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Original article

Preovulatory progesterone secretion terminates the duration of reproductive behavior during heat in the bitch

K. Bladowska, W. Barański, T.E. Janowski

Department of Animal Reproduction with Clinic, Faculty of Veterinary Medicine, University of Warmia and Mazury, Oczapowskiego 14, 10-719 Olsztyn, Poland

Abstract

To evaluate the role of preovulatory progesterone on canine sexual behavior and the course of proestrus and estrus, seven bitches in spontaneous cycles were treated with aglepristone for temporary elimination of progesterone action. Aglepristone was administered at the dose 10 mg/kg b.m., two times 24 hours apart, beginning in early proestrus when progesterone concentration was <0.5 ng/ml. Seven untreated bitches served as a control group. Reproductive sexual behavior (standing behavior, display vulva, tail deviation) was evaluated according to behavioral score. Cytologic, clinical and vaginoscopic examinations and progesterone measurements were used for the determination of proestrus and estrus and estimation of ovulation time.

Although, a similar pattern and magnitude of sexual behavior were observed in both groups, the duration of a total reproductive behavior was significantly extended (28.71 ± 2.06 vs 17.00 ± 2.45, $p < 0.05$) in experimental group; similarly, the length of cytologic estrus (23.86 ± 3.02 vs 11.14 ± 2.41, $p < 0.05$) was prolonged in treated bitches. In contrast, ovulation rate, duration of proestrus did not differ between the groups ($p > 0.05$). We conclude, that during the canine estrus cycle the preovulatory progesterone terminates the duration of reproductive behavior and cytologic estrus.

Key words: bitch, preovulatory progesterone, aglepristone treatment, reproductive behavior, estrus

Introduction

Bitches are monoestrus, non-seasonal and spontaneous ovulators. The physiological canine cycle is divided into four phases – 5 to 20 days of proestrus, 5 to 15 days of estrus, 50 to 80 days of metestrus and an anestrus typically lasting 80 to 240 days (Concannon 2011). Endocrine regulation for dog cycles is in many aspects different from those of females in other domestic species. Among numerous endocrine particularities such as a prolonged LH secretion, luteotrophic roles for both LH and prolactin, a prolonged luteal phase and the lack of uterine luteolytic mechanism, a pronounced preovulatory luteinization of follicles associated with progesterone increase is considered an important factor influencing reproductive events during the perio-

Correspondence to: T.E. Janowski, e-mail: jantom@uwm.edu.pl

vulatory period (Hoffmann et al. 1996, Groppetti et al. 2015). This latter phenomenon has also been documented in humans and rodents (Concannon 2011). The results of previous studies in the bitch suggested that a declining estrogen:progesterone ratio during proestrus-estrus is a trigger for ovulation and the appearance of specific sexual behavior; however, these mechanisms have been poorly understood until now (Concannon et al. 1979b, de Gier et al. 2006). Normal reproductive behavior, characterized by increased male seeking and proactive receptivity to mounting by males, is required for physiological fertility. Although during recent years several clinical and laboratory techniques such as vaginoscopy, cytology and measurements of progesterone have been implemented in the control of canine estrus cycle, the evaluation of sexual behavior is still very important for the monitoring of reproduction (Kustritz 2012). Attractivity towards males remains high throughout estrus, whereas receptivity increases over the first 3 days of estrus and continues at a high level until the last 3 days, during which it decreases and finally drops nearly to zero (Beach et al. 1982, Kustritz 2005).

The monitoring of sexual behavior is not an easy task, due to the very individual pattern of estrus displayed between individual bitches. Sexual behavior is scored in the presence or absence of males and definitions of estrous onset vary between "receptive reflex", "standing heat" and "sexual reflex". Sexual reflexes of the vulva, tail and hindquarters follow the same course of changes described for attractivity (Christie and Bell 1972, Beach et al. 1982, Concannon 2011).

Information on hormonal mechanisms controlling canine sexual behavior is limited, particularly with regard to the preovulatory release of progesterone (Concannon et al. 1977). Serum progesterone, reflecting follicle luteinization, increases very slightly during mid-proestrus, rising from 0.2-0.4 ng/ml to reach above 1-3 ng/ml during the preovulatory LH surge; then it rapidly increases further, reaching 10 to 25 ng/ml after ovulation and during formation of a new corpora lutea. At the same time, estradiol continues its decline from peak values of late proestrus. Sexual behavior onset is facilitated synergistically by the above described rapid rise in progesterone resulting from the LH surge and the decline in estrogen after an ovulatory collapse of follicles, and their change into corpora lutea (Concannon et al. 1977, Wildt et al. 1979, Hoffmann et al. 1996, de Gier et al. 2006, Concannon 2011).

The essential role of progesterone for ovulation, establishment and maintenance of pregnancy and mammary gland development in the humans, rodents and bitches is rather well documented, whereas its involvement in the expression of sexual behavior in the bitches has not been studied intensively. It has been reported that in ovariectomized bitches, objectively scored sexual behavior induced by estrogen injections was more intense, rapid and synchronous when progesterone was administered at the time of estrogen withdrawal (Concannon et al. 1979b). This finding underlines the importance of preovulatory progesterone for the onset of normal behavioral estrus. In older studies performed in rodents, progesterone and its metabolites seemed to have some common stimulating effects on reproductive behavior in females, e.g., sexual motivation, receptivity and proceptivity (Birke and Sadler 1983, Fryea et al. 1998). Moreover, inhibition of sexual behavior in female guinea-pigs was possible via blockage of the progesterone receptor, using its antagonist RU 384486 (Brown and Blaustein 1984).

Treatment with aglepristone, which is a well-known progesterone receptor antagonist, allows the field to develop a less-invasive experimental model for temporary elimination of endogenous progesterone biological action (Reynaud et al. 2015). In numerous studies, administration of aglepristone was reported to antagonize the biological progesterone function in non-pregnant and pregnant bitches (Galac et al. 2004, Gogny and Fiéni 2016).

To investigate the influence of preovulatory progesterone on the behavioral estrous, aglepristone, a progesterone antagonist, was administered to bitches who had entered a state of spontaneous heat prior to the endogenous increase in circulating progesterone.

Therefore, the aim of our study was to simultaneously evaluate behavior as well as course of estrus of bitches, using cycles of temporary inhibition of endogenous progesterone's action prior to ovulation and cycles without this treatment.

Materials and Methods

Animals and study design

Fourteen clinically healthy adult bitches of various breeds and cross-breeds, aged 2 to 8 years and weighting from 8 to 30 kg, were included in this study. Before the experiment all females had physiological cyclicity and displayed normal sexual behavior. The bitches were housed in indoor-outdoor runs, fed a complete standard dry diet twice a day and provided with water ad libitum.

All procedures were conducted according to the rules of normal veterinary practice with owner permission and ethical approval number 44/2013/DTN of the local ethical commission of the Faculty of Veterinary Medicine, Warmia and Mazury University in Olsztyn, Poland. The funding institution was Department

Table 1. Sexual behavior elements observed after manual stimulation of flanks and perineal area during proestrus and estrus and the corresponding scores used for evaluation of sexual behavior of aglepristone treated and control bitches.

Behavior elements	Scores			
	0	1	2	3
Standing behavior	nil	crouch	stand poorly	stand firmly
Vulva display	nil	minimal reaction	moderate reaction	strong reaction
Tail deviation	nil	minimal reaction	infrequent reaction	rapid and constant

of Animal Reproduction with Clinic, Faculty of Veterinary Medicine, University of Warmia and Mazury in Olsztyn.

The experiment began when the bitches entered spontaneous heat and displayed the first signs of proestrus (vulval oedema, bleeding). The bitches were randomly assigned into two groups: experimental group (I, n=7) and control group (II, n=7). Group I included bitches treated during proestrus with aglepristone (Alizin®, Virbac) at the dose of 10 mg/kg body weight subcutaneously two times 24 hours apart, according to the manufacturer recommendations for dogs. This treatment started when progesterone concentration was at levels of approximately 0.5 ng/ml. The effectiveness of anti-progestin treatment was controlled by cytologic response of vaginal epithelium according to Hoffmann and Gerres (1989).

In turn, to monitor proestrus and estrus phases as well as ovulation time, experimental bitches were examined clinically, vaginoscopically and cytologically every other day. Simultaneously, the serum progesterone concentration in peripheral blood was measured once a day during the entire experiment.

Control animals (group II) were injected with placebo according to the same protocol. They underwent the same examination procedure and were matched with the treated bitches based on the methods described above.

The examination procedure included documentation of sexual behavior. Standing reflex and the display of vulva and tail deviation were evaluated according to the behavioral score modified after Concannon et al. (1979b). On a group basis, a total behavioral score was calculated as the mean ±SD of the three scores above. The duration of the sexual behavior was compared between groups and analyzed statistically.

The cytologic criteria but also clinical and endocrine criteria of canine estrus cycle were used as a "gold standard" for the determination of proestrus and estrus stages as well as the estimation of ovulation time in the individual bitches. On this basis, an average duration of proestrus and estrus as well as estimated ovulation rate was determined in both groups of bitches. Moreover, the intervals from the first aglepristone injection to the estimated ovulation and interval from the beginning of proestrus to ovulation were also calculated.

In both groups the 1st Day of proestrus was considered as Day 1 of experiment whereas the last day of experiment was 1 st day of diestrus.

Monitoring of estrous behavior

The sexual behavior patterns for individual bitches of both groups were assessed by evaluation of intensity and duration of the three behavioral reflexes: standing posture, display of vulva and tail deviation. Bitches were tested by manual stimulation of the flanks and perineal areas and evaluating the intensity and length of time the sexual reflexes were held.

Sexual behavior tests were conducted by the same investigator, who was unaware of the treatment used in experimental animals. Behavioral tests were carried out every day between 08:00 and 10:00 am and continued till all bitches were nonresponsive.

Standing behavior was defined as the female is standing still, flagging tail and tolerate mounting and deflected loins as well as well tolerating the press. Displaying the vulva was defined as a characteristic vulva movement reaction after mechanic stimulation. Deviation of tail was defined as a characteristic reaction after mechanic stimulation near the vulva, in which the tail was moved aside and female presented her vulva.

To monitor behavioral estrous, the sexual behavior score from zero to three outlined in Tab. 1 was used. A bitch was considered sexually receptive if she showed standing posture accompanied by additional signs at least for a few seconds during two consecutive tests. An animal was considered no longer sexually receptive when she failed to respond to manual stimulation for 2 consecutive tests (mean sexual behavior score ≤2).

Progesterone measurements

The peripheral progesterone levels were measured in samples obtained by venipuncture of the cephalic or saphenous vein. The blood was centrifuged immediately (1200x g, x 15 min) and the obtained serum samples were stored at -18°C until assayed. The concentration of progesterone was evaluated via radio

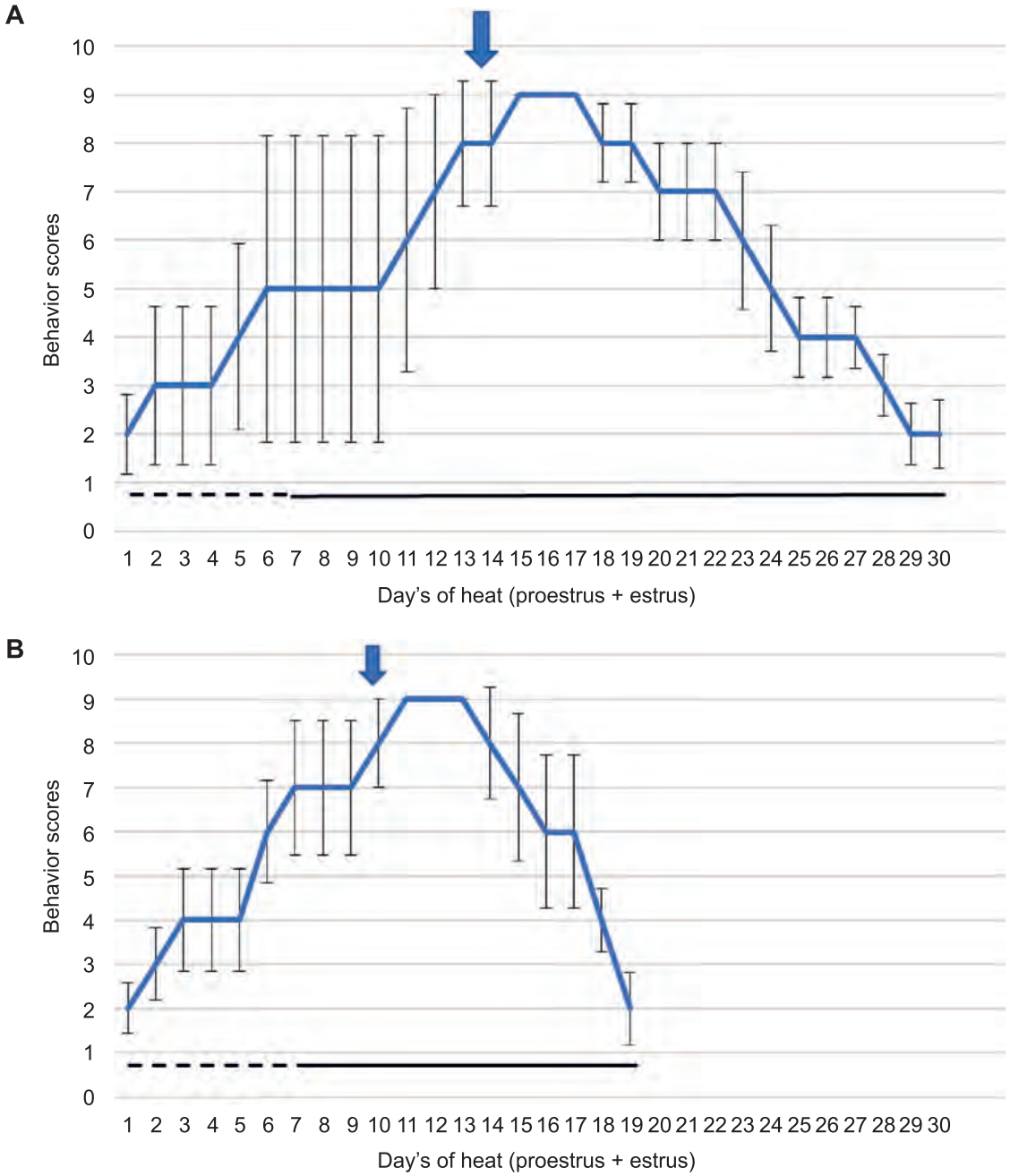

Fig. 1. Mean (±SD) sexual behavior scores of experimental (A, n=7) and control (B, n=7) bitches during proestrus and estrus. The arrow indicates the average time of ovulation. Data shown in A are from aglepristone treated group, whereas data shown in B are from placebo treated group. The dotted line shown duration of proestrus, the solid line shown duration of estrus.

immunoassay with antibody AS-Gi-P4-IV received as gift from Prof. Dr. B. Hoffmann from J Liebig-University in Giessen, Germany. The inter-assay coefficient and the intra-assay coefficient were 5.2% and 10.4%, respectively. Sensitivity of test was 350 pg/ml.

Evaluation of estrus cycle phase and ovulation time

All clinical and laboratory procedures were performed as routine methods used in our lab. Detailed descriptions of these procedures such as clinical examination, vaginoscopy, cytologic examination and related evaluation criteria are based on those by England (2013) and were described in our previous studies (Socha et al. 2012, Jurczak et al. 2016).

Cytologic proestrus was defined as a period when in the vaginal smears a mixture of parabasal, intermediate and superficial cells appeared and the number of large intermediate cells increased. There was an constant increase in the proportion of cells which appeared large and irregular in outline and became keratinized. Eryth-

Table 2. Reproductive performance during proestrus and estrus in aglepristone treated (n=7) and control (n=7) bitches. The data in columns represent mean value with standard deviation. Different letters a,b indicate significant differences between groups ($p<0.05$).

Ovulation rate (%)	Experimental group (n=7)	Control group (n=7)
	100%	100%
Interval from the first injection to estimated ovulation (days,mean ±SD)	6.86 ± 1.86	7.57 ± 1.99
Interval from the beginning of proestrus to estimated ovulation (days, mean ±SD)	13.86 ± 3.53	11.71 ± 3.30
Duration of cytologic proestrus (days, mean ±SD)	6.86 ± 1.35	7.43 ± 2.57
Duration of cytologic estrus (days, mean ±SD)	23.86 ± 3.02 a	11.14 ± 2.41 b
Duration of bloody vagindischarge (days, mean ±SD)	9.00 ± 2.08	8.43 ± 2.50
Duration of vulval turgid oedema (days, mean ±SD)	12.57 ± 6.29	10.43 ± 2.94

Secretion of progesterone during the entire experiment was similar (data not shown) and there were no significant differences between experimental and control groups ($p>0.05$).

rocytes, polymorphonuclear neutrophils and bacteria were present in small numbers.

Cytologic estrus was defined as a period when in the vaginal smears the superficial anucleated squamous cells dominated clearly (>80%) over the other epithelial cells, together with a decreasing number of erythrocytes and usually absent neutrophils.

Vulval oedema was defined as a state when the vulval lips were strongest enlarged and hard, turgid because of swelling.

The estimated time of ovulation was defined as a period when the progesterone level exceeded at least 5.0 ng/ml. Moreover, standing posture combined with pale and dry mucosa, shrinking of the vaginal fold and the highest eosinophilic index (>90% of the superficial cells) were used for estimation of ovulation.

Statistical analysis

All of the data were calculated as mean ± SD. The values of reproductive performance measures, duration of sexual behavior and progesterone concentration between groups were the statistical significance of differences in the duration of behavioral score display and the progesterone concentration between the groups were analyzed with t-Student test using Sigma Plot 6.0 ® Systat Software Inc.(SSI), San Jose, California, USA. The level of significance was set at <0.05.

Results

The results of the mean sexual behavioral scores for the experimental and control groups are presented in Fig. 1. In both groups of bitches, a similar pattern and intensity of sexual behavior were observed. The only difference between treated and non-treated bitches was a significantly prolonged duration of expression of estrus signs in treated females (28.71 ± 2.06 vs 17.00 ± 2.45, $p<0.05$). In both groups the maximal sexual behavior occurred around the ovulation time.

The reproductive performance for the experimental and control groups is presented in Table 2. The evaluated major parameters of estrus cycle were similar, with the exception of the average duration of cytologic estrus stage (23.86 ± 3.02 vs 11.14 ± 2.41, $p<0.05$). Ovulation rate (100%), interval from time at which the treatment started to the ovulation time (6.86 ± 1.86 vs 7.57 ± 1.99) and interval from the beginning of proestrus to ovulation (13.86 ± 3.53 vs 11.71 ± 3.30) did not differ between groups ($p>0.05$). Moreover, the average duration of bleeding (9.00 ± 2.08 vs 8.43 ± 2.50) and vulva edema (12.57 ± 6.29 vs 10.43 ± 2.94) were similar in both groups ($p>0.05$) (Table 2).

Discussion

Treatment of bitches with the antiprogestin aglepristone prior to ovulation changed the sexual behavior

in part. Although the general pattern and intensity of sexual behavior in treated animals were similar to normal sexual behavior, its duration was significantly extended. Behavior scores in both groups peaked around the estimated ovulation time; however, in aglepristone treated bitches there was a trend towards slightly delayed ovulation.

To our knowledge this is the first study investigating the role of preovulatory progesterone for canine sexual behavior using an experimental model with temporary elimination of endogenous progesterone action. In accordance with the well-documented effects of aglepristone (Galac et al. 2004, Gogny and Fiéni 2016), we significantly reduced the biological effects of progesterone at least 7 days before ovulation. Although in our study peripheral progesterone concentration in treated bitches was not lowered, the aglepristone has an effect on the tissue level, which is independent of the circulating progesterone level (Hoffmann and Schuler 2010). The dose of aglepristone (10 mg/kg b.w.) applied in our study has been well documented as an effective treatment in numerous previous papers dealing with physiological and pathological conditions related to a high progesterone concentration (Gogny and Fiéni 2016).

In the literature, there are only a few older studies on the relationship between steroid hormones secreted around ovulation and sexual behavior in the bitch. Those studies were performed using ovariohysterectomy followed by injectable supplementation of estrogens and progesterone, mimicking the endocrine status around ovulation (Concannon et al. 1979a,b). These studies suggested preovulatory progesterone involvement in the control of sexual behavior, because progesterone injected during the estrogen withdrawal caused more intense and synchronous estrous signs. It was hypothesized that the onset of natural mating behavior is due to the synergic action of estrogens and progesterone, wherein the estrogens secreted during proestrus prime the system, while the progesterone secreted just before and during estrus activates it. Subsequently, termination of estrous reflex is thought to be caused by a marked decline in estrogens, with inhibition due to high concentrations of progesterone or to a combination of both these factors (Concannon et al. 1977, Wildt et al. 1979, Beach et al. 1982, Hoffmann et al. 1996).

Our behavioral findings are in line with the above mentioned hypothesis, and suggest an important role of preovulatory progesterone for termination of sexual behavior. Although in treated animals we could not observe an alteration of the general pattern and magnitude of sexual behavior, its significantly longer duration was clearly documented. However, our initial hypothesis based on the earlier studies by Conncanon et al. (1979b), predicting that preovulatory progesterone action might be related to more intense displays of sexual behavior, was not confirmed. In previous studies on rodents, rabbits and guinea pigs, inhibition of sexual behavior in females was observed after progesterone receptor antagonist treatment (Brown and Blaustein 1984, Beyer et al. 1995, Hoffman et al. 2009). However, in those studies other treatment protocols and experimental models based on ovariohysterectomy and hormonal supplementation were applied. The interpretation of the data on relationship between progesterone and magnitude of sexual behavior is difficult, however, it should be noted that species-specific regulatory differences are possible. The exact contribution of preovulatory progesterone to the intensity of estrus expression in the bitch remains still to be elucidated.

It has been documented in rodents that progesterone and it's metabolites modulates neuroendocrine functions in the central nervous system, resulting in alterations in physiology and behavior (Fryea et al. 1998, Fryea 2001, Mani and Oyola 2012). In female mammals, progesterone and its receptor modulate many components of sexual behavior but the progesterone may have both stimulatory and inhibitory effects. These effects depend on the cycle phase and related estrogen-priming (Debold et al, 1976, White et al. 2007, Hoffman et al. 2009). These latter authors suggested that progesterone impacts on sexual behavior via progesterone receptor dependent and independent mechanisms. It should be stressed that in our experimental model we were not able to inhibit progesterone action via its non-genomic mechanism. It has been suggested that cross-talk between the classical receptor and non-genomic signaling pathways also promotes or inhibits progesterone-depended behavior in mammals (Fryea 2001, Mani et al. 2006, Hoffman et al. 2009).

Another interesting aspect of our study was a comparison of sexual behavior and estrus length determined as cytologic and clinical event. Interestingly, in treated bitches a longer duration of sexual behavior was accompanied by a prolonged period of cytologic estrus. Simultaneously, these cytologic outcomes, according to the opinion of Hoffmann and Gerres (1989), demonstrate the antigestagenic activity of aglepristone. These authors developed bioassay for testing the antigestagenic effect of RU 38486 in the bitch, based on the reaction of the vaginal epitelium on both progesterone and estrogens. To our knowledge this is the only direct method to confirm effectiveness of aglepristone treatment under in vivo conditions.

Our above mentioned findings on the general biological effects of preovulatory progesterone withdrawal are in line with the results of Reynand et al. (2015), who

used a similar experimental model based on preovulatory aglepristone treatment for studies on oocytes maturation. These authors did not observe a profound effect of preovulatory progesterone elimination on reproductive physiology, because only altered secretory activity of the oviduct and uterus and delayed oocyte maturation were confirmed. Similar to our outcomes, those authors observed a nearly physiological course of late proestrus-early estrus, including normal ovulation rate occurring in the antigestagens treated bitches. Our finding that the reduction of preovulatory progesterone action had limited effect on course of estrus cycle and sexual behavior score, with the exception of its duration, shows that progesterone receptor blockage one week before ovulation induces only an incomplete subset of events associated with periovulatory phase. Similarly, only a change of limited spectrum of processes related to normal parturition has been confirmed in the study based on the use of aglepristone to characterize the role of progesterone withdrawal for parturition in the cow (Shenavai et al. 2010).

The search for a profound understanding of the hormonal mechanism of reproductive behavior in the bitch is hampered by the lack of comparable studies. In dogs, information about the impact of gonadal hormones on sexual behavior has been largely gained from the older studies based on behavioral observation in spayed or castrated females or males (Hart and Eckstein 1997) or from the studies with ovariectomy and steroid hormone supplementation (Concannon et al. 1979a,b). However, this type of study has many drawbacks and very often gives unclear results. Thus, our data extend previous knowledge regarding a relationship between preovulatory progesterone and sexual behavior displays, showing its limited effect on not only the typical pattern and magnitude of canine reproductive reflexes, but rather it's involvement in termination of sexual behavior. However, it is difficult to compare the data obtained in previous studies and our outcomes due to the different methodological approaches applied in specific studies. These differences include, primarily, dissimilar methods of progesterone elimination or supplementation, as well as using ovariohysterectomized versus intact bitches. It seems that our experimental model was less invasive because a temporary elimination of preovulatory progesterone action was the only difference between experimental and physiological conditions. The physiological duration of proestrus, quite normal sexual behavior pattern and ovulation rate in treated bitches support this suggestion.

In conclusion, our data highlight the role of preovulatory progesterone in the termination of sexual behavior and cytologic remodeling of mucosa during estrus rather than for the control of basic reproductive events such as a general pattern and intensity of sexual behavior, as well as ovulation rate.

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