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Original article

The efficiency of synthetic sex pheromones in sexual arousal stimulation in domestic dogs

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Abstract

The aim of this study was to evaluate the suitability of synthetic bitch sex pheromones (Eau' De Estrus®, Synbiotics USA) for the stimulation of the reproductive reflexes in adult male dogs. In experiment I: anoestral bitches were applied synthetic (n=6) or natural (n=6) pheromones and their attractiveness was compared to the attractiveness of bitches in natural oestrus. In experiment II: swabs soaked in natural estrual discharge or Eau D'Estrus were presented to male dogs and behavioral arousal (Exp. II a) and changes in blood flow in the penile artery reflecting erection (Exp. II b) were recorded. In experiment III the time dedicated for sniffing in experimental dogs was analyzed in relation to the type of attractant presented (natural estrual discharge or Eau D'Estrus). In all three experiments we observed the signs of sexual arousal, increased blood flow in the penile artery and increased time of sniffing only with natural pheromones. No signs of excitation or changes in penis perfusion were noted after the use of artificial pheromones. Our results did not support the suitability of Eau D'Estrus for male dog sexual stimulation.

Key words: bitch, oestrus, sex pheromones, sexual behavior, color Doppler

Introduction

Communication through chemical signals occurs in all taxas, from prokaryotes to eukaryotes and is the oldest and most widespread means of communication. In animals pheromones play a crucial role in formation of specific sexual behavior. Female attractiveness to males is created mainly by semiochemical substances emitted to the environment and subsequently detected by the male recipient. Pheromones not only behaviorally attract males, but also trigger a sexual response, which alters various physiological reactions, leading to sexual arousal. One of the noticeable changes during arousal is the intensity of the blood flow in the penile artery. Measurement of the magnitude of the arterial flow is a useful tool for sexual arousal estimation and has been previously described both in humans and animals (Sachs 1997, Bertolotto 2008, Dzięcioł et al. 2012b). The specificity of this reaction was indicated by Sachs et al. (1994) who declared that nonsexual arousing stimuli do not readily evoke erections.

Although bitch synthetic sex pheromones have been commercially available on the market for some time and used to be recommended for sexual stimulation in dogs, independent, reliable and unambiguous information about its usefulness has not been verified (Kustritz 2005, Kutzler 2005). Hence, the aim of our study was to evaluate the suitability of synthetic bitch sex pheromones (Eau' De Estrus®, Synbiotics USA) for the stimulation of the reproductive reflexes in adult male dogs. The study was conducted in the Clinic of Reproduction of the local Faculty of Veterinary Medicine and was approved by the Local Ethical Committee for Experiments on Animals.

Materials and Methods

Location and animals

For the experiment dogs from the Local Experimental Kennel (dogs and bitches) belonging to the University as well as patients of the Department of Reproduction (bitches) were used. Nine adult males (3-6 years old) and ten females (2.5-4.5 years old) of different breeds were used (Table 1).

During the experiment the same females were randomly allocated to the different groups, if the phase of the reproductive cycle correlated with the particular parts of the experiment. Such group formation minimized the risk of interindividual aversion behavior between particular animals (males and females) (Martens 2006) (Table 1).

The source of natural and artificial pheromones

Synthetic pheromones (Eau' De Estrus®) were commercially purchased from the manufacturer (Synbiotics Corporation, Kansas City, Missouri, USA). According to the manufacturer's description "odor attractant/synthetic pheromone" should be helpful during the semen collection procedure.

Samples of natural odors were obtained by swabbing the vulvar area of estrual bitches with a cotton swab. Samples collected from the bitches were taken by direct contact of the swab with the vaginal environment (Kustritz 2006). The phase of the cycle was determined according to the protocol described below. Samples of oestral odors were taken from bitches only at their optimal time for mating.

Application of synthetic and natural pheromones:

According to the manufacturer's instructions, a few drops of the solution containing artificial pheromones were applied to the vulva of the bitches from group B. In group C natural oestral pheromones were applied to the vulva with the use of swabs soaked in the bitches' oestral discharge. In the case where the same bitches were part of the groups B and C, the interval between the administration of the two types of pheromones was approximately 30 (± 2) days. At least four bitches from each group were presented to each male. The reaction of the male dogs during contact with the females from particular groups was analyzed according to the protocol based on behavioral analysis, described below.

Swabs containing oestral discharge and swabs soaked in Eau D'Estrus (this method of presentation is also suggested by the manufacturer in their instructions) were presented to the stud dogs. The reaction of the males to the samples presented was analyzed by behavioral analysis.

Experiment I

In this part of the study we compared the reaction of adult male dogs ($n=6$) to the bitches in oestrus versus the bitches in anoestrus to which the artificial (Eau' De Estrus®, Synbiotics USA) or natural pheromones were applied. The females were divided as follows: Group A – six bitches in oestrus, Group B – six bitches in anoestrus which received Eau D'Estrus and group C – six bitches in anoestrus which received natural pheromones.

Experiment II

For this part of the experiment we used two adult males (both 3 years old, 11 kg, Beagles) and four

Table 1. Dog females and males used in the experiment.

| No. | Groups * | Age | Gender | Breed | Used in Experiment |
|-----|----------|-----|--------|--------------------|--------------------|
| 1 | A, B, C | 3 | Female | Beagle | I, II a,b, III |
| 2 | A, B, C | 3 | Female | Beagle | I, II a,b, III |
| 3 | A, B,C | 3 | Female | Beagle | I, II a,b, III |
| 4 | A, B, C | 3 | Female | Beagle | I, II a,b, III |
| 5 | A,B | 3.5 | Female | Yorkshire Terrier | I, III |
| 6 | A | 2.5 | Female | German shepherd | I, III |
| 8 | B | 4 | Female | Mix breed | I |
| 9 | C | 4.5 | Female | Dachshund | I |
| 10 | C | 3.5 | Female | Mix breed | I |
| 11 | | 3 | Male | Beagle | I, IIa,b, III |
| 12 | | 3 | Male | Beagle | I, IIa,b, III |
| 13 | | 6 | Male | Bedlington terrier | I |
| 14 | | 4 | Male | Yorkshire Terrier | I |
| 15 | | 3 | Male | Labrador | I |
| 16 | | 3.5 | Male | Mix breed | I |
| 17 | | 1.5 | Male | Beagle | III |
| 18 | | 4.5 | Male | Beagle | III |
| 19 | | 2.5 | Male | Beagle | III |

* Concerns experiment I

females (3 years old, 10±1 kg, Beagles) from the Local Experimental Kennel. Those dogs were previously trained and familiarized with the repeated presentation of sterile dry swabs and ultrasound scanning.

Experiment IIa

Samples of odors on the cotton swabs were presented to the dogs from a distance of about two cm from the nose (Hirano et al. 2000). The presentation of the artificial (Eau D'Estrus) and natural pheromones (collected from bitches in oestrus) was preceded by presentation of sterile swabs and swabs from bitches in anoestrus. The samples collected from the females were used fresh: collected and used on the same day. In all experiments the presentation of the samples to the males were done in a well-ventilated and air-conditioned room at the same environmental temperature and at the same time of day. The animals' behavior was analyzed according the protocol described below.

Experiment IIb

During this part of the study two adult males from the Local Experimental Kennel (3 years old, 11 kg, Beagles) were used and the intensity of the blood flow

in the penis artery was analyzed. The intensity of the blood flow was evaluated before and after artificial and natural pheromone presentation. The artificial pheromones were presented first examined signal and the natural pheromones were presented second with an interval between the two trials of at least half an hour.

For ultrasonographic examinations, the MyLab25 Gold ultrasound system (Esaote, Italy) equipped with a 12 MHz- linear probe was used. The investigated vessels were the ramifications of the dorsal penile artery and arcuate artery. The dorsal penile artery supplies those body organs (penis) that should be targets for sex pheromones, whereas the arcuate artery is the vessel of a control organ- the kidney (Dzięcioł et al. 2012b). To visualize the examined vessel, color coded ultrasonography was used (Gunzel-Apel et al. 2001). For the evaluation of the results Pixel Flux[®] software (Chameleon Software, Germany) dedicated for dynamic sonographic tissue perfusion measurement was used (Dzięcioł et al. 2014, Scholbach et al. 2004, Wiczorek et al. 2011). Each male was examined 6 times.

Experiment III

During the third part of the experiment we focused on the time spent by dogs on sniffing the vulva of the bitches in anoestrus, in oestrus and in anoestrus but

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

| Behavior/ Males and groups | Approaching | Sniffing the vulva | Licking the vulva | Temporary Immobilisation | Pawing | Mating |
|----------------------------------|---------------|-----------------------|----------------------|-----------------------------|------------|------------|
| 1A | 100%* (4/4)** | 100% (4/4) | 100% (4/4) | 100% (4/4) | 100% (4/4) | 100% (4/4) |
| 2A | 100% (4/4) | 100% (4/4) | 100% (4/4) | 100% (4/4) | 75% (3/4) | 75% (3/4) |
| 3A | 100% (4/4) | 100% (4/4) | 100% (4/4) | 100% (4/4) | 100% (4/4) | 100% (4/4) |
| 4A | 100% (4/4) | 100% (4/4) | 100% (4/4) | 100% (4/4) | 100% (4/4) | 75% (3/4) |
| 5A | 100% (4/4) | 100% (4/4) | 100% (4/4) | 100% (4/4) | 100% (4/4) | 100% (4/4) |
| 6A | 100% (4/4) | 100% (4/4) | 100% (4/4) | 100% (4/4) | 100% (4/4) | 100% (4/4) |
| 1B | 100%* (4/4)** | 100% (4/4) | 25% (1/4) | 25% (1/4) | 100% (4/4) | 0% (0/4) |
| 2B | 100% (4/4) | 100% (4/4) | 50% (2/4) | 50% (2/4) | 0% (0/4) | 0% (0/4) |
| 3B | 100% (4/4) | 100% (4/4) | 25% (1/4) | 25% (1/4) | 0% (0/4) | 0% (0/4) |
| 4B | 100% (4/4) | 100% (4/4) | 50% (2/4) | 0% (0/4) | 0% (0/4) | 0% (0/4) |
| 5B | 100% (4/4) | 50% (2/4) | 25% (1/4) | 0% (0/4) | 0% (0/4) | 0% (0/4) |
| 6B | 100% (4/4) | 100% (4/4) | 25% (1/4) | 25% (1/4) | 0% (0/4) | 0% (0/4) |
| 1C | 100%* (4/4)** | 100% (4/4) | 100% (4/4) | 75% (3/4) | 0% (0/4) | 0% (0/4) |
| 2C | 100% (4/4) | 100% (4/4) | 100% (4/4) | 100% (4/4) | 0% (0/4) | 0% (0/4) |
| 3C | 100% (4/4) | 100% (4/4) | 100% (4/4) | 75% (3/4) | 0% (0/4) | 0% (0/4) |
| 4C | 100% (4/4) | 100% (4/4) | 100% (4/4) | 100% (4/4) | 0% (0/4) | 0% (0/4) |
| 5C | 100% (4/4) | 100% (4/4) | 100% (4/4) | 100% (4/4) | 25% (1/4) | 25% (1/4) |
| 6C | 100% (4/4) | 100% (4/4) | 100% (4/4) | 100% (4/4) | 0% (0/4) | 0% (0/4) |

* Percent of reactions ** observed reactions/examined females

with vulva smeared with Eau D'Estrus. Sniffing time of dry swabs, swabs soaked in natural oestral discharge and swabs soaked in Eau D'Estrus were also measured. The trials were repeated 6 times for each male. Five males have been used.

Determination of the bitches' reproductive cycle phases.

Exact detection of the stage of oestrus cycle was determined by history taken from the owners, clinical examination and laboratory tests (vaginal cytology and progesterone concentration analysis). Progesterone concentration in peripheral blood was determined using the enzyme-linked fluorescence assay (ELFA) (mini VIDAS® Biomerieux, France) (Brugger et al. 2011).

Behavioural analysis

Analysis of male dogs behaviour during contact with the females from groups A, B and C (Experiment I).

The reaction of the stud dogs to the female was analyzed, with reference to the modified method proposed by Kruse and Howard (1983). During that test, the presence or absence of typical sexual behaviour

(sniffing the vaginal and anal region, temporal immobilization, licking the vulva, pawing, mating attempts) were analyzed (Kruse and Howard 1983, Kustritz 2005). To avoid any aversive reaction of the female which could influence the male behaviour, the bitches in all cases were gently held by the owners/staff during the testing procedures. The presence or lack of behaviour was described by a person who could observe the male behaviour but who was not informed about the status of the female (to which group she belonged). The observation was carried out for 2 minutes (Kruse and Howard 1983).

Analysis of male dog behaviour during contact with swabs containing natural and artificial pheromones (Experiment II).

Presentation of the swabs to the males took place within the kennel area and was always done by the same person who always remained in a standing position. According to the modified method proposed by Kruse and Howard (1983), who also used swabs as a pheromone carrier, the male behavior was assessed during swabs presentation by the presence or absence of the following behavior: sniffing, licking the swabs, temporary immobilization, following the swabs, pawing and jumping on the person presenting the swab. Each male was tested four times with the samples

Table 3. The behaviours observed in the male dogs (expressed as a percentage) during the presentation of the four types of swabs: sterile swabs, swabs from the bitches in anoestrus, from bitches in oestrus and swabs soaked with Eau D'Estrus (Experiment IIa).

Sterile swab

| Male behaviour | Sniffing | Licking the swab | Immobilization | Following the swabs | Pawing/Jumping |
|----------------|----------|------------------|----------------|---------------------|----------------|
| 1* | 100% ** | 33% | 0% | 0% | 0% |
| 2 | 100% | 0% | 0% | 0% | 0% |

Swabs from bitches in anoestrus

| Male behaviour | Sniffing | Licking the swab | Immobilization | Following the swabs | Pawing/Jumping |
|----------------|----------|------------------|----------------|---------------------|----------------|
| 1 | 75% | 75% | 75% | 25% | 0% |
| 2 | 75% | 75% | 25% | 25% | 0% |

Swabs from bitches in oestrus

| Male behaviour | Sniffing | Licking the swab | Immobilization | Following the swabs | Pawing/Jumping |
|----------------|----------|------------------|----------------|---------------------|----------------|
| 1 | 100% | 100% | 100% | 100% | 100% |
| 2 | 100% | 100% | 100% | 100% | 75% |

Swabs soaked with artificial pheromones

| Male behaviour | Sniffing | Licking the swab | Immobilization | Following the swabs | Pawing/Jumping |
|----------------|----------|------------------|----------------|---------------------|----------------|
| 1 | 100% | 25% | 25% | 0% | 0% |
| 2 | 100% | 25% | 25% | 0% | 0% |

* (1, 2) male dogs ** percentage of observed positive behaviours

collected from different females. Each trial took no longer than two minutes.

Statistical analysis

Statistical analyses were performed using Statistica 5.0 (StatSoft Inc., Tulsa, OK, USA). The level of significance was set at $p < 0.05$. For evaluation of the results of experiments I and II a, the Wilcoxon test were used. For the evaluation of the results from experiment II b the t-Student test for dependent samples was used. Normal distribution of data was confirmed using the K-S test (Kolmogorov-Smirnov test) (Wieczorek et al., 2011). For evaluation of the results from experiment III the paired t-Student test was used. Normal distribution of data was tested by Gaussian function.

Results

Experiment I

During presentation of the females from group A (females in oestrus) the males, in all cases, showed

a high level of interest, which was expressed by intensive sniffing of the vulva area, licking the vulva, pawing the female and almost always an attempt of mating (Table 2). During the males' contact with the females from group B the dogs presented a moderate interest. After approaching, they usually sniffed the vulva, but the percentage of males licking the vulvar region, presenting immobilization posture, pawing the females and mating attempts was very low, or such behaviors were not observed at all. During the contact of the males with the females from group C an increased interest and signs of sexual arousal were clearly observed, but the percentage of mating attempts was significantly lower than when the males were in contact with the females in oestrus.

Experiment IIa

Statistical analysis confirmed the presence of significant differences among the levels of interest in the males testing different samples (Table 3). Generally, the males were very interested in swabs containing the natural pheromones (licking of the swabs, immobilization, following the swabs and pawing were strongly demonstrated), while presentation of the sterile

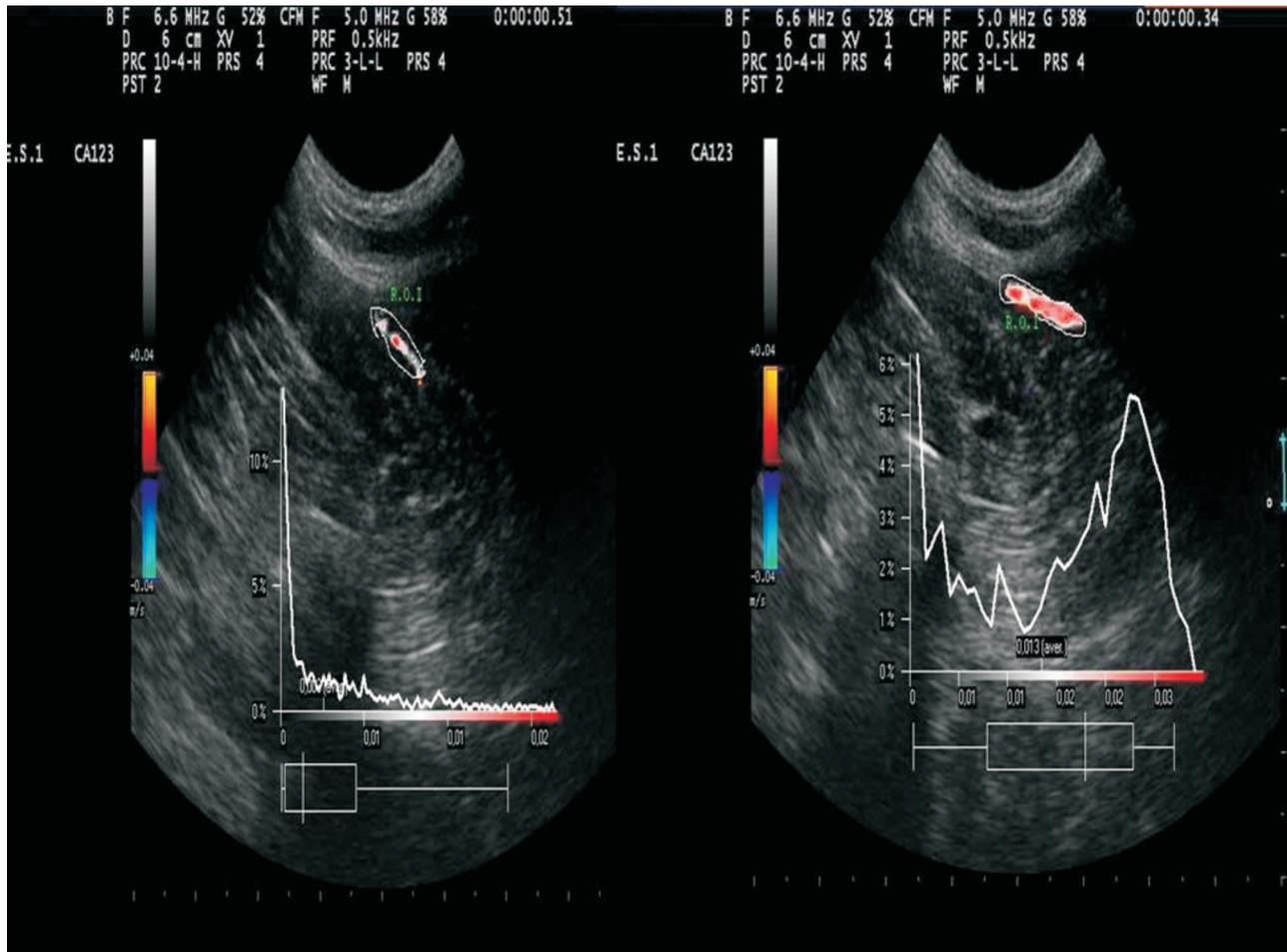


Fig. 1. Analysis of the blood flow in the penile artery during artificial (left) and natural pheromone (right) presentation. Areas with high perfusion (in the ROI – Region of Interest) are shown red, middle perfusion is indicated by white and low perfusion is shown in black.

Table 4. Comparison of the blood flow intensity (I) in the ramification of the penile artery and in arcuate artery (control) before and after synthetic and natural pheromones presentation (Experiment IIb).

| Male | Penile artery | P.a. I | P.a. II | P.a. III |
|------|----------------------------|------------------|-------------------|-----------------|
| | Parameter | Mean I mix (SD) | Mean I mix (SD) | Mean I mix (SD) |
| 1 | | 0.0013 (±0.0005) | 0.00143 (±0.0008) | 0.006 (±0.0006) |
| 2 | | 0.0017 ±(0.0015) | 0.00181 (±0.0015) | 0.008 (±0.0016) |
| | Kidney artery (A. arcuate) | A.a. I | A.a. II | A.a. III |
| | Parameter | Mean I mix (SD) | Mean I mix (SD) | Mean I mix (SD) |
| 1 | | 0.0019 (±0.0005) | 0.0018 (±0.0006) | 0.002 (±0.0005) |
| 2 | | 0.0031 (±0.0015) | 0.0022 (±0.0015) | 0.003 (±0.0010) |

P.a. I – blood flow intensity in the penile artery before pheromones presentation
 P.a. II – blood flow intensity in the penile artery after artificial pheromones presentation
 P.a. III – blood flow intensity in the penile artery after natural pheromones presentation
 A.a. I – blood flow intensity in the arcuate artery (kidney) before pheromones presentation
 A.a. II – blood flow intensity in the arcuate artery (kidney) after artificial pheromones presentation
 A.a. III – blood flow intensity in the arcuate artery (kidney) after natural pheromones presentation
 Mean I mix – mean mixed blood flow intensity (cm/s)
 1, 2 – males

Table 5. The average value in seconds (standard deviations) of dogs' times sniffing the vulvar area and the swabs containing different attractants (Experiment III).

| Samples/ males | Females in anoestrus | Females in oestrus | Females wit Eau D'Estrus | Dry swabs | Swabs with oestrus discharge | Swabs with Eau D'Estrus |
|-------------------|-------------------------|-----------------------|-----------------------------|--------------------|---------------------------------|----------------------------|
| 1 | 3.1 (± 0.27) | 7.2 (± 0.32) | 2.7 (± 0.29) | 2.3 (± 0.31) | 6.5 (± 0.39) | 2.3 (± 0.16) |
| 2 | 2.9 (± 0.34) | 6.8 (± 0.30) | 2.5 (± 0.25) | 2.2 (± 0.31) | 5.6 (± 0.35) | 2.2 (± 0.18) |
| 3 | 3.0 (± 0.26) | 8.2 (± 0.44) | 2.4 (± 0.14) | 2.3 (± 0.22) | 5.8 (± 0.32) | 2.4 (± 0.14) |
| 4 | 3.1 (± 0.19) | 6.8 (± 0.35) | 2.6 (± 0.1) | 2.3 (± 0.19) | 6.3 (± 0.43) | 2.3 (± 0.17) |
| 5 | 3.0 (± 0.3) | 6.9 (± 0.35) | 2.4 (± 0.06) | 2.4 (± 0.25) | 6.4 (± 0.32) | 2.4 (± 0.20) |

swabs, as well as swabs containing artificial pheromones, did not cause any kind of excitation (described by the above-mentioned behavior). Swabs from bitches in anoestrus caused moderate excitation: the immobilization reflex as well as following the swabs occurred in two trials while pawing and jumping were not observed at all.

Experiment IIb

Analysis of the pheromones influence on the blood flow in the penile vessel by color-coded Doppler ultrasonography showed, in all trials, an increased intensity of blood flow in the penile vessels during presentation of the natural pheromones, while this kind of changes was not observed when artificial pheromones were presented (Fig. 1). The mean intensity (cm/s) of the blood flow in the penile artery did not significantly change before and after artificial pheromone presentation, while contact with natural pheromones caused a significant increase in this parameter. In all trials, during different sample presentations, there were no changes in blood flow in the control vessel (*arcuate artery*) (Table 4).

Experiment III

A significant difference was observed between the average time of sniffing depending on which samples the experimental dogs were presented with. A significantly longer sniffing average time was noted when females in oestrus and swabs collected from females in oestrus were presented to the males, compared to the presentation of dry swabs, females in anoestrus, swabs soaked in Eau D'Estrus and females in anoestrus marked with Eau D'Estrus (Table 5).

Discussion

Recently a tendency to use synthetic pheromones to influence animal behavior can be observed and progress in that field can be noted (Gunn-Moore and Cameron 2004, Tod et al. 2005, Falewee et al. 2006, Frank et al. 2010, Landsberg et al. 2015). In domestic animals, except for appeasing pheromones which are commercially available for many species, among others research was also focused on sex pheromones (Izard and Vandenberg 1982, Stevens et al. 1982, Nishimura et al. 1991, Rekwot et al. 2001, Sankar and Archunan 2008).

In our study the reaction of the males to the natural pheromones confirmed that the presence of the female in oestrus is not necessary to obtain signs of sexual interest and arousal in a male dog and the pheromones by themselves are able to stimulate (however, usually without a full range of behavior) the adult males. In the present study, this kind of change in behavior, as well as in physiological reaction (blood flow intensity), was not observed after the use of the artificial pheromones.

Observations of the nature of pheromone activity assumes that these kind of semiochemical substances act mostly as a groups of substances and not as a single, individual substance (Kashiwayanagi 2003, Pageat 2003). Moreover, in the context of sex pheromones, the significance of other sources of semiochemically active substances except the vulvar discharge should be considered as a component of the full oestral signal in oestral females (e.g. substances produced in anal glands) (Pageat 2003, Zhang et al. 2008). In experiment II we observed some kind of confusion of the experimental male dogs in response to presentation of females in anoestrus marked by natural pheromones (oestral vaginal secretion). They led to decreased percentage of mating attempts. This could be connected with the fact that single vomeronasal sensory neurons respond selectively to

only one class of pheromones (Kashiwayanagi 2003). The lack of fully expressed semiochemical information (especially for males experienced in oestrus detection) could be a reason for the lack of expression of a full range of sexual behavior (Novotny et al. 1985, Kustritz 2005, Zhang et al. 2008, Dzieciol et al. 2012a, Dzieciol et al. 2013)

Considering the usefulness of the commercially available artificial canine sex pheromones for male dog stimulation, it is worth mentioning that regarding Goodwin et al. (1979) the probable main sex pheromone in dogs is the methyl paraben (methyl 4-hydroxybenzoate). However, Kruse and Howard (1983) in their study concluded that methyl p-hydroxybenzoate cannot be considered as a key sexual attractant for male dogs. Santos et al. (2013), using methyl 4-hydroxybenzoate (cat. H5501; Sigma-Aldrich®, Saint Louis, MO, USA) in their study also stated that “there was no sexual behavior observed between the dogs and the bitch (...), even though she was impregnated with artificial pheromone”. Other authors evaluating the composition of canine urine did not confirm the presence of methyl paraben at all in the collected material (Schultz et al. 1985, Dzieciol et al. 2014).

It is not clear what the complete composition of Eau D’Estrus is. The producer do not describe it. However it is very probable (and the use of GC/MS analysis confirmed this thesis) that methyl paraben is the component of this product. It is worth noting that even though methyl paraben would be indeed, as a single substance, a canine sex pheromone, for the proper acting of the synthetic attractant, and to achieve the expected effect, probably the proper carrier, vehiculum must be used, and the synergistic action of both compounds should take place. Taking into account the experience of other researchers in this matter, water solution without other biological substrates could not give a expected effect (Novotny et al 1985). Thus, taking into account these considerations and the results of the above-mentioned studies, the lack of efficiency of the examined product in stimulation of the males should not be surprising.

The fact that the complete composition of the active substances presented in the bitches’ secretions is still not fully established, means that it is very difficult to create an effective, synthetic equivalent of bitch sex pheromones, which would be fully recognizable to male dogs and which would be able to stimulate the full range of sexual reflexes. Therefore, we have to conclude that in this model of experiment the Eau D’Estrus did not confirm its usefulness in canine reproduction.

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