Characterization of the Preovulatory Period in Dairy Cows

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ABSTRACT---- ***The periovulatory period of productive Holstein cows was studied, through their hormone levels, ovarian dynamic, characteristics of the cervical flow and their behavior; observed and registered throughout two consecutive periods. Results showed that during ovulation, the most evident changes were: animal behaviour (from being restless and unquiet to being quieter and allow to be mounted); the reduction of the size of their vulva together with a loss of the labia turgidity; a decrease in the quantity of flow and this one appeared as a transparent and noticeable secretion, one which kept the vulva humid and lubricated. The pH of the flow got higher (7-20- 7.50) and the degree of crystallization was reduced to 1.5-2, among the kinds of estrogenic flow, ovulation coincided with the presence of certain arrangements corresponding to type S3, P2 and P6. P flow is associated to the time of maximum fertility. The peak of the luteinizing hormone was detected on the ovulation day (1.90mUI/mL) and 24 hours afterwards the level was low; estrogens increased on the preovulatory days (0.80-0.97 pg/mL) causing the sexual behaviour, the stimulating follicle hormone accompanied the follicular growth and progesterone reached its highest increase consistent with the establishment of the corpus luteum (1.45ng/mL). The vulvar characteristics and the analysis of the cervical flow become complementary tools in order to determine the ideal moment for insemination.***

Keywords--- sexual behaviour, cervical flow, follicular dynamics, estrous hormones

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1. INTRODUCTION

Fertility must reflect the capacity of the cow to get pregnancy, at this point it is really important to differentiate the physiological function itself. Such a function constitutes the basis for common reproductive practices in production systems such as: lack of observation of the animal in oestrus, inadequate insemination technique, insemination at the non ideal moment, lack of exhibition of primary signs of oestrus, among others. In milking premises, the reproductive efficiency becomes essential in production; the association between milk production and pregnancy is controversial and is nowadays being studied by several investigators, some of whom claim that the metabolic demand of production and reproduction is reaching its biological limit. The criteria for genetic selection, nutrition and general handling practices must lead to optimize fertility and therefore encourage milk production; considering that managing so as to achieve efficient production may be compatible with a good reproductive performance (12). The oestrus cycle represents the cyclicity of ovarian activity and lets the cow go through a period of receptivity, mating and later establishing the process of pregnancy (6). In bovines, during each cycle (17 to 21 days) there are two or three follicular waves and the main hormones engaged with the folliculogenesis are the FSH (stimulating follicle hormone) and LH (lutenizing hormone) which determine as final destiny of a follicle the ovulation. Then, when the corpus luteum is formed, there is an important increase of P4 (progesterone) (21).

The dynamic of reproduction then, turns to be a multi factor problem, and nowadays such problems focus –for example- on the duration of the oestrus cycle, the signs of oestrus and its inference at the moment of ovulation (11). These reproductive phenomena are regulated by the axis hypothalamus-hypophysis-ovaries, growth factors, metabolites and neurotransmisors among other biomodulators, all together, responsible for animal behaviour, vulvar turgidity, flow secretion and others. That is why the aim of this work is to describe the periovulatory period, associating it with hormonal levels, ovarian dynamic, characteristics of the cervical flow and animal behaviour; claiming that oestrus observation and analysis of the cervical mucus are essential tools to make reproduction more efficient.

2. MATERIAL AND METHDS

This work was carried out during Spring 2016 at the milking premises of the Faculty of Veterinarian Sciences- UNR, situated in the city of Casilda, province of Santa Fe – Argentina (33º 02´ 39´´ south latitude, 61º 10´ 05'' west longitude). The premise in use is representative of the milking systems of the area under study: 80 – 100 milking cows, average annual production of 18 litres per cow per day, continuing parturing system, using artificial insemination. Nutrition during the period mentioned was 22 kg of MS including pastures, conserved and concentrated forages.

Animals. Two sexual cycles were recorded using a Holstein cow, multiparturing, 550 kg heavy, fit and healthy (free from brucellosis, tuberculosis, campilobacteriosis, tricomoniasis, under control of leptospirosis, bovine infectious rinotraqueitis and viral bovine diarrhea), with a body condition ≥ 2.5 (rank 1-5. (5) and between ≤ 90 and ≥ 120 days of interval in open days. The reproductive system of the used cow was checked in order to test its functionality and integrity.

Feeding and handling. During the period of these evaluations, the cow consumed forages under direct pasturing (mixed pastures and annual winter grassing) or conserved (silages of whole plants of maize and sorghum, meadow hays) and concentrated (maize and sorghum grains). The cow used was kept in the rodeo with all the other cows of the premises, following their same routine.

Design of sampling. Day 0 of each period started with the presence of the corpus luteum in ovaries through an ultrasound and 500 µg of Cloprostenol (Ciclase DL, Syntex SA, Argentina) was applied intramuscularly and from that point, a daily register was carried out according to the moment of the cycle. Samples were always taken during the afternoon milking (4.30pm)

variables under study

a) Behaviour. It was classified as restless (nervious – more active, lack of appetite, mating, higher self-cleaning) or quiet / normal (common behaviour with no visible modifications)

b) Vulva. Aspect (Turgid or flabby) and color of the mucus (bright red, reddish and light pink)

c) Uterine horns. Inspection was carried out through rectal palpation and muscular tone or consistency, presentation (windings), size and symmetry or lack of it was registered in both horns.

d) Ovarian dynamic. In both ovaries, it was determined the presence of follicles (diametre in mm), hemorrhagic corpus and corpus luteum. A portable ultrasound Welld model WED-3000 with transrectal probe of 5.0, 6.5 y 7.5 MHz was used.

e) Cervical mucus. The gathering of this secretion was carried out by aspiration using a sanitary plastic sheath for insemination and a plastic syringe of 60 ml. First a macroscopic evaluation was carried out and pH was measured, afterwards, the sample obtained was aliquoted into micro vials (1ml) and stored in -20ºC till the moment of evaluating degree of cristalization and ferning. At that moment it was thawed (to 20ºC). In each sample a smear was carried out placing one drop of mucus on one extreme of a clean and properly labeled slide; the material was then spread softly and firmly with another slide. The content was let to dry out for about 30 minutes and was then observed through a binocular microscope of clear field (Leica DM750), with camera (Leica EC3).

e1) eye observation of the flow:

- quantity (scarce or null, moderate, abundant or copious)

- consistency (solid or gellatinous, medium, liquid)

- appearance (opaque, translucent, transparent)

The three variables mentioned above were evaluated by eye and summarize the macroscopic characteristics or those observable at simple sight.

-pH. It was measured using a reactive tape Rank 6-8.9 in roll (Macherey Nagel, Germany), letting a drop fall into such material.

e2) Flow analysis with optic microscope

- Degree of crystallization. A 0 to 4 scale was used, being 0: no crystal formation; 1: all crystals have atypical fern leaves; 2: mainly atypical crystal forms and only some typical, 3: mostly typical crystals and only some atypical in well defined fern leaves, 4: all the arborization corresponds to typical crystals. (Fig.1)

-Types of estrogenic mucus. The type of mucus was also classified according to the location of the secretory cells within cervix, differentiating the estrogenic types: L, Pand S. (15, 16)

f) Hormone levels in serum. Blood was withdrawn from the coccyx vein, and then centrifuged to 3000 rpm/10 minutes. The serum obtained was stored in micro vials and kept in -20ºC until its later use for hormone determination. The concentrations of each hormone were determined through electrochemiluminescence (EQLIA) (Laboratory Alkemy, Santa Fe) (13). The hormones measured were:

* Stimulating follicle hormone: FSH (mUI/mL)

* Estradiol: E17B (pg/mL)

* Progesterone: P4 (ng/ml)

* Luteinizing hormone: LH (mUI/mL)

3. RESULTS

Each phase of the cycle could be differentiated according to the different variables observed, and it was particularly focused on the periovulatory period. Table 1 shows –according to the sample day and during two consecutive cycles, the characteristics referring to the animal behaviour, vulva observation, condition of the horns and ovaries and physical properties of the cervical flow.

Besides the macroscopic description of the flow, its pH and degree of arborization, the kinds of estrogenic mucus found were identified in each preparation, using some of them as a proof of the closeness of the ovulatory process. In that way, during the periovulatory period, each type of mucus found was identified: preovulatory days (Fig.2), the day itself (Fig. 3) and the days after ovulation (Fig. 4).

Through the horns and ovarian palpation and the ultrasound carried out on daily basis, the follicular growth could be confirmed within the ovary, and through the formation of the hemorrhagic and later corpus luteum, ovulation was confirmed and it contributed to detect the moment it was produced. (Table I)

The serum levels of FSH, LH, estrogens and progesterone determined for each sampling day, showed that they were within the normal rank and also its fluctuations in time confirm the progress of the oestrus (Fig. 5).

4. DISCUSSION

It has been proved that the variation of the estrous cycle length of cattle occurs mainly individually, per cow, and not generally in all bovine cattle (17). In this essay, each cycle studied lasted 22 days, oestrus showed up for 3 days, and ovulation took place within the 24 hours of the observation of the physical signs of oestrus and 48 hours from the beginning of the behavioural change, showing restlessness in the corral waiting to be milked, matching what has been accepted for Bos Taurus of 21 days long, with a normal rank between 18 and 24 days (6, 8, 20). After ovulation, the hemorrhagic corpus was immediately observed and 48 hours later the corpus luteum had already been formed. Both ovaries acted as one; the dominant follicle prevented the growth of the subordinated ones and that would lead to the emergence of a new follicular wave. Only one follicle from both ovaries was selected to be dominant and its development was observed in one ovary as well as in the other.

Sexual behaviour is the combination of acts, rites and courtship which leads to the mating of the cow and the bull to reproduce and preserve the specie; that includes attractiveness, proceptiveness and receptivity from the heifer or cow as well as courtship and copulation from the bull (9, 10, 18). During the days before ovulation the cow showed some changes in its behaviour, which changed from being normal, that is to say, quiet, docile and respectful of its routine in the milking room, to showing restlessness, nerviousness, headbutting other cows, having a passive attitude when mounted and consuming 20% less balanced food in the milking room. These results coincide with a work which studied the behaviour of Holstein-Friesian milking cows during spontaneous cycles of oestrus (24), where the secondary most frequent signs were: smelling of the anogenital area, accepting the leaning of the chin on the rump, chin leaning accepted, smelling received, mounting other cows, headbutting, mounting received, trying to mount other cows, ramming received, game with physical contact, mounting rejected, chasing initiated, threaten received, flehmen, escape, bellow and licking received.

The vulva appeared turgid, bigger in size and its pale mucus turned reddish or bright pink; the horns were felt firm, rolled and symmetrical. Some hours before the ovulation, the vulva lost turgidity and size, then, immediately after its occurrence, the vulva got its normal size back and its labia were seen flabby, and the mucus turning to a really light pink.

The cervical flow was seen clean, growing in quantity as ovulation was nearer and its consistency was liquid during the previous days, turning stringy (hanging from the vulva unable to cut in order to be laid on the floor) after the ovulation. Then, its appearance becomes translucent (loss of transparency) and eventually – days far from the ovulation- becomes hard to collect. And if it happened to be collected the sample was scarce; its consistency was gelatinous or thick and opaque. The mucus was abundant and fluid during the preovulatory and ovulatory phase, on the other hand, during the luteum was scarce and opaque. The pH of this secretion was higher when confirming ovulation (7.70 -7.80) and its value decreased as this event became further in time (6.70-7.60=; the degree of crystallization decreased as ovulation got closer, the crystals observed corresponded mostly to atypical fern leaves, while on the days further from ovulation crystals formed typical fern leaves. These results coincide with previous publications and with results announced by other work groups who analyzed the moment of ovulation in different bovine races (2, 11, 25)

The periovulatory period –between 48 hours before ovulation and 24 hours afterwards; confirming it by means of an ultrasound- was characterized by the study of the estrogenic mucus classified according to the type of gland participating in its secretion. In that way, it could be observed that the days before ovulation, about 75 % of crystals corresponded to mucus type L with mostly atypical palm leaves, also in the shape of short and markedly curved leaves; and lastly there were observed –although in a low percentage- rosette or four-point star shaped formations together with typical fern leaves. On the day before ovulation, besides mucus L, there is between 10 and 15 % of mucus P, irregular shaped patterns and laid in concentric stacked layers. On the ovulation day itself between 3 and 5 % of the mucus show type S, crystals in parallel axis, with few ramified thorns which start on both sides; this mucus was always accompanied with different subtypes of mucus P, mainly those observed in 6-point star shapes (30ºangles). This kind of mucus is the one that lubricates the vulva and keeps it humid enough, and makes mucus L more liquid. Mucus P stays for one or two days after ovulation. Those types of mucus have been described firstly in humans and are also used to detect the high peak day of fertility (14, 15, 16). In bovine cattle different types of crystal patterns have also been described, corresponding to each type and subtype of estrogenic mucus (4, 11), in this case there was not a daily monitoring throughout the cycle nor an emphasis on the periovulatory cycle. The presence of these types of mucus secretion prepares and optimizes the cervix to receive the sperm; the concentration of estrogens is at its peak and the vulva looks slippery and bulky but less turgid.

Finally the hormone levels confirmed each event observed, so, the increase of estrogens during the days before ovulation coincided with the behavioural change of the animal which became restless, nervious, with lack of appetite, headbutting and mounting its pairs. Moreover, the influence of estrogens caused water retention swelling the vulvar labia and folds of skin, -and due to the increase of the irrigation in the folds, these looked more coloured. The increase of the follicular diameter coincided with the increase of FSH in serum allowing the follicle reach 20 mm diameter, big enough to become the oocyte donor. Progesterone made a small ascending pulse at the moment the estrogens were diminishing, a hormonal situation which coincides with the short-term peak of LH, responsible for liberating the oocyte. Twenty-four hours later, through an ultrasound, a hemorrhagic corpus was observed which confirmed the occurrence of the ovulatory process. The behavioural oestrus was observed about 48 hours before ovulation and was more evident in the second cycle observed than in the first one. This could be due to environmental factors, among others, such as soil or ground conditions -since the first cycle studied coincided with a period of copious and recurrent rain which caused the ground to become muddy and the size of the sexually active group was smaller than in the second cycle observed. These modifications due to environmental factors were stated and proved by other authors previously (6).

What was observed as regards hormone variations, coincide with had already been published by other authors (1, 3) when studying the follicular waves of the oestrus cycle of cows. They claim that the increase of oestradiol and the decrease of progesterone are responsible for the evident oestrus behaviour and the preovulatory peak of LH. The selection of the dominant follicle is associated to a blood decrease of the concentrations of FSH during the three first days of the wave. The lowest peak in the concentration of FSH is reached four days after the emergence of the wave, and concentrations keep low for the next two or three days. An increase in the concentration of LH in blood precedes ovulation. This pulsating increase of LH is accompanied by low levels of circulating progesterone, the corpus luteum is the main source of progesterone: the morphology of the corpus luteum and the plasma concentration of progesterone are good indicators of the synthesis of progesterone within the corpus luteum.

On the other side, in studies carried out in order to compare the ovarian hormone profiles between cows and nulliparous heifers (21, 23), even though differences between both categories were detected (being lower the values corresponding to the cows), the curve described for estrogens and progesterone coincides with our results. They showed that the estradiol reaches its maximum concentration in the preovulatory period and that the levels of P4 reach their peak in the postovulatory period, making the corpus luteum responsible of such an increase, since that structure gets a bigger diameter in cows coinciding with a higher concentration of P4 if compared to nulliparous heifers. By discovering the smaller hormone levels for cows, despite the bigger size of their follicles and the corpus luteum, it could be stated that the high impact of reproductive anomalies such as low conception rates, anovulations and other failures, could be attributed to the low concentrations of estradiol despite the size of the follicles and the volume of the luteal structures of cows.

Several groups have investigated about the sexual behaviour -natural as well as induced- in bovine cattle; in Hosltein especifically, it was observed that the quiet acceptance of mounting and the mounting itself were not consistent as oestrus indicators, proposing more use of other signs in order to detect oestrus (19). This concept generates the need to check in which productive system the cow is and which kind of cow is used. These authors coincide with other groups of investigators (5, 7, 22) who state that despite the great scientific and technological advances in the field of animal reproduction and specially in the pasture systems, oestrus detection keeps being the main problem of bovine cattle because it requires the combination of the cow to express the oestrus and the timely presence of man and his technique in order to detect it,

5. CONCLUSSIONS

Modern systems of exploitation tend to interfere with the natural sexual behaviour due to the difficulties found when trying to carry out insemination at the most adequate moment. This situation forces a bigger demand of time and effort on the comprehensive / integral evaluation of the cow which, eventually will result in a higher precision when determining the closest moment to ovulation, optimizing reproduction.

In this sense, it is concluded that ovulation can be inferred using the following tools as a complement:

* The restless and unmanageable behaviour turns into a quieter, less nervous one.

* The vulva looks smaller, loses turgidity, the swelling decreases and also does the coloring of the mucus.

* The flow turns stringy (egg white) –from being abundant and liquid- it decreases in quantity and is always clean and transparent.

* The mucus pH loses acidity, getting close to 7-7.50.

* The presence of mucus type S3 and P6 in low percentages together with typical and atypical fern leaves (mucus L).

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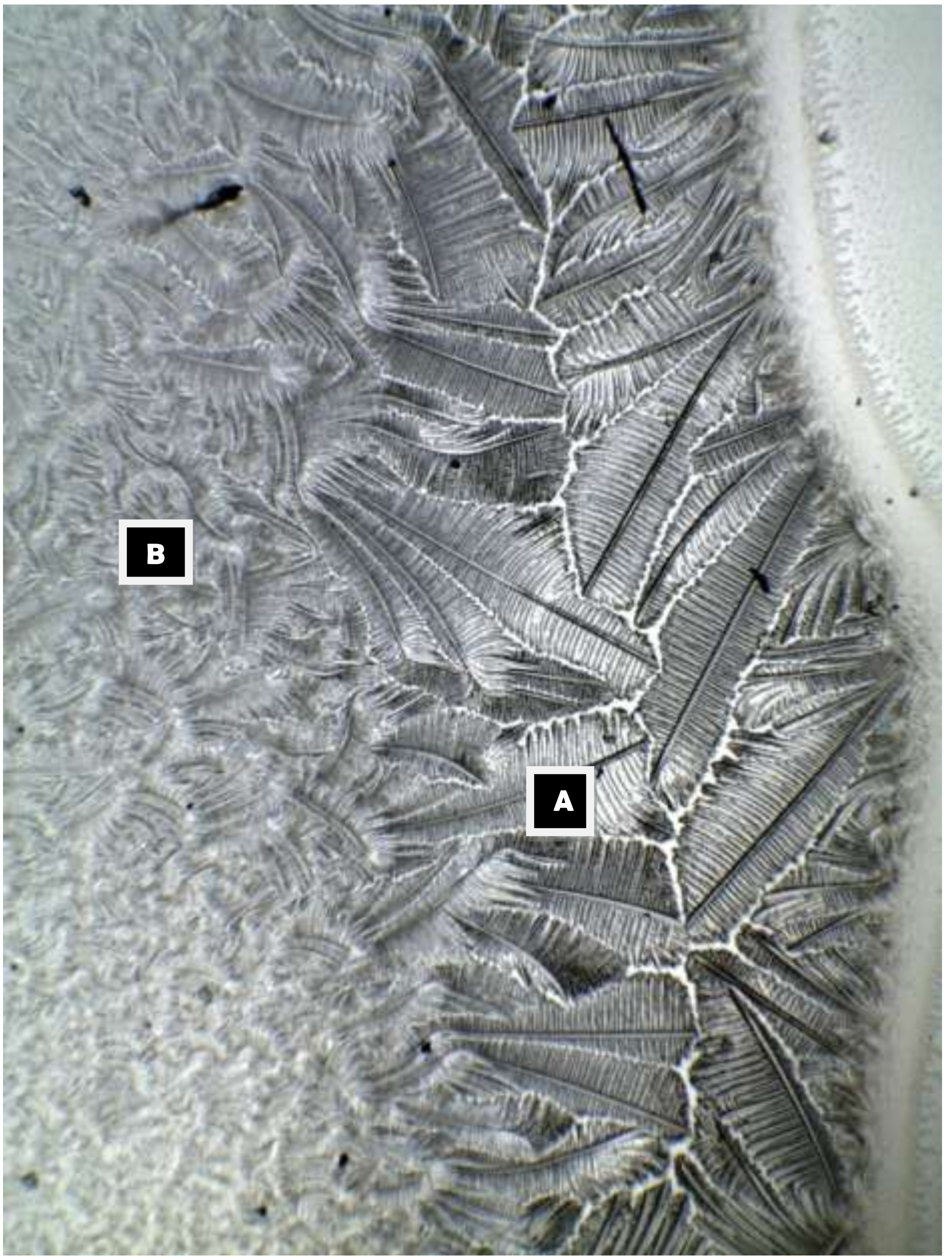
Table 1. Parametres observed in the bovine oestus cycle: animal behaviour, horn conditions, ovarian ultrasound, cervical flow.

Day	Behaviour	Vulva	Horns	Ovaries		Cervical flow		
				Left	Right	Aspect	Crist degree	pH
0	Normal	Normal and pale folds	Symmetrical and winding, semi-soft in consistency	Medium Fol. 12 mm	Leuteun Corpus 30 mm	none	--	-
4	A bit unease	Turgid bright reddish	Symmetrical and winding - Firm	Big follicles 18 mm	No formations	Transparent and liquid	3	7.00
5	unease, nervous, indocile, headbutting	Turgid bright reddish		Big follicles 18 mm	No formations	Transparent and stringy	2	6.70
6	oestrus behaviour and physical signs	Size decrease, turgidity loss and becomes paler	Symmetrical and more winding – very firm	Big follicle 20 mm	No formations	Abundant, transparent and stringy	1.50	7.20
7	Normal	Normal, flabby, soft pink	Symmetrical and winding - Firm	Big follicles, **hemorrhagic corpus**	No formations	Moderado, gelatinoso y traslúcido	2	7.50
14	Normal	Normal, flabby, soft pink	Symmetrical and winding – moderate tone	2 corpus luteum 30 mm, follicle 15 mm	Small follicle	Escaso, espeso y gelatinoso	3	6.80
25	Normal	Normal, flabby y a bit pinker	Symmetrical and winding – Very firm	corpus luteum of 19 mm, follicle 13 mm	follicle 19x15 mm	Abundant, transparent and stringy	1,50	7.00
26	Normal	swollen, very turgid, bright pink		corpus luteum of 19 mm, follicle 13 mm	follicle 19x16 mm	Abundant, transparent and stringy	1,50	7.00
27	indocile, nervous, unquiet	Turgid and bright pink	Symmetrical and winding – Very firm	luteum corpus follicle 13 mm	follicle 20x19 mm	Abundant, transparent and stringy	2	6.80
28	quieter, physical signs of oestrus	Vulvar swelling decrease, not so evident	Symmetrical and winding – Very firm	follicle 13 mm	follicle 21x19 mm	Abundant, transparent and stringy	1,50	7.30
29	Normal	Normal, flabby, soft pink	Symmetrical and winding – firm and elastic	follicle 13 mm	**Hemorrhagic corpus**, absence of follicle	Abundant, transparent and stringy	2	7.50
35	Normal	Normal, flabby, soft pink	Symmetrical, little winding. Soft	Small follicles	corpus luteum with central pond and médium follicle	Scarce, thick and traslucent	3	6.60

Figure 1. Crystalizaton of bovine cervical flow (4X)



Mucus L: **A.** Typical fern leaves. **B**. Atypical fern leaves

Fig. 2. Types of estrogenic mucus of the preovulatory period

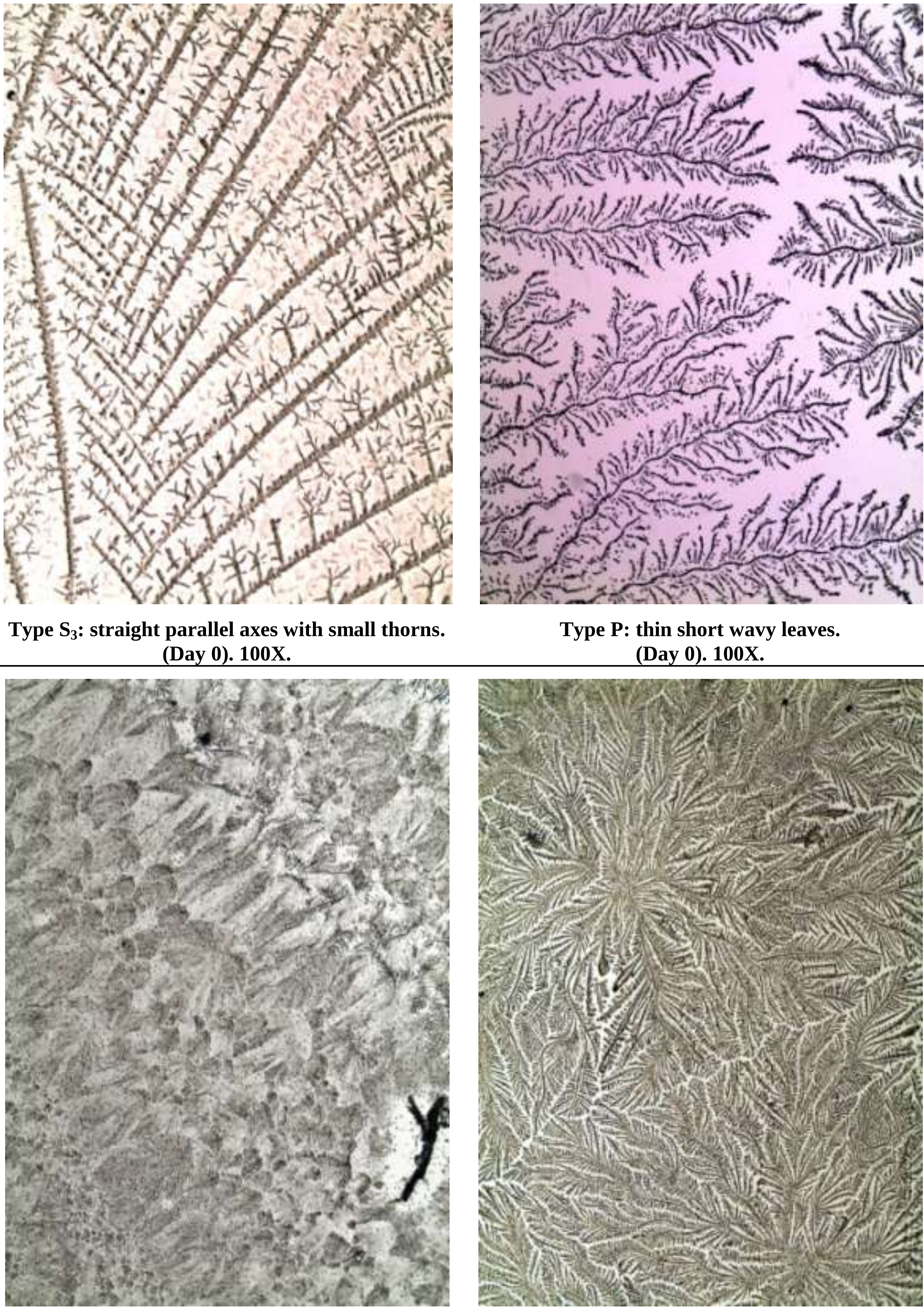
Type L: palm leaves (- 3 days). 100X.

Type L: 4 - 90º axis roseettes (-2 days). 40X.

Type L: short leaves with curve lines (-2/-1 days). 100X.

Type P_6: 4-to-6-axis - rossettes (Day -1/0). 100X.

Figure 3. Types of estrogenic mucus: Ovulation Day



Type S_3: straight parallel axes with small thorns. (Day 0). 100X.

Type P: thin short wavy leaves. (Day 0). 100X.

Type P: irregular patterns (-1 day/ 0). 100X.

Type P (Day 0/+1). 40X.

Figure 4. estrogenic Mucus. Postovulatory Period

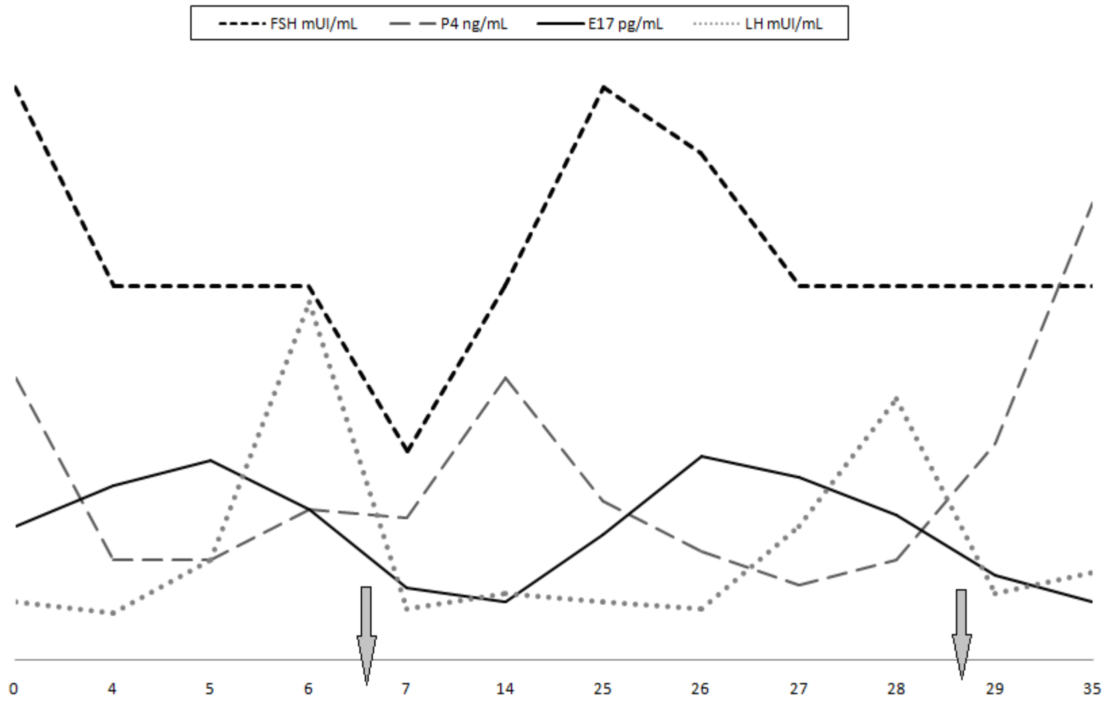
Type P (Day +1). 40X.

Type P_t (Day +1). 100X

Figure 5. Hormonal levels and curves during two consecutive cycles

Day	FSH mUI/mL	P4 ng/mL	E17 pg/mL	LH mUI/mL
0	6.2	1.45	0.55	0.10
4	5.0	0.35	0.8	0.03
5	5.0	0.35	0.95	0.35
6	5.0	0.65	0.65	1.90
7	4.0	0.60	0.18	0.05
14	5.0	1.45	0.10	0.15
25	6.2	0.70	0.50	0.10
26	5.8	0.40	0.97	0.05
27	5.0	0.20	0.85	0.55
28	5.0	0.35	0.62	1.32
29	5.0	1.05	0.26	0.15
35	5.0	2.50	0.10	0.27

Note: Highlighted line indicates ovulation day



Note: Arrows indicate the moments of ovulation