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Original article

The influence of antibiotic treatment of bitches in oestrus on their attractiveness to males during mating

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Abstract

The aim of this study was to evaluate the influence of the antibiotic treatment, including the mode of drugs administration, on bitches' attractiveness to the stud dogs during mating. Moreover, we tried to estimate the possibility of aversive effect of the drug vehicle on the male behavior. In experiment I, four bitches in oestrus without antibiotic treatment (group A), four bitches treated with intravaginal antibiotic (group B) and four bitches treated with intramuscular antibiotic (group C) were presented to four stud dogs. In experiment II, bitches in oestrus (n=5) were presented to the males (n=2) before and after the application to the females' vulva the antibiotic carrier – Miglyol 840 (Sasol, Germany). In both experiments the presence of the typical sexual behavior of the males (sniffing, licking the vulva and anal region, mating attempts) was evaluated. In experiment III the reaction of the males to the samples containing oestrual discharge from the bitches untreated and treated with antibiotics was evaluated. In the last part of study the aversion reaction to the samples containing antibiotic and the antibiotic carrier was evaluated. The results of experiments showed that females treated with the antibiotics were less attractive to males than untreated females, regardless of the method of administration. We did not observe adverse effect of the antibiotic carrier but samples from the bitches treated with antibiotics were significantly less attractive to the males. We concluded that the reason for reduced attractiveness of the bitches in oestrus after antibiotic treatment was the changes in semiochemical signal emitted by treated females as a consequence of elimination of the vaginal bacterial flora, which seems to be involved in creation of the typical, recognizable by the stud dogs, oestrual signal but also by the possible covering effect of used drugs.

Key words: bitch, pheromones, oestrus, reproductive behavior

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Introduction

Odour signals play an important role in initiating and modifying many types of behaviour, including sexual behaviour (Stevens et al 1982, Raymer et al 1986, Lindsay 2000, Vázquez and Orihuela 2001, Levy et al. 2004, Tod et al. 2005, Dzięcioł et al. 2012b). In animal reproduction female attractiveness to a male is essential for a proper conduct of the subsequent sexual act and this is determined mostly by the presence of specific semiochemical substances emitted into the environment (Kustritz 2005, Martens 2006).

The role of bacterial flora in the development of semiochemical substances has been confirmed by many authors in both animals and humans (Dravnieks et al. 1970, Michael et al. 1974, Michael et al. 1975, Albone et al. 1978, Bird and Gower 1982, Jackman and Noble 1983, Gower et al. 1994, Kohl et al. 2001).

Although olfactory signals play a significant role in a formation of specific animals' behaviour, under some circumstances that way of communications could be disturbed (Goodwin et al. 1979, Gower et al. 1986, Merkx et al. 1988, Nishimura et al. 1991, Pageat and Gaultier 2003, Kustritz 2005, Ungerfeld and Silva 2005, Martens 2006, Ungerfeld et al. 2006). The aim of this study was the estimation of the influence of the antibiotic treatment of bitches in oestrus on their attractiveness towards experienced stud dogs during mating procedures. We also investigated the influence of the route of antibiotic administration (intramuscular or intravaginal) and whether the expected reduction in attractiveness might be explained by vaginal flora elimination and/or by a possible aversive reaction to the drug and its vehicle possibly present in vaginal discharge.

Materials and Methods

Location and animals

The study was carried out over a period of 48 months in three German Shepherd Dog Breeding Kennels located in Poland and Germany and in the Clinic of Reproduction of the Wroclaw University of Environmental and Life Sciences. In the experiment a total number of 21 dogs (stud dogs and bitches – patients of the Clinic and dogs belonging to the Local Experimental Kennel) were used. (Table 1). All males used in the experiment were highly experienced in mating and in oestrus detection. We focused on the experienced dogs since they showed moderate excitement during mating: they are more focused and usually did not show additional behaviour (playing, jumping etc.) (Kustritz 2005, Dzięcioł et al. 2012a). The males from the Local Experimental Kennel were routinely used for oestrus detection. The stud dogs (German Shepherds) were top ranking males which are used for mating tens times per year (in Germany according to SV Rules the stud dogs of German Shepherd Breed are allowed up to a maximum of 90 mating per calendar year.

Experiment I

During this part of the study, we assessed the influence of antibiotics and route of administration (intramuscular or intravaginal) during oestrus, on the females' attractiveness during mating.

Animals and antibiotic treatment

Four males were used. Eight bitches were allocated to three groups: A (n=4), no antibiotic treatment; B (n=4), intravaginal antibiotics; C (n=4) intramuscular antibiotics. Same bitches were allocated to various groups in the different cycles (i.e. one year the female was in group A and in the following year she was included into the group B or C (Table 1). The same bitches were also presented to various males in the different cycles.

The bitches used in the experiment were classified for the treatment according the individual medical indications (reduced fertility in previous cycles, but without any pathological discharge from vagina). Bitches were treated with a broad spectrum antibiotic Synulox® (Amoxicillin with clavulanic acid, Pfizer) at a dose 12.5 mg/kg b.w. at 24 h intervals for 7 days. The drug was administrated intravaginally or intramuscularly in group B and C respectively. In all cases the sensitivity of the vaginal flora was previously confirmed by laboratory tests and the treatment was completed at least 2 days before mating.

Test of the bitches' sexual attractiveness

The observations were done during the commercial mating or during the arranged meetings. The bitches from all groups were tested according to the same procedures described below. When a meeting was arranged solely for this experiment (i.e. not for commercial mating) introducing of the penis was disallowed. The reaction of the male to the female were analyzed, based on the modified method proposed by Kruse and Howard (1983).

Bitches used in this study were exposed to a male when progesterone concentration reached

Table 1. The females and males used in experiment.

No.	Groups *	Age	Gender	Breed	Used in Experiment
1		3	Female	Beagle	II, III
2		3	Female	Beagle	II, III
3		3	Female	Beagle	II
4		3	Female	Beagle	II
5	A,B	3,5	Female	German shepherd	I
6	A	2,5	Female	German shepherd	I
7	B	4	Female	German shepherd	I, II, III
8	AC	4,5	Female	German shepherd	I
9	B	4	Female	German shepherd	I
10	A, C	4,5	Female	German shepherd	I
11	C	3,5	Female	German shepherd	I
12	B, C	6	Female	German shepherd	I
13		2	Female	Yorkshire terrier	III
14		7	Female	Golden retriever	III
15		7,5	Female	Bavarian mountaindog	III
16		4	Male	German shepherd	I
17		3	Male	German shepherd	I
18		3,5	Male	German shepherd	I
19		3	Male	German shepherd	I
20		3	Male	Beagle	II, III, IV
21		3	Male	beagle	II, III, IV

* in experiment I

10-15 ng/ml, which has been reported as the optimum time for mating (Manothaiudom et al 1995). The females were transported to the habitual residence of the stud dog. Shortly before exposition the bitch was let free in a restricted area where she could urinate, and the male could observe her behaviour. Then, the bitch was situated in the place which was known to the male as a place for mating (copulative point). The male was introduced to the female from behind. To avoid the adverse reaction of the female, in all cases the bitches were gently held by their owners during testing. During the contact a complete behavioural reaction of the male to the female was recorded. The presence or absence of the typical sexual behaviour (sniffing the vaginal and anal region, temporal immobilization (more than 5 seconds), licking the vulva, pawing and mating attempts) were analysed (Kruse and Howard 1983, Kustritz 2005).The observation was continued by 5 minutes (Kruse and Howard 1983).

Experiment II

In this part of study, the influence of the antibiotic carrier (Miglyol 840, Sasol Germany GmbH) on female attractiveness was evaluated. The bitches in oestrus were presented to the males two times, before and after marking the vulva with the examined substance. The antibiotic carrier was applied to the vulva of the females in oestrus with the use of swab soaked in Miglyol 840 (Kruse and Howard 1983). The intervals between both trials was approximately 20 min. The behaviour of the male dogs during the contact with the females was evaluated during both presentations. The presence or absence of the aforementioned typical sexual behaviour as well as the duration of interest of the male to the female was analysed. Five females and two males were used (Table 1). During particular trials the same bitches were presented to both males in about 10 min intervals.

Experiment III

During this part of study we evaluated the response of the males to swabs soaked in different substances (Kruse and Howard 1983). From the distance of about two centimeters a sterile swabs (n=4), swabs collected from bitches in oestrus (progesterone level = 10-15 ng/ml, n=4) and swabs collected from the bitches in oestrus treated with antibiotic (n=4) were presented to two Beagle males (Hirano et al. 2000). Each male was tested four times with the different kind of sample. The presentation took place within the kennel area and was always done during the same part of a day (morning) by the same person who always remained in a standing position. We assessed the male behavior during the swab presentation according to the presence of following behavior: sniffing, licking the swabs, temporary immobilization, following the swabs and pawing, jumping on the person presenting the swab.

Experiment IV

In this experiment we tried to analyse the possibility of the aversive reaction to the particular substances. We focused on the observation of the males' response to the sterile swabs, swabs soaked in Miglyol 840, swabs soaked in Synulox® and samples collected from the bitches in oestrus. We analysed the average time of sniffing the samples. Each male was tested four times with the different kind of samples.

Determining the optimal time for mating

The advance of the oestrus cycle of the all females was evaluated according to the measurement of the progesterone level in the blood serum (Manothaiudom et al 1995, Niżański et al 2003, Kustritz 2006).The tests were performed with the level of progesterone at 10-15 ng/ml.

Blood sampling, progesterone assay and timing of mating

Eight ml of blood were taken by venipuncture from the cephalic vein into heparinised tubes. Plasma was separated 60 min after blood collection by centrifugation for 15 min at 2000 g. Progesterone concentration was determined on the same day with the use of a commercial radioimmunoassay (RIA) kit (progesterone Coat-a-Count kit, Diagnostic Products Corporation, Los Angeles, CA, USA) validated for dog plasma (Srikandakumar 1986). Blood samples were taken from each bitch at 48 h intervals starting from the 5-6 day of the oestrus cycle. Bitches were mated when plasma progesterone concentration reached values between 10-15 ng/ml.

Bacterial analysis of samples collected from the vagina

Samples from the anterior vagina, were taken by a direct contact, avoiding rubbing; from each bitch, starting on the first day of oestrus, followed by a control check after treatment.

The study was approved by the Local Ethical Committee for the Experiments on Animals of Wrocław University of Environmental and Life Sciences.

Statistical analysis

For the statistical analysis of the obtained results the ANOVA test (experiment I, II and III) and t-Student test were used (exp. IV), with the $p \ll 0.05$ (Statistica).

Results

The males used in experiment I found as attractive, and decided to mate, almost all bitches from group A. During contact with the bitches treated with antibiotics (group B and C), the dogs sniffed the females and tried to recognize their status, but did not decide to mate them (Table 2). The results of experiment I showed that the bitches treated with antibiotic were less attractive to the males than untreated bitches. That applied to the females from both groups B and C. The way of antibiotic administration has been found as not important as there were no significant differences observed between the level of attractiveness of the females from both mentioned groups.

In experiment II there were only a few cases of reduced interest in the male dogs when they were tested with samples taken from the bitches treated with the antibiotics' carrier (Table 3). Analysis of the males' behavior did not shown significant differences between both presentation trials. Some kind of "confusion" expressed by more intensive sniffing behavior towards the females marked with Miglyol 840 and also by attempts of sniffing another regions of the females' body (the flanks) were observed in a few cases.

In experiment III, reduced interest in the samples obtained from the bitches treated with the antibiotic during the heat was noted (Table 4).

Table 2. The behaviours of the male dogs during the presentation of the bitches from group A, B and C – (Experiment I).

	Sniffing the vulva	Licking the vulva	Temporary Immobilisation	Pawing	Mating
	A* B C	A B C	A B C	A B C	A B C
1**	(4/4)(4/4)(4/4)***	(4/4)(3/4)(3/4)	(4/4)(2/4)(2/4)	(4/4)(0/4)(0/4)	(4/4)(0/4)(0/4)
2	(4/4)(4/4)(4/4)	(4/4)(2/4)(3/4)	(4/4)(2/4)(3/4)	(3/4)(1/4)(1/4)	(4/4)(0/4)(1/4)
3	(4/4)(4/4)(4/4)	(4/4)(3/4)(3/4)	(4/4)(3/4)(2/4)	(4/4)(0/4)(0/4)	(4/4)(0/4)(1/4)
4	(4/4)(4/4)(4/4)	(4/4)(3/4)(3/4)	(4/4)(3/4)(3/4)	(3/4)(1/4)(0/4)	(3/4)(1/4)(0/4)

* A,B, C – females from different groups; ** 1,2,3,4 – different males; *** exp. (3/4) three times observed behaviour during the contact with four bitches

Table 3. The behaviours observed in the male dogs during the presentation of the females in oestrus and the same females after antibiotic carrier (Miglyol 840) application to the vulva. (experiment II).

Male behaviour	Sniffing	Licking the swab	Immobilization	Mating	Average time (sec.) [SD] from sniffing to matting attempt or to leaving
Females/males	I* II**	I II	I II	I II	I II
1***	(5/5) (5/5)****	(5/5) (5/5)	(5/5) (4/5)	(5/5) (4/5)	(15 [5]) (13 [4])
2	(5/5) (5/5)	(5/5) (5/5)	(5/5) (4/5)	(5/5) (3/5)	(14[5]) (12 [4])

* I – females in oestrus; ** II – the same females in oestrus after Miglyol 840 application; *** (1, 2) male dogs; **** (4/4) number of behaviour observed/number of examined females

Table 4. The behaviours observed in the male dogs during the presentation of the three types of swabs: sterile swabs, swabs from the bitches in oestrus and swabs from the bitches in oestrus treated with the antibiotic (experiment III).

		Sterile	swab		
Males' behaviour	Sniffing the swabs	Licking the swab	Temporary immobilisation	Following the swabs	Pawing, jumping
Samples/males	I II III IV *	I II III IV	I II III IV	I II III IV	I II III IV
1**	P P P P ***	N N N N ****	N N N N	N N N N	N N N N
2	P P P P	N P N N	N N N N	N N N N	N N N N

	Swabs	from the	bitches in	oestrus	
Males' behaviour	Sniffing the swabs	Licking the swab	Temporary immobilisation	Following the swabs	Pawing, jumping
Samples/males	a b c d *****	a b c d	a b c d	a b c d	a b c d
1	P P P P	P P P P	P P P P	P P P P	N P P P
2	P P P P	P P P P	P P P P	P P P P	P P P P

Swabs	from the	bitches in	oestrus treated	with the	antibiotic
Males' behaviour	Sniffing the swabs	Licking the swab	Temporary immobilisation	Following the swabs	Pawing, jumping
Samples/males	a b c d	a b c d	a b c d	a b c d	a b c d
1	P P P P	P P N P	N P N N	N N N N	N N N N
2	P P P P	N P N N	N N P N	N N N N	N N N N

* (I, II, III, IV) different samples of the sterile swabs; ** (1, 2) male dogs; *** (P) Positive – behaviour observed; **** (N) Negative – the lack of the behaviour; ***** (a, b, c, d) samples presented to the males from four different bitches

In experiment IV we did not observe any significant differences in time of sniffing of the sterile swabs and swabs containing the antibiotic (Synulox®, Pfizer), while the swabs containing antibiotic carrier alone were more attractive and the average time of sniffing was similar to the time spent on sniffing samples contained oestrual attractant (no significant differences between these both trials was found).

Performed control bacteriological examination were negative in all treated animals.

During the control clinical examination no side effects in the bitches which received the Miglyol 840 has been observed (Exp. II).

Discussion

The vagina of a bitch is not sterile throughout the all reproductive cycle, but during the proestrus and oestrus the number of bacteria in this region significantly increases. According to many reports, the type of microflora present at this time in all bitches is very similar (Allen et al. 1982, Janowski et al. 2006). A substantial reduction of the amount of bacteria in the vagina is observed at the time of transition from estrus to dioestrus (increased number of neutrophils), and this is correlated with the abrupt reduction of the attractiveness of the bitches to the dogs. (Allen et al. 1982, Baba et al. 1983, Watts et al. 1996, Grundy et al. 2002, Janowski et al. 2006, Zduńczyk et al. 2006, Ulgen et al. 2010).

The significant reduction of the attractiveness of the females with reduced vaginal flora (females from the both group B and C in the experiment I) suggests the importance of the presence of the bacterial flora for the creation of the semiochemical signals emitted to the environment by the bitches during the oestrus. This conclusion is consistent with the observations made by Merkx et al. (1988) and Ungerfeld and Silva (2005) who also observed reduced females' attractiveness after antibiotic treatment.

The Miglyol 840, a carrier of the antibiotic used in this experiment, is a non-toxic substance. It contained (in different concentrations) the fatty acids: capronic acid, caprylic acid, capric acid and lauric acid. The results of experiment II showed the specific influence of the presence of this substance on the males' sexual behaviour. Although the time spent on checking the females did not differ significantly, some kind of confusion expressed by intense investigation of the other parts of the bitches' body (flanks) were observed. That could suggest that dogs recognise the difference between the scent of the bitches from both trials but it did not caused an aversive reaction. This suggestion seems to be confirmed by the observation obtained in experiment IV, where increased interest in swabs soaked in Miglyol 840 (the time spend on sniffing) was noted.

These results suggest that, as well as the effect of elimination of vaginal bacterial flora which may affect the production of specific semiochemical signals, treatment with antibiotics may also disrupt the natural mating behaviour of dogs by the presence of additional substances, the antibiotic carrier. These observation seems to be interesting in the light of findings by Novotny et al. (1985) who reported that the reaction of the recipient to the same substances (pheromones) could be different when the substance is presented in different vehiculum. In our opinion also the duration of the treatment could influence the clear results of the experiment I. In clinical practice antibiotics are very often administrated for a shorter period of time or just ones before mating (in that case sometimes it used to be done by the breeders).

The role of learning in the process of sensitization to the pheromonal signals has been previously described by many authors (Gelez and Febre-Nys, 2004, 2006, Chanvallon and Febre-Nys 2009). The fact that the most experienced stud dogs expressed reduced interest in bitches receiving the antibiotic treatment could be explained by the presence of an unexpected signal emitted by these treated females. The lack of typical semiochemical signal resulting from the elimination of the vaginal microflora (suspected for the help in production of the pheromones) as well as the additional chemical signal (presence of antibiotic or its metabolites) even without aversive reaction could influence the stud dog behavior. The meaning of the accuracy of the pheromone signal for the male dog behavior could be also taken into account considering the observation of Kustritz (2005) who mentioned that some dogs "are able to determine not just if the bitch is in estrus, but if she is at the optimal time for breeding". In that case some experienced stud dogs seem to be able to distinguish between particular phases of the reproductive cycle (proestrus, oestrus), probably recognizing specific pheromonal signal.

Comparing the average time of sniffing sterile swabs and swabs with Synulox® (Experiment IV) in both cases the noticed time was extremely short (Table 5). That makes interpretation very difficult, because it cannot be stated if the males stop sniffing the samples contained the Synulox® scent because of the unpleased smell or just because it was not interesting for them (samples did not contain any attractive scent that dogs used to look for when they were working as a heat detectors at the Kennel). However, the significantly longer time spent on sniffing samples contained antibiotics' carrier could suggest that the scent of Synulox® was unpleasant.

Tabl 5. The average time in seconds (standard deviations) of dogs' times of sniffing the four type of swabs: sterile swabs, swabs soaked in Miglyol 840, soaked in Synulox® and from bitches in oestrus.

Samples/males	Sterile	Soaked in Miglyol 840	Soaked in Synulox®	Soaked in estrus discharge
1	3,1(0,27)	7,2 (0,32)	2,8(0,12)	8,2 (0,31)
2	2,9 (0,34)	6,8 (0,30)	2,6(0,18)	7,3 (0,3)

We were not able to clearly explain the reason for the observed reduced attractiveness of the females in oestrus treated with antibiotic. In our opinion complex analysis using gas chromatography and mass spectrometry techniques of the changes of the chemical signal emitted by the female in oestrus before and after treatment could give additional important information which would be helpful in solving this problem.

In summary, we concluded that antibiotic treatment during the oestrus reduced the attractiveness of bitches to males. The reason for that phenomenon could be the change in semiochemical signals emitted by the treated females which could result from bacterial flora elimination (observed in all cases) but also by the presence of the additional chemical signals.

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