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STUDY OF ESTRUS INDUCTION IN THE FEMALE DOG BY PMSG AND HCG

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ABSTRACT : *Our study, spread over four (4) months (February to June), aims at heat induction and monitoring within a population of 10 female dogs of different breeds (German shepherd, Rottweiler and Pit-bull). Aged from 2.5 to 4.5. Two of them were used as controls. To do this, we carried out the following investigations: Monitoring the sexual cycle of the female dogs, prior to heat induction, by gynecological and cytological examination (vaginal smears) to confirm the sexual rest phase (anestrus); hormonal induction of heat by PMCG and hCG; monitoring the induced heat by the vaginal smear method (Giemsa stain); and a breeding of female dogs in estrus confirmation of the pregnancy of the mated female dogs (palpation and ultrasound) by comparing the vaginal smears of the treated female dogs with those of the control female dogs. At the end of our study, the results obtained were encouraging, since we succeeded in obtaining the appearance of heat in 60% of the treated female dogs.*

KEYWORDS: Female *dogs, sexual cycle, vaginal smears, heat induction, PMSG, HCG, gestation*.

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INTRODUCTION

The female dog presents a continuous ovarian cycle lasting between 150 and 250 days that is not interrupted by gestation (Locci D., 1983). It is a non-seasonal mono-oestrous species with spontaneous ovulation, which means it has only one period of heat per cycle during which ovulation occurs independently of the season and in the absence of a male dog (Barone R., 1978). Regarding puberty, studies (Colin M., 2004) have shown that small breeds reach puberty earlier (5 to 8 months) than large breeds (10 to 18 months). The dog's reproductive pattern is different from other mammalian species. It is characterized by a long inter-oestrus interval (diestrus and sexual rest) for which it has been described as a monocyclic animal. Studies (Bisen et al., 2021) have also depicted apparent evidence that geographic conditions and breed influence reproductive function. The sexual cycle of a female dog is divided into 4 phases: pro-oestrus, oestrus, metestrus and anestrus. The ovulation period is between the 2nd and 5th days following the LH surge. The sexual cycle is under hormonal control. This regulation involves the central nervous system, the anterior pituitary and the gonads (Nathalie A., 2007). *The oestrogen* secreted by the ovaries is responsible for congestion and oedema of the vulva and vagina as well as hyperplasia of the uterus. They increase uterine contractility which promotes the cervix's opening. Moreover, they are responsible for the growth of the vaginal epithelium accompanied by keratinisation and desquamation of the cells of the superficial epithelial layer. They conduct the behavioural manifestations of heat (Nathalie A., 2007) whereas *progesterone* is mainly secreted by the corpus luteum and in lower percentage by the ovarian follicles (Leroyer C., 2000; Nathalie A., 2007).

Pre-ovulation luteinisation is a peculiarity specific to female dogs. Progesterone secretion begins a few days before ovulation. After 40 days, progesteroneemia gradually declines to a basal level with a sharp reduction from 24 to 48 hours before farrowing (Nathalie A., 2007). Concerning the diagnosis of anoestrus in female dogs in particular, the etiological diagnosis is based on several methods, four of which are mentioned as follows (Attaba A., 2006): Pap smear developed in the 1980s is based on changes in the vagina walls (epithelium) during the oestrous cycle (Malandain E., Fontbonne A., 2006; Taradach C., 1980). Interpretation should be based on the five elements—presence of blood cells (WBC and/or RBC), shape and size of epithelial cells, presence or absence of nuclei in its cells, shape and size of nucleus if it exists and the dye affinity of the cells (Harris-Shorr and May Grunwald-Giemsa's staining) (Mimouni Ph. & Dumon C., 2005; Barone R., 1978). Ultrasound (Bouchard G. F., Gross S., 1993) and the endoscope first provides additional details on ovulation compared to pap smears and hormonal assays (Mimouni Ph. & Dumon C., 2005); meanwhile, the second allows to observe the appearance of the vaginal mucosa during the cycle. This method makes it possible to highlight the modifications of contours, the secretion present and the colour of the mucous membrane (Attaba A., 2006; Nathalie A., 2007). Another method is based on assay of progesterone (Bourdelle B. & Bressou C., 1953); the latter is measured using rapid kits (i.e., ovulation test) (Colin M., 2004). The projection or the insemination will be practiced only when the progesterone reaches values higher than 15 to 20ng/ml (Mimouni Ph. & Dumon C., 2005). Finally, the resistivity of the vaginal mucus—this test consists of measuring the electrical conductance of the vaginal mucus, which changes during heat substantially in parallel with the keratinisation of the vaginal epithelium (Nathalie A., 2007). Numerous protocols use PMSG (Pregnant Mare's Serum Gonadotropin) for a period of 8–10 days at a daily dose of 20–500 IU/Kg S/C or I.M, followed by a single injection of 500 IU of hCG (human Chorionic Gonadotropin) by I.M route (**Buff S**., 2001) since 1939. The first signs of oestrus are observed

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on an average of 10–15 days after the start of treatment; however, the fertility rate rarely exceeds 50% (Buff S. & Halesse H., 2000).

In the case of female dogs having received anabolic treatment, the prior use of diethylstilbestrol (D.E.S.) until the first signs of pro-oestrus are obtained at a dose of 5mg/day (> 7 days) seems to improve the results (Buff S., Fontbonne A. & Buff-Vogt C., 1999; Buff S. & Halesse H., 2000). The administration of GnRH (Gonadotropin Releasing Hormone) or a GnRH agonist is possible when the functioning of the pituitary-ovary axis is preserved because GnRH directly stimulates the secretion of pituitary hormones.

Contrary to all expectations, the establishment of an uninterrupted infusion for 14 days of a GnRH analogue (luteolin) made it possible to obtain nearly 50% of pregnancy. The administration of Triptorelin by S/C route at a dose of 0.5 µg/kg three times a day for 11 days, would have made it possible to obtain 80% of pregnancy in the treated females. Indeed, similar observations have recently been reported during the use of deslorelin implants at a dose of 4–8 ug/kg/d initially intended to obtain a lasting contraceptive effect (Buff S. & Halesse H., 2000; Concannon P. W., 2006).

More recently, the use of prolactin inhibitors with dopaminergic effect (Bromocriptine, Metergoline and Cabergoline) has revealed a new possibility of estrus induction. Regardless of the type of treatment or control, the control of canine reproduction remains difficult (notably in countries where there are heterogeneous populations formed by pure breeds, crossed-breeds and mongrels). The main objective of treatments for the artificial induction of estrus in the female dog is to control the reproductive function, which makes it possible to voluntarily trigger heat and to shorten the phase of anestrus. The main objective of artificial estrus induction treatments in the female dog is to restore reproductive function, thereby voluntarily inducing estrus and shortening the anestrus phase.

MATERIAL AND METHODS

For heat induction and monitoring of the oestrous cycle by the vaginal smear method, the experiment was carried out in private homes in the Mitidja region (Central Algeria). Ten female dogs aged over 2 years and regularly cycled were selected. Two were taken as controls and eight divided into three batches—Batch 1: two German Shepherds and a Pit bull, Batch 2: a German Shepherd and two Rottweilers and Batch 3: a German Shepherd and a Pit bull. These three batches underwent heat induction treatment, applying a hormonal protocol based on PMSG and hCG accompanied by rigorous monitoring of the estrus cycle by gynaecological and cytological examination (vaginal smears). The female dogs were monitored during several visits, from 28/02/2019 to 10/06/2019 (4 months). Additional information was requested using material safety data sheets (MSDS) (anamnesis and analysis of vaginal smears).

Gynecological examination was conducted during each visit, ending with a vaginal smear in order to specify the phase of the sexual cycle and to detect any genital disease. In total, 20 vaginal smears (2 smears per female dog and at an interval of 3 days) were made and their interpretation was executed at the clinic of the Veterinary Department of Blida 1, Algeria. Finally, after insemination, a pregnancy diagnosis was conducted using an ultrasound to confirm the results.

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Samples for Vaginal Smears

After immobilizing, the animal with a good restraint, the female dog's tail, is lifted to perform a rubbing act on the vaginal walls using a swab. The latter moistened with physiological saline is aimed at the upper part of the vagina in order to avoid the urinary meatus and the clitoris. First, we gently rub the walls of the vagina by making a rotational movement. In the second step, we immediately roll the swab on a degreased blade to dry it in the open air afterwards.

Principle of Coloring

It is based on the combined action of two neutral dyes: The first is May-Grünwald's neutral dye, containing an acid dye, eosin, and a basic dye (methylene blue). The second is Giemsa's, also containing eosin, and a basic dye (methylene azure).

1. **Heat Induction**

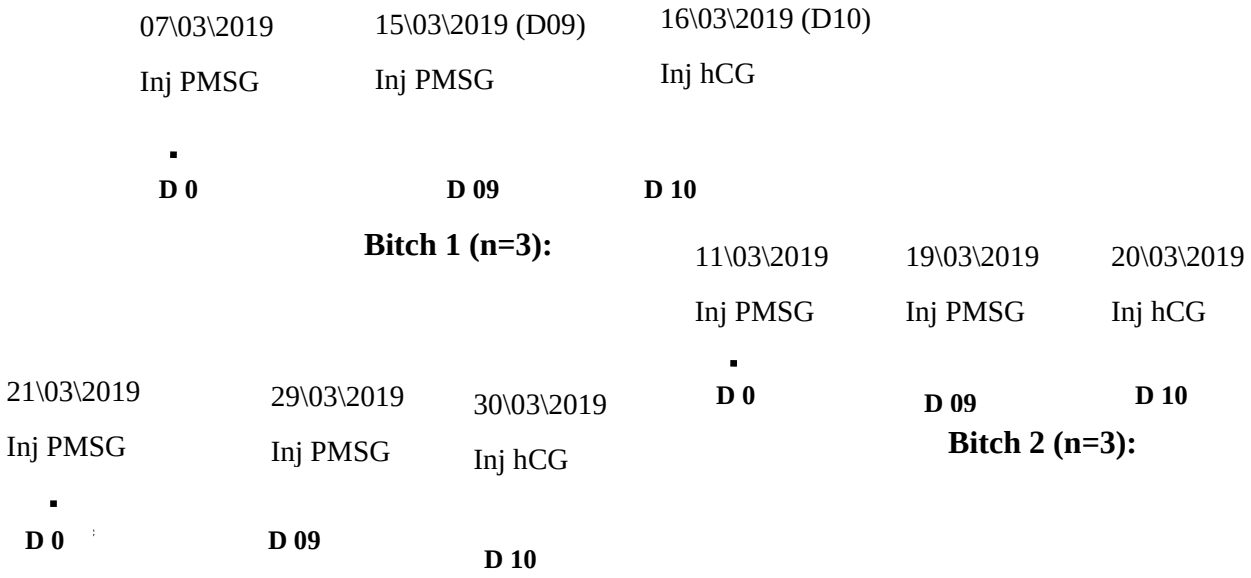

Figure 01: Protocol for heat induction in female dogs

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PMSG (Folligon® - INTERVET): One daily intramuscular injection of 500 IU per female dog, corresponding to a dosage of 2.5 ml for nine days.

** hCG (Chorionic Gonadotropin Endo 5000® - Organon): Intramuscularly on the tenth day of treatment at a dose of 500 IU per female dog, corresponding to a dosage of 0.1ml.

Statistical Analysis

The data was organised in an Excel file allowing the statistical analysis performance. Two types of analyses were executed. The first one was descriptive, highlighting the percentages of the different parameters:

1- The results of the observation of the vulvar region.

2- The results of the onset of heat after the induction treatment.

3- The appearance of heat after induction treatments depending on breed and age.

4- Acceptance of the male.

The second was an analysis of variance conducted using the ***XLSTAT VERSION 2021 software***. Concerning the appearance of heat after induction treatment, according to age and race, the error threshold used was 5%.

RESULTS AND DISCUSSION

Based on the records and gynecological examination, taking as criteria the vulva swelling and the flow of blood, all the female dogs were in the sexual resting phase (anestrus). Taking into consideration the first day of onset of blood flow to be the first day of heat, more than 50% of the induced female dogs came into heat manifesting blood flow, while only 50% manifested a swollen vulva (Table 1).

Table 1: Vulvar swelling and blood flow after heat induction

Female dog's number	**01**	**02**	**03**	**04**	**05**	**06**	**07**	**08**	**%**
Vulvar Swelling	0	0	1	1	1	0	1	0	50
Blood flow	1	1	1	1	1	0	1	0	62, 5

***(0): Absence of clinical signs** ***(1): Manifestation of clinical signs**

The state of swelling vulva is a subjective criterion depending on the judgment of the examiner. For these reasons, pictures of the vulva and perineum were taken during each visit and for each female dog to make the examination more critical. In 50% of the cases (Nos. 03, 04, 05 and 07), the change in size was noticeable. Similarly, 50% of the female dogs indicated a continuous bright red blood flow without any interruption until the end of heat, with a stringy

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consistency and a characteristic blood odour. Thus, 60% of the cases studied revealed the external signs of heat.

Figure 2: Swelling of the vulva on an experimental female dog

The monitoring of the oestrous cycle by the vaginal smear technique in female dogs has shown a large presence of red blood cells, superficial cells and large intermediate cells which are preponderant proofs of a state of pro-oestrus.

Swelling of the vulva

Vaginal Smear Results:

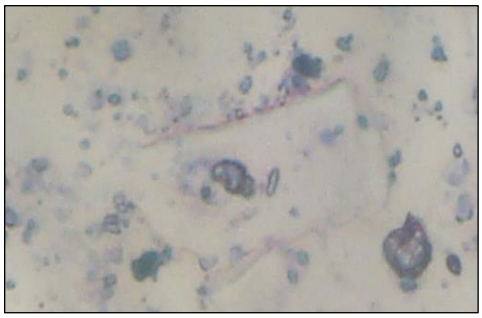

Figure 3.1: treated female

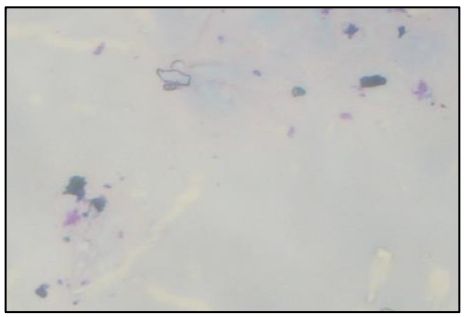

Figure 3.2: controls female

Performing vaginal smears as soon as bleeding begins every 2–3 days enables the identification of the oestrus' onset moment: more than 60% of large superficial keratinised cells (red cytoplasm in Harris Shorr staining), with nuclei more or less pyknotic.

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Table 2: Onset of heat according to the number of days following the treatment by breed.

Breed	Heat	Num. of days after the beginning of treatment
German Shepherd	+	13
German Shepherd	-	17
Pitbull	+	12
German Shepherd	+	12
Rottweiler	+	14
Rottweiler	-	17
German Shepherd	+	12
Pitbull	-	17

Table 3: Variation in heat response according to breed

Bitch	DDL	R^2	Equation	P
Heat and Breed	14	0.947	Y = 14,25-13,625*Breed-Heat	**<0,0001**

On the report of the results about the onset of heat according to the number of days following treatment by breed, it was found that 3 out of 8 revealed a negative response after 17 days, while the analysis of variance revealed a very significant effect ($p<0.0001$) of the onset heat according to the breed and also a very strong coefficient of determination ($R2 = 0.947$) (Table 3).

Table 4: Heat onset by breed

Breed	Num.	Positive Heat	%
German Shepherd	**4**	**3**	**75**
Rottweiler	**2**	**1**	**50**
Pitbull	**2**	**1**	**50**

Regarding the breed factor, it was observed that German Shepherds responded the most to treatment with 75%, followed by the other breeds (Rottweiler and Pitbull) in Table 4, whilst the age effect showed an absolute positive effect in female dogs aged between 3 and 3.5 years (Table 5).

Table 5: Onset of heat according to age

AGE (year)	Total	Positive Heat	%
2,5	**2**	**1**	**50**
3	**2**	**2**	**100**
3,5	**1**	**1**	**100**
4	**1**	**0**	**0**
4,5	**2**	**1**	**50**

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Figure 6: Ultrasound displaying pregnancy

The ultrasound in Figure 6 was taken 30 days after the fertilization and the outline of the fetus, which is still only a few millimeters long, can be seen.

Given the importance of reproductive management in the canine species, the induction of heat in this species has been widely studied in recent years. Satisfactory results have been published by many authors. However, the various factors such as age, breed and the detection methods used by veterinarians can give varying results from one study to another. The duration of pro-oestrus and oestrus is highly variable from one female dog to another; it is therefore arduous to determine the period during which a female dog is fertile and the dates of mating. Our study revealed several results which are more or less close to those which were quoted in the bibliography. Concerning the rate of onset heat is 60%. This figure is comparable to that found by Buff S. et al. (1999) and Kutzler (2005) which is respectively 50%, 60% and 64%. In addition, an average duration of onset of heat of 12.6 days after the start of treatment was noted. This value is included in the interval adopted by Buff S. et al. (1999) and Buff S. (2000) of 10–15 days. Moreover, the analysis of the variation in heat responses according to breed claimed a significant effect ($p < 0.0001$), thus explaining the variations in the estrus cycle from one breed to another.

The age factor of the female dogs did not have an influence on the results of onset heat since there are intrinsic individual variations. During the breeding of female dogs in heat, it was observed that the average duration of male acceptance was of 11.2 days after the appearance of the first blood flows. The percentage of pregnancies following the breeding of treated female dogs is 60%. This rate is relatively higher than that obtained by Buff S. et al. (1999) and Buff S. (2000) reporting a rate of 20 to 30%. All the female dogs visited manifested dry discharge. However, their vulvas were normal or atrophied with a pale coloring of the vaginal mucosa, which justifies their phase of anestrus (sexual rest). Vulva swelling was recorded in 50% of the cases. Nonetheless, its appreciation remains relative to the observer; therefore, the use of this

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parameter can only have an indicative value. Also, a detected presence of blood flow in 60% of cases was made. The use of this parameter enabled the recognition of the beginning of the heat. Before the start of the protocol, it revealed an evident background on which there were few isolated parabasal cells and sometimes grouped in clusters.

These findings respond to results reported by different authors (Buff S. & Fontbonne A. et Buff-Vogt C., 1999). However, Mialot J. P. (1984) and Mimouni Ph. and Dumon C. (2005) reported the existence of red blood cells in variable numbers in this phase of the cycle, which was not found in our smears. After heat induction, and during hormonal treatment, no change in the smears was noticed. Nevertheless, from the beginning of heat, there was a significant variation in the results of the observed smears. The bottom has become dirty and on which there are numerous erythrocytes. These results confirm those reported by these same authors (Buff S. & Fontbonne et Buff-Vogt C, 1999; Mialot J. P., 1984; Mimouni. Ph. & Dumon. C, 2005). However, the number of red blood cells was variable in the smears, since they attach with considerable efforts to the blade than other cells. During the oestrus phase, a clear background on which there are various parabasal, intermediate and superficial cells accompanied by minor red blood cells were revealed. Howbeit, in a portion of vaginal smears, it was found that a high number of red blood cells, which is expected according to Attaba A. (2006), to uninterrupted blood loss in some female dogs which can last until the phase of metestrus. A dirty bottom, full of cellular debris due to the desquamation of the vaginal epithelium, on which there are numerous parabasal, intermediate cells scattered and sometimes grouped in clusters, was noticed during this period, occasionally accompanied by red blood cells. These results are consistent with those reported by various authors (Buff S. & Fontbonne A. et Buff-Vogt C., 1999; Mialot J. P., 1984; Mimouni Ph. & Dumon C., 2005). The staining technique used (Giemsa stain) does not allow determination of the tinctorial tendency of the vaginal cells (basophilic or acidophilic). To calculate the eosinophilic index, there are two essential elements to forecast when interpreting vaginal smears. This can be obtained using Harris-Shorr staining.

CONCLUSION

This work represents an essential step in the restoration of reproduction and control of the estrous cycle in female dogs. To induce heat, it was established to use a hormonal protocol based on PMSG and hCG in anestrus female dogs, accompanied with an investigation by the vaginal smear method (Giemsa staining) associated with an appropriate gynecological examination.

Vaginal smear examination is a reliable method that requires careful preparation of the samples and accurate knowledge of the cell types present. Progesterone testing could not be performed due to its high cost. The rate of female dogs in heat was 60% with an average onset of 12.6 days after treatment. The pregnancy rate of induced female dogs was 60% (3/5 of them admitted to breeding), which represents a considerable recovery of reproductive losses. The results of the study were small and therefore cannot be generalised to the general dog population. In the future, studies on representative populations will undoubtedly consolidate these results.

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