly to determine the incidence of pregnancy loss in mares which failed to develop SCL between the period of 35 and 120 days of pregnancy, and, thirdly, to determine the incidence and timing of SCL formation in recipient mares with differing ovarian and reproductive status at the time of embryo transfer. It was hypothesized that (1) more mares becoming pregnant during the breeding season would develop SCL than those impregnated during the non-breeding season; (2) the incidence of pregnancy loss would be higher in mares with an absence of SCL; and (3) anoestrous recipient mares receiving an embryo would be less likely, and would take longer to, develop SCL than cyclic recipients.

2. Materials and methods

The data from Experiment 1 was obtained from mares examined for routine pregnancy diagnosis (Day 15, Days 35–40, and Days 100–120) and whose owner JN, co-author of the manuscript, gave informed consent to use the data from the mares. Experiment 2 was a controlled study and was approved by the Ethics Committee on Animal Use of the School

of Veterinary Medicine and Animal Science, Universidade Estadual Paulista (CEUA-115/2009).

2.1. Experimental design

This study was carried out in two locations (UK and Brazil), using two sets of data, and therefore it is separated into two Experiments. In Experiment 1, performed in the UK, retrospective data was analysed from mares carrying their own pregnancy. In Experiment 2, data were collected from an embryo transfer centre in Brazil from pregnant recipient mares at differing reproductive status at the time of embryo transfer. In both Experiments, the ovaries of the pregnant mares were examined thoroughly by ultrasonography, in two periods of pregnancy, to determine the number of corpora lutea. The first period examined was prior to the development of the endometrial cups and secretion of eCG into the maternal circulation. Hence, mares were examined at ovulation, at Day 15 of pregnancy and again between 30 and 35 days of pregnancy. Therefore, the number of primary CL could be determined. The second period in which mares were examined was between 100 and 120 days of pregnancy, coinciding with the decline and disappearance of eCG from the mare's blood. The total number of CL at this time point was recorded so that the percentage of pregnancies with the development of one or more SCL during the time of eCG secretion could be calculated. Evidence shows that the primary CL and any other CL(s) that develop before Day 35 are visible up to Day 180 of pregnancy [17]. Hence, it was assumed that a mare with no visible SCL by Day 120 of pregnancy, had failed to develop any SCL by the end of Period 2.

Supplementary CL were defined as the development of a newly formed CL as a result of either ovulation of a pre-ovulatory follicle (secondary CL) or luteinization of an anovulatory follicle (accesosory CL). However, no attempt to distinguish between the two was made, only to calculate the difference in the total number of luteal structures between Period 1 and 2 of pregnancy. Mares with the same number of CL in Periods 1 and 2 were classified in the group of absence of SCL formation, while mares with one or more extra CL in Period 2 compared with Period 1 were classified in the group of mares with the presence of SCL formation.

In Experiment 1, the pregnancy status (pregnant or not pregnant) at the end of Period 2 was determined for mares with or without SCL by transrectal utrasonography. The incidence of pregnancy loss at the end of Period 2 was calculated and compared between groups. In Experiment 2 the pregnancy loss was not taken into account for data analysis as the recipient mares were maintained with altrenogest to maintain pregnancy in the non-cycling recipient mares.

2.2. Animals and ultrasound examinations

2.2.1. Experiment 1

Retrospective breeding records of 45 mares and 199 pregnancies during 10 breeding seasons were analysed. The number of pregnancies from each individual mare ranged from 2 to 6. Mares aged 3 to 18 years old, of a variety of breeds but mostly riding horse type, were resident at an equine fertility clinic in the UK (52° 37' N). All mares were kept in paddocks on a diet based on grass hay, concentrate, trace mineral salt, and water ad libitum. The majority of pregnancies were conceived during the physiological breeding season months of March to September ($n = 178$ pregnancies). The rest of the pregnancies ($n = 21$) were from mares conceiving during the non-breeding season (October to February). The decision to inseminate mares at different times of the year was based on mare owners̕preference.

Once in oestrus and inseminated, mares were checked for ovulation, with all examinations undertaken using transrectal ultrasonography. The day of ovulation was considered as Day 0 of pregnancy, and the development of the primary CL was confirmed the following day. In mares with double or triple ovulations, the development of multiple CL was noted within 2 days of the first ovulation and confirmed at the time

of the first pregnancy diagnosis at Day 12 to 16 post-ovulation. Pregnancies were confirmed at Days 30 to 35, and the ovaries were examined thoroughly to determine the total number of CL present at Period 1. These CL consisted of the primary CL and other CL from twin or dioestrous ovulations, assumed to have occurred as a result of the action of endogenous LH, and not from eCG, which is not present in the peripheral circulation until after Day 35 to 40 of pregnancy [11]. Pregnant mares were examined again at least once between Day 100 and 120 of pregnancy, to confirm the viability of pregnancy and examine the ovaries to determine the number of CL. New CL found at this period of pregnancy (Period 2) were termed as supplementary CL (SCL).

For each pregnancy the following data were recorded; mare ID, the month of ovulation (Day 0, beginning of pregnancy), presence or absence of SCL, and pregnancy status at Day 120 (pregnant or not pregnant).

2.2.2. Experiment 2

Data from this experiment was used to determine the effect of month and reproductive status (anoestrus, transitional, cyclic) on the development of SCL in recipient mares. The experiment was performed in a commercial breeding farm in Brazil (19° 58' S). Twenty-two crossbred pregnant recipient mares, ranging in age from 4 to 15 years and weighing between 300 and 450 kg were used. All animals were maintained in paddocks with free access to water and fed a diet based on hay, concentrate, and trace mineral salt. Pregnant recipient mares were selected based on their reproductive status at the time of embryo transfer and classified into three groups: cyclic ($n = 7$), spring transitional ($n = 7$) and anoestrous recipients ($n = 8$). Mares were examined by transrectal ultrasonography to evaluate ovarian activity, uterine oedema and tone so their reproductive status could be charcterised prior to the experiment. Following examintion of their reproductive tracts mares were categorized into anoestrus (absence of a CL and follicles < 20 mm; $n = 8$), transitional (absence of a CL and follicles > 20 mm in diameter; $n = 7$), or cyclic (presence of a CL; $n = 7$). All mares were monitored for at least 21 days to confirm their reproductive status before the beginning of the study.

Anoestrous and transitional recipient mares were treated with a single dose of oestradiol benzoate (2.5 mg/kg, i.v.; Estrogin®, Farmavet, São Paulo, SP, Brazil) between the first and third day after donor ovulation. The day after oestradiol treatment, recipients were examined by ultrasound to evaluate the presence of uterine oedema. Once oedema was confirmed, non-cyclic mares were supplemented daily with 33 mg of altrenogest according to the manufacturer's recommendation (0.044 mg/kg p.o.; Progestal®, 2.2 mg/mL altrenogest, ProSer, Buenos Aires, Argentina), until embryo transfer and pregnancy diagnosis on Day 15 (with respect to the embryo age; i.e. 15 days after detection of ovulation in the donor). Recipient mares that were confirmed pregnant were maintained on altrenogest until 120 days of pregnancy.

Donor mares from which the embryos were obtained were managed in the following manner. After detection of a 35 mm follicle and uterine edema, ovulation was induced in donor mares with 2500 IU of hCG (Vetecor®, Hertape Calier, Juatuba, MG, Brazil). Insemination with fresh or cooled semen from stallions of known fertility was undertaken 24 h after hCG administration. Uterine flushes were performed eight days post ovulation by a non-surgical procedure using 4L of Ringer Lactate solution (Sanobiol®, Pouso Alegre, MG, Brazil). After identification, embryos were washed and maintained in a holding medium (TQC Holding Plus® Nutricell, Campinas, SP, Brazil) and immediately transferred to recipient mares. Embryo transfers were performed between Day 4 and 6 after beginning of altrenogest treatment for non-cyclic recipient mares, and between Day 4 and 6 after ovulation for cyclic mares. Embryo transfers were performed between July and January in the Southern Hemisphere.

The day of pregnancy was calculated from the day of the donor's ovulation. All pregnant recipients were examined between Day 30 to 35 of pregnancy and again at Day 120 of pregnancy, to determine the

number of CL and SCL, respectively, as in Experiment 1. Furthermore, in order to determine the timing of the first development of a SCL, recipients were examined every 5 days between Day 35 and Day 120. For each pregnant recipient, the following data were recorded: month of embryo transfer, reproductive status at the time of embryo transfer, presence or absence of SCL by Day 120 of pregnancy, and Day of pregnancy in which the first SCL developed.

2.3. Statistical analyses

For each Experiment, data were computed in a statistical software programme (Systat 13, California, USA) to create a binary logistic regression with the presence/absence of SCL by Day 120 of pregnancy as the dependent variable, and month of beginning of pregnancy and season (non-breeding [October – February, Northern hemisphere; April–August, Southern hemisphere] vs. breeding season [March–September, Northern hemisphere; September–March, Southern hemisphere]), and for Experiment 2 reproductive status at embryo transfer, as independent variables. The Kruskal–Wallis non-parametric test was used to test the difference in the median Day of pregnancy at which the first SCL developed amongst reproductive status of recipient mares (anoestrus, transitional and cyclic) in Experiment 2. In Experiment 1, the incidence of pregnancy loss in mares with or without SCL was compared by Fisher's exact test. Non-parametric data were presented as median and interquartile range (IQR).

3. Results

3.1. Experiment 1

The overall percentage of pregnancies with no SCL development was 28.6 % (57/199). The month of year in which the mare conceived did not influence ($P > 0.1$) the likelihood of developing a SCL, but it was affected by season ($P = 0.0001$; OR = 6.279). Mares conceiving during the non-breeding season (October to February) were less likely to develop SCL (7/21, 33.3 %) than mares conceiving during the breeding season (March to September) (135/178, 75.8 %). The distribution of pregnancies per month with no SCL development is shown in Fig. 1.

The overall pregnancy loss between Day 35 of pregnancy and term was 7.5 % (15/199). Three pregnancies from mares with no SCL were lost (3/57, 5.2 %), from which, 2 pregnancies failed between Day 35 and 120 of pregnancy (2/57, 3.5 %), while the remaining mare aborted on the 9th month. On the other hand, 12 pregnancies in mares with SCL were lost (12/142, 8.4 %), from which, 10 mares aborted after Day 120 of pregnancy, and the remaining two failed between Day 35 and 120 (2/142, 1.4 %). The incidence of pregnancy loss during the eCG stage (Day 35 to Day 120 of pregnancy) in mares with no SCL was not different from that of mares with SCL (1.4 % vs. 3.5 %, respectively; $P > 0.1$; Table 1).

3.2. Experiment 2

Recipient mares in anoestrus at the time of embryo transfer were less likely ($P < 0.01$; OR = 13.0) to develop SCL by Day 120 of pregnancy (4/8, 50 %) than transitional or cycling recipient mares (14/14, 100 %). Furthermore, recipient mares to which embryos were transferred during the winter months (July to September, Southern Hemisphere) were less likely ($P < 0.05$; OR = 0.102) to develop SCL (6/10, 60 % of pregnancies with at least one SCL) than recipients transferred during the breeding season (October to January; 12/12, 100 % of pregnancies with at least one SCL). The Day of pregnancy on which the first SCL developed was influenced by the reproductive status of the recipient mare at the time of embryo transfer ($P = 0.006$). The median (IQR) day of first formation of SCL (and interquartile range) was 67.5 (17.5), 55 (21.2), and 40 (3.75) days for anoestrous, transitional, and cyclic recipient mares, respectively (Fig. 2).
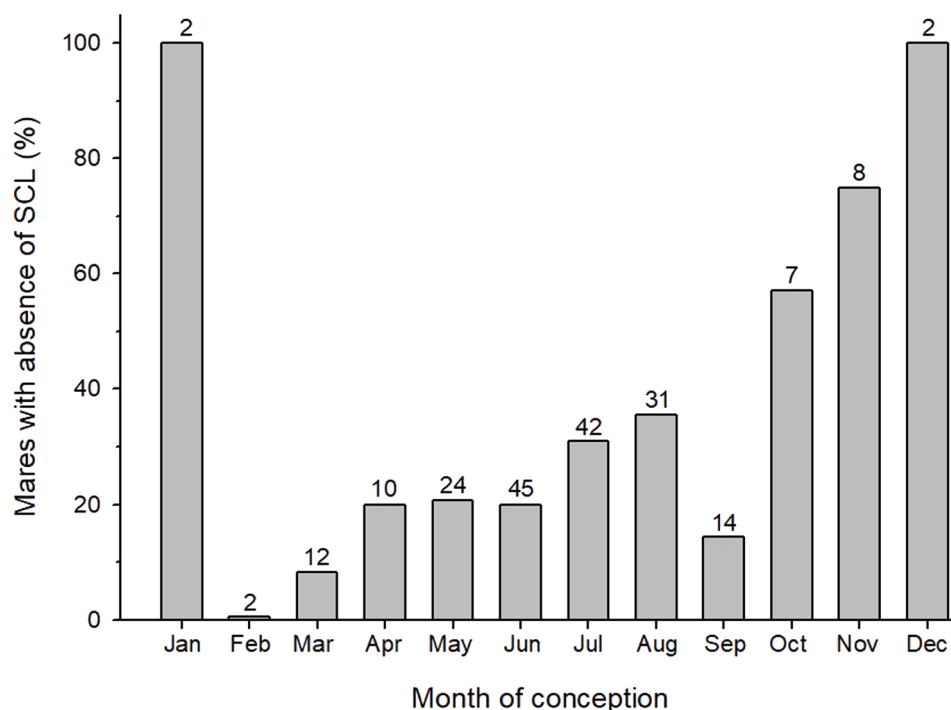
Fig. 1. Percentage of mares with no supplementary corpora lutea (SCL) conceiving at different months of the year. Data are from mares that were resident in the Northern Hemisphere. The number of pregnancies for each month is shown above each bar.

Table 1

Pregnancy loss in mares carrying their own pregnancy with or without the development of supplementary corpora lutea (SCL).

Presence of one or more SCL	Pregnancy losses between Day 35 and 120 (%)	Pregnancy losses between Day 120 and term (%)	Overall pregnancy loss after Day 35 (%)
NO (n = 57)	2 (3.5)	1 (1.8)	3 (5.2)
YES (n = 142)	2 (1.4)	10 (7.1)	12 (8.4)

No significant difference ($P > 0.1$) existed in pregnancy loss rate at different stages of gestation between those mares with or without a SCL.

4. Discussion

The main hypothesis of the study that more mares becoming pregnant during the breeding season would develop SCL than those impregnated during the non-breeding season is substantiated by the results of this study. Therefore, it appears that seasonal variation in follicular selection and growth, driven by endogenous gonadotrophins (LH and FSH), play an essential role on the development of supplementary CL. This agrees with the results of Allen [29] who saw that there was a marked seasonal effect on ovarian size, follicle numbers and ovulation rate during early pregnancy in Pony mares, with the frequency of secondary ovulations being higher during the physiological breeding season. Likewise, in mares aborting which still had active endometrial cups and, hence, eCG in their peripheral circulation, it was noted that the month of abortion influenced the likelihood of them having SCL [15]. These studies paralleled earlier ones showing the relationship between season and the occurrence of ovulations and/or follicular development recorded in non-pregnant mares, both in the Northern [30, 31] and Southern hemispheres [32]. Experiment 1, in the present study, used ultrasonography of the ovaries to show a seasonal influence on the incidence of supplementary CL (SCL) during early pregnancy in the mare. Significantly more SCL were noted in mares which became

pregnant during the physiological breeding season versus those becoming pregnant at other times of the year. Furthermore, Experiment 2 demonstrated that the status of a mare (anoestrus, transitional or cyclic) at the time of embryo transfer influenced the timing at which the first SCL developed.

The ability of a mare to produce SCL in early pregnancy depends on two major factors. Firstly, she needs follicles of a sufficient size present on her ovaries to respond to the luteinising or ovulating effects of pituitary LH or the LH-like component of eCG. Secondly, she needs these luteogenic hormones to be present in the peripheral circulation.

So, what seasonal influences are there on the development of follicles within the mares' ovaries? The mare is a long-day seasonal polyoestrous animal, with the transition from reproductive quiescence (anoestrus) to cyclic activity during the breeding season driven primarily by seasonal changes in the hypothalamic-hypophyseal axis mediated by the actions of gonadotrophin-releasing hormone (GnRH) [27,33]. During the winter months there is a reduction in both the amount of GnRH in the hypothalamus and its secretory rate [33,34]. Mean concentrations of LH [26, 35–37] and FSH [33] in serum are low close to the winter solstice. Although Turner and coworkers [26] showed no seasonal variation in plasma FSH concentrations throughout the year when mares were sampled every 3 days, the secretory pattern (frequency of pulsatile release, determined by every 6 h sampling) has been shown to be reduced as the autumn transition into winter anestrus approaches [28], which may explain the lack of follicular development and hence SCL formation in mares conceiving during the autumn.

Pituitary FSH is believed to be entirely responsible for production of follicles in the ovaries during early pregnancy [11,12,38]. The appearance of eCG in maternal blood from around 40 days does not induce further follicle growth despite having an FSH-like component, as it binds weakly to receptors in equine ovarian tissue [39]. So, in early pregnancy each follicular wave is preceded by an increase in FSH secretion from the pituitary [40,41]. Once sufficiently mature, the follicles ovulate or luteinize in response to pituitary LH and/or the LH-activity of eCG to form SCL.

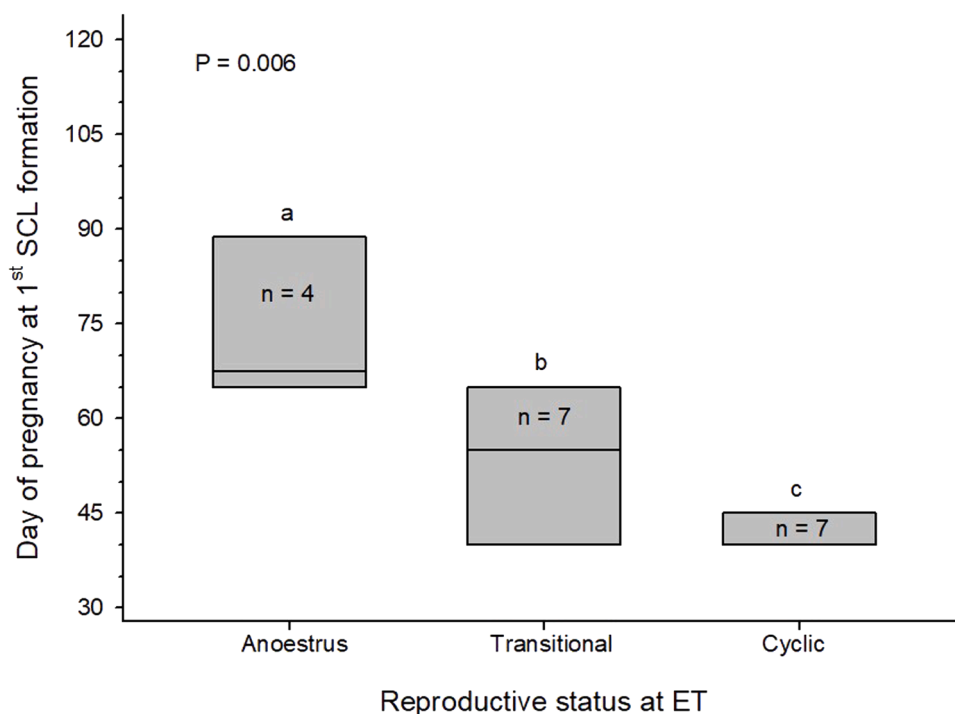The first ovulation of the season, as the mare moves from transitional

Fig. 2. Box plot distribution (first quartile, median and third quartile) of Day of pregnancy when the first supplementary corpora lutea (SCL) developed in recipient mares that were resident in the Southern Hemisphere with differing reproductive statuses at the time of embryo transfer (ET). Different letters (a,b,c) indicate a significant difference ($P < 0.05$) in the median Day of pregnancy on which the first SCL appeared. The median Day of pregnancy in cyclic recipients at which the first SCL appeared was 40 days.

to cyclic, is usually an indicator that sufficient levels of FSH are present to result in repeated FSH waves which stimulate follicular growth to a certain "maturity" threshold after which the follicle can respond to LH, and this will continue until the autumn transition. It is logical that these same FSH waves will stimulate follicular growth in early pregnancy if the mare conceives during the physiological breeding season In Experiment 1, the presence of SCL in >60 % of mares bred in the Northern hemisphere during physiological breeding season (March–September) illustrates that follicles were present on the ovaries and FSH waves occurred to drive their formation during early pregnancy. Further evidence that FSH is the primary factor driving follicular growth and its ovulatory competence in anoestrous mares is the fact that daily treatment with recombinant eFSH alone in deep anestrous mares resulted in the development of several (6.4 ± 0.8) pre-ovulatory sized follicles within 7 days of treatment, which ovulated in response to hCG administration in 80 % of treated mares [42].

In Experiment 2, in which embryo transfer recipients were either anoestrus, transitional or cyclic, the timing of the first SCL formation post transfer was related to their reproductive status at the time of transfer, with anoestrous and transitional mares taking longer than cyclic recipients. This difference in the timing of SCL formation may reflect the fact that anoestrous and transitional recipients were 'behind' in terms of entering the spring transition period, hence stimulation of ovarian follicular development driven by secretion of FSH was yet to occur. Likewise, the transitional mares were faster than the anoestrus, but slower than the cyclic ones, in the appearance of their first SCL, as they were further along the endocrine pathway to the physiological breeding season but not yet through the spring transition like the cyclic mares.

Few mares in Experiment 1, as opposed to the majority of those in Experiment 2, conceived in the autumn transition period or over winter when FSH secretion and, hence, the potential for follicular development, differs from that in the spring transition and the physiological breeding season. During the autumn transition, although pituitary FSH content

shows no seasonal variation [26,27,43,44], FSH waves continue for some time [28,45,46] albeit with lower amplitude pulses before ceasing altogether [45]. Although, in some mares FSH secretion continues throughout anoestrus [40,47]. However, despite the presence of FSH during this autumnal transition period follicular growth to a stage >25 mm ceases [40,47]. Hence, mares conceiving to one of the last ovulatory cycles prior to the autumn transition are unlikely to have the capacity to develop follicles of a size that can respond to the luteinising action of either pituitary LH or eCG. This is supported by the findings of both Allen [29], Cuervo-Arango et al. [15] and those of Experiment 1 in the present study.

Although there was a significantly lower incidence of SCL in the autumn and winter months in the Northern hemisphere mares in Experiment 1, some mares, albeit a low percentage, did produce SCL. This likely indicates variation in the serum FSH profiles of individual mares, as noted earlier [40,47]. Furthermore, any mare becoming pregnant in Experiment 1 outside of the physiological breeding season must have had an endocrine profile that allowed her to develop, mature and ovulate a follicle with a viable oocyte at a time when many mares would be transitional or in anoestrus and unable to do so. Hence, it is likely that conceiving mares that did produce SCL had a later decline in FSH waves, and other endocrine changes that orchestrate the autumn transition, compared with those mares which did not produce SCL. Indeed, if those animals that produced SCL had not been mated and conceived, they would potentially have never entered a period of anoestrus over the winter months. It has previously been noted that not all mares enter a period of anoestrus, with around 27 % of mares at latitudes between 30 and 50° continuing to cycle throughout the year [48–50]. This is a percentage which is akin to that of mares in Experiment 1 (33 %; 7/21) which produced SCL when conceiving in the non-physiological breeding season.

So, what influence does the season have on the luteogenic hormones LH and eCG? There is a marked depletion of pituitary LH during the autumnal transition and into winter anoestrus [26,27,43,44]. Follicular

activity on the mares' ovaries during early pregnancy and the production of LH are influenced by several factors, but predominantly these are seasonal. In contrast, eCG will be secreted in all pregnancies in which a conceptus is present and develops normally up until the time of invasion of the chorionic girdle (approx. 35 days) [51] and secretion of eCG into the peripheral circulation (approx. 40 days) [52], with concentrations not dependent on the season [29]. Although, a previous study [15] reported lower concentrations of eCG in mares in November versus January in the Southern hemisphere. One study has also suggested that the production of eCG is influenced by altrenogest treatment during early pregnancy [53], although the experimental mares varied in reproductive status (maiden, lactating or barren), age and parity, all factors known to potentially influence eCG production [54]. Unfortunately, eCG levels were not assessed in the present experiment to determine if the findings of Allen [29] or Cuervo-Arango et al. [15] could be confirmed. However, levels of eCG are tremendously variable in individual mares [54], yet only relatively low levels are required to provide enough stimulus to luteinize and/or ovulate ovarian follicles. This is evidenced by mares losing their pregnancies before the demise of the endometrial cups in which eCG continues to be secreted into the maternal bloodstream. In such mares re-breeding them is often challenging due to the rapid luteinization of any follicles even when low levels of eCG are present [15,55,56]. Hence, it is unlikely that in the present experiment eCG levels would have influenced the incidence of SCL.

That being said, when horse mares are inseminated with donkey semen to produce mule foals eCG levels are significantly lower and the secretion period shorter with eCG undetectable by 80 days of gestation [11,20,57]. In such pregnancies it has been reported that the occurrence of SCL is less and pregnancy loss is higher in comparison with mares inseminated with horse semen to produce horse foals, with the hypothesis that low eCG levels are, at least in part, responsible [19–21]. A further study found that pregnancy loss in mares carrying mule foals was affected by the season of conception, with loss rates significantly higher in those mares conceiving at the end of the physiological breeding season [58]. On the other hand, Carluccio et al. [59] found no difference in pregnancy loss rate between mule pregnancies and those reported for horse pregnancies. Furthermore, it should be noted that the FSH:LH ratio varies between mule and horse eCG in maternal serum (0.64 vs. 1.45, respectively) [60] making it difficult to extrapolate the findings of Boeta and Zarco [19–21] on any influence the levels of eCG might have on SCL formation in normal horse x horse pregnancies, such as in this study.

The findings in the aforementioned mule studies [19–21] found a higher level of pregnancy loss which the authors potentially attributed to a reduction in SCL occasioned by lower eCG and progesterone levels. However, in the present study in normal horse x horse pregnancies we found no evidence for an increase in pregnancy loss in mares with no SCL. In the cyclic mare the lifespan of the CL is only around 14–15 days, however if PGF2a is not released from the mare's endometrium to cause its demise, the CL may have an extended lifespan in non-pregnant mares [61]. This occurs commonly in non-pregnant mares during the breeding season often as a consequence of a later dioestrous ovulation which is unresponsive to PGF2a, early embryonic death after Day 12 when the maternal recognition of pregnancy signal has already been transmitted, reduced synthesis or secretion of PGF2a, or for idiopathic reasons [61, 62]. In such cases prolonged luteal function may persist for a long time [62]. For example, when luteal prolongation was caused experimentally by embryo reduction in 11 pony mares after transmission of the MRP signal all remained in prolonged dioestrus with 10 of them maintaining progesterone levels >1 ng/mL for a mean of 82 ± 13 days (range 64–109 days), and the eleventh outlier for >223 days before she was administered PGF2a [63]. Similar periods of luteal prolongation have been reported in non-pregnant mares given exogenous oxytocin [64,65], or when an intrauterine device has been placed in the uterine lumen [66–68]. Hence, even without the support of eCG to boost its

steroidogenic activity [7,8] the primary CL is capable of remaining functional for almost the entire period up until the time the placental unit would take over progesterone production between 70 and 120 days of pregnancy [18]. However, with the support of eCG the primary CL does not regress until between 140 and 210 days of gestation [17]. Therefore, it is perfectly possible for the primary CL to provide enough support to maintain a pregnancy without the assistance of SCL, especially when the primary CL has extra luteotropic support from eCG. The fact that SCL appear not to be essential for pregnancy maintenance, does not imply that eCG is not necessary for normal pregnancy development. In fact, the donkey-in-horse pregnancy model, which has no detectable circulating eCG is seldom carried out to term [69].

In conclusion, the incidence of SCL in mares carrying their own pregnancies is affected by the time of year when the mare is mated, with a lower incidence of SCL when conception occurs in the non-physiological breeding season. Recipient mares that are anoestrus or transitional can form SCL, but their appearance is delayed and would appear dependent on how advanced the seasonal changes towards spring cyclicity are in the hypothalamic-hypophyseal axis at the time of embryo transfer. There is no evidence from this study that a lack of SCL results in a higher incidence of pregnancy loss in the mare.

Ethical statement

The authors declare this manuscript is adhered to the ethical guidelines of this journal. The data from Experiment 1 was obtained from mares' resident to or owned by the Veterinary Clinic "Equine Fertility Clinic, Warren House Farm" whose owner is John Newcombe, co-author of the manuscript, giving permission to use the data from the mares. These mares were examined for routine pregnancy diagnosis (Day 15, Days 35–40, and Days 100–120). Experiment 2 was a controlled study and was performed under a valid research experiment license: ref. number CEUA-115/2009.

Declaration of Competing Interest

None of the authors has any financial or personal relationships that could inappropriately influence or bias the content of the paper.

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