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Discrimination of estrus odor in urine by male dogs in different experimental settings

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ABSTRACT

This study assessed the most suitable behavioral test for discrimination of the odor of female dog urine during estrus and anestrus, to identify chemical compounds acting as female pheromones. Twelve male dogs were used for testing. Urine samples were collected from 16 females in estrus, 5 females in anestrus, 5 spayed females, and 8 males. In test 1, five samples were arranged randomly in a lineup outdoors, and the dogs showed no spontaneous interest or preference toward the estrus samples. In test 2, urine samples from a female in either estrus or anestrus were spilled on the grass in two diverging tracks. The dogs seemed to be unable to localize the estrus urine sample, and when found accidently, they showed only a weak interest. In test 3, two urine samples from a female either in estrus or in anestrus were placed into artificial vaginas of two dog models. The dogs sniffed the samples longer ($P = 0.004$) than in test 2 and were able to distinguish estrus versus anestrus samples by longer sniffing of estrus samples ($P < 0.02$); however, their interest decreased in consecutive trials. Test 4 involved responses of dogs that had been previously trained in the scent lineup to discriminate cancer odor. The trained dogs indicated the estrus urine sample correctly amidst four anestrus and one male samples in 98.6% and 74.6% of trials, respectively, with a probability of correct indications by chance of 50% and 20%, respectively. Longer sniffing of estrus samples was observed only during the first trial. It was concluded that the lineup using trained dogs was the most useful method to discriminate estrus odor if multiple testing is required. Although not all typical sexual behaviors could be observed with this method, quantification of the dogs' responses was helpful for future studies on the chemical structure of pheromones.

Introduction

Studies on semiochemical communication between dog sexes require proper, exact methods of assessing behavioral reactions of animals toward pheromones present in urine or excreted by the skin, anal glands or present in saliva or in feces.

Two anatomically separate but physiologically complementary chemosensory organs, the main olfactory epithelium and the vomeronasal organ (VNO) also called Jacobson's organ or the accessory olfactory system, play important roles in the biology of terrestrial vertebrates (Baum and Cherry, 2015; Cooper and Burghardt, 1990; Keller et al., 2009; Stowers and Kuo, 2015).

It was believed that in pheromonal communication, the VNO located in the base of the nasal cavity plays a crucial role. However, Keller et al. (2009), in a review of the literature, indicated that the main olfactory epithelium and the VNO may function synergistically in evoking or sustaining some pheromone-dependent behaviors. These authors suggested that the mutual roles played by both systems in detecting chemosignals and in regulating chemosensory-dependent behaviors is a central feature of olfaction.

Pheromones are chemosignals for communication within a species. Pheromones can have releaser effects (i.e., directly affecting the central nervous system and causing an immediate behavioral

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response) or priming effects, which stimulate the neurohormonal system, altering hormonal activity and physiological processes and, consequently, modulate or change an animal's behavior, not immediately, but after some time has elapsed (Baum and Cherry, 2015; Benny and Kimchi, 2014; Haga-Yamanaka et al., 2014; Hummer and McClintock, 2009; Keller et al., 2009). Some pheromones have both effects (Wyatt, 2017).

Various methods for evaluating the influence of sex pheromones on the physiology of the signal recipient have been proposed, from very simple ones up to sophisticated, technically complicated approaches that are difficult to use routinely, for example, methods based on evaluation of changes in the penile blood flow (Dzięcioł et al., 2012).

On the other hand, the simplest method applied by Goodwin et al. (1979) or by Kruse and Howard (1983), evaluating male behavior during contact with a female in anestrus and scented with sex pheromones, often in practice gives very disappointing results and difficult to explain, mostly due to the interindividual interactions resulting from different sizes, ages, social status (dominant/subdominant), lack of tolerance reflex of the female, or just individual aversion. Moreover, the most recent results of chemical analysis of signals emitted by bitches in various stages of the ovarian cycle suggest that the often observed ambiguous reaction of males sniffing a female in anestrus but scented with the urine from an estrual female could be associated with the simultaneous presence of chemical signals characteristic of estrus and anestrus (Dzięcioł et al., 2018).

Taking into account the abovementioned characteristics of pheromones, and disadvantages of previously used protocols aimed at their detection, new appropriate methods for pheromone assay have to be applied. The testing methods should enable assessment of immediate or long-term behavioral or physiological effects of pheromones or of material containing pheromones such as urine. The methods should be replicable and free from a "novelty effect" to enable multiple testing of odorous material collected from many females or under different physiological conditions. The methods should also be not vulnerable to various external or internal factors like weather conditions, distraction by other attractive odors, et cetera. Multiple testing giving clear and unequivocal responses of particular subjects is also important for receiving quantitative data that can be analyzed statistically.

In studies on olfaction, including those involving pheromones, problems associated with generating, controlling, presenting, and measuring of odors, make experiments more difficult and less advanced compared to studies on other sensory modalities (Fendt et al., 2017). Problems with experiments on olfaction are associated with the facts that odor molecules may be differently volatile, may either diffuse freely or their concentration decreases with distance from the odor source, or because of turbulence the odor plume may follow an unpredictable path. Another complication is the fact that there are tens of thousands of odors that can be detected by animals at different vapor pressures, diffusion rates, and solubility. A simple question is whether an animal can detect an odor or can discriminate between two or more odors (Fendt et al., 2017). However, if quantitative measures of odor detection or discrimination are required, more exact tests and control of odors are necessary.

Fendt et al. (2017) classified olfactory tests into nonassociative and associative. Nonassociative tests are simple observations of an animal's response to odors presented to it. Associative tests are conditioning tests in which animals are trained to make a specific behavioral response to one type of odor and another response to a different odor or the absence of an odor (Fendt et al., 2017).

Dog responses to releaser pheromones can be distinguished as full behavioral repertoire of sexual behaviors (approaching the source of odor, intense sniffing, and in the case of urine licking and splashing with the tongue onto the palate, which serves for introducing an appropriate amount of urine into the VNO) or as a simple discrimination between pheromonal and nonpheromonal odors. The latter could be labeled as pheromone detection without a full behavioral repertoire.

The aim of our study was to compare different olfactory tests for detection and discrimination of female natural sex pheromones by male dogs, to provide a basis for identifying compounds that actually act as pheromones and to find a suitable test for assaying artificial analogues of natural pheromones.

Materials and methods

Ethics statement

According to the country's statute law on animal experimentation, the procedures involving observations of natural behaviors of dogs toward odor or conventional dog training are not animal experimentation; the Local Ethical Commission for Animal Experimentation (resolution no. 67/2014) stated that no special permission for the use of dogs in such noninvasive studies was required.

Animals

A total of 12 adult male dogs (2-10 years old) of different breeds (German shepherds, Belgian shepherd/Malinois, Border collie, Rhodesian ridgeback, and mongrels) were used for testing. The dogs were sexually experienced, however, to different degree. The German shepherds, Belgian shepherd/Malinois, and Border collie being pedigree males were occasionally and successfully used as sires; the Rhodesian ridgeback was systematically used as sire, and according to the owner, demonstrated extreme interest in females in heat. Three of the mongrels (of German shepherd type) showed excellent performance in the scent lineup and were also used for mating to obtain progeny suitable for training in the lineup. The remaining two mongrels showed a strong sexual drive and, on encountering females in heat, were difficult to control. Urine samples, as a source of natural pheromones (Doty and Dunbar 1974), were collected during natural voiding from 16 females in estrus, 5 females in anestrus, 5 spayed females, and 8 males.

Four kinds of tests were conducted to assess the most applicable one for further research. All tests were video-recorded.

Test 1: Five glass containers covered with lids with holes to prevent direct contact with the dog's nose were placed in pots arranged randomly in a lineup outdoors. Open Eppendorf tubes with 2 ml urine were placed in the glass containers. Each dog was led toward the lineup upwind and then unleashed and encouraged to sniff the containers. The assumption was that this experimental setup would use the natural sniffing behavior of dogs toward novel objects. For each trial, the samples in the lineup were arranged in a random order. The dog handler was blind to the position of the estrus urine sample.

Test 2: Five 2 ml urine samples from a female either in estrus or in anestrus were spilled in an open area of grass of circa 5 cm in height, in the form of two diverging traces 4 meters apart at the end. The spots where the urine samples were spilled had no special characteristic features to attract the dogs' interest visually. The assumption was that this experimental setup would use the natural sniffing behavior of dogs. Each dog was led toward the spots upwind of where the urine samples were spilled and then unleashed and encouraged to sniff around without directing it toward the urine samples. The behavior of the dogs was video recorded for 10 minutes.

Test 3: Two samples of 2 ml of urine, one from a female in estrus and the other in anestrus, were placed in open Eppendorf tubes that were put in an artificial vagina of two separate dog models (Hot Doll, France), located outdoors 4 m apart. The assumption was that

dogs would be attracted visually by the dog-shaped models resembling a bitch in a squatting position and thus would sniff the anal and vaginal region of the models. This variant of experimental setup seems to be the most similar to the natural conditions and should allow expression of a full range of behavior by the male without the negative impact of intraindividual disturbing interactions. Each dog performed three trials within the tests 1-3. The dog models were thoroughly washed/cleaned after each trial.

Test 4: This test involved trained responses of dogs that had been previously trained in the lineup to detect cancer in humans on the basis of exhaled breath odor. This test was started with three trained dogs, but after one dog had to be euthanized because of a malignant incurable disease, the tests were continued with two dogs. The dogs underwent a one-week retraining to indicate urine odor from females in estrus instead of cancer odor. According to the training method routinely applied in the first author's laboratory, the operant conditioning response of dogs was reinforced by a treat using a continuous reinforcement schedule (Jezierski et al., 2010; Walczak et al., 2012). After the dogs were able to faultlessly indicate the estrus urine sample in 20 consecutive trials in a lineup of five samples, they were considered to be trained enough for testing in the present experiment. Five glass containers of the same kind as used in the test 1, with open Eppendorf tubes containing 2 ml urine, covered with lids with holes to prevent direct contact with the dog's nose, were placed in pots arranged randomly in a lineup on the floor of an isolated sniffing room. For each trial, the samples in the lineup were arranged in a random order. The dogs were trained to indicate one target odor sample (estrus urine sample) by sitting down in front of it. One of the five samples contained water to check if the dogs have no tendency to indicate a sample that is only different from the others. The dog handler was blind to the position of the estrus urine sample. With each of the dogs, six trials (runs along the lineup) were conducted within one testing day. Altogether each dog performed 1100 trials within 1 year using urine samples collected several times from females during different phases of ovary cycle, from castrated females and from males.

In each test, the following parameters were assessed:

- sniffing duration
- frequency of approaching to the urine sample
- sniffing order (which sample was sniffed first)
- urination/defecation on or near the sample
- licking/salivation on the sample
- erection of the penis
- for Test 4, correct indication of the estrus sample, false positive alerts/indications on the anestrus or male urine sample, and false negatives/misses (not indicating the estrus sample) were recorded. Correct indications were calculated in two ways: as a yes/no response to each sniffed sample in the lineup (50% probability of correct indication by chance) and as a choice of one sample out of 5 in the lineup (20% probability of correct indications by chance in one trial).

Since the sniffing duration, especially in test 4, was very short (<1 sec), the video recordings were played in slow motion mode (4x), for measuring the sniffing duration, and the results were then multiplied by 4 to obtain the final results in seconds.

Statistical analysis

For the trait that demonstrated a normal distribution (sniffing duration), ANOVA (mixed model with repeated measures) was used where the fix effects were estrus status, consecutive trial and place (spot vs. phantoms vs. lineup), and individual urine donor as a random effect. In the test 4, as placement of the sample, the position in the lineup was taken. For traits demonstrating a non-normal distribution (frequencies of licking, urination in the tests 1-3 as well as correct indications, false positive alerts/indications and false negatives/misses in the test 4), the Chi-square test was applied.

Results

In test 1, male dogs showed no spontaneous interest in containers with urine samples, even being encouraged by the handler. None of the samples in the outdoor lineup was clearly preferred, and the dogs mostly went away from the samples and sniffed spots elsewhere, thus no comparable results could be obtained, and the results were not analyzed statistically.

In test 2, a weak interest in urine samples spilled on a grass area was observed in some dogs, whereas the other dogs ignored the spots with urine. The dogs seemed to be unable to localize precisely the spot where the estrus urine samples were spilled, passing several times as closely as less than 50 cm from the spot. The mean sniffing duration of estrus urine samples in the grass was nonsignificantly longer than for the anestrus urine, and the total number of approaches as well as the total number of urinations were also nonsignificantly greater for estrus versus anestrus samples (Table 1). No licking of urine samples in the grass was observed. More intense sniffing and licking in some dogs was observed when the dogs accidently found the spot where the urine samples were spilled. However, no other behaviors characteristic toward sex pheromones such as salivation were observed. When repeatedly tested, the interest of the dogs in urine samples spilled in the grass

Table 1
Behaviors of dogs toward estrus versus diestrus urine samples spilled on grass or placed in artificial vaginas of dog models (mean ± s.d.)

Behaviors	Type of test	Urine sample from female in estrus (E)	Urine sample from female in anestrus (A)
Total number of approaches within the test duration	Test 2 Spots on grass (S)	2.82 ± 1.52	2.18 ± 1.33 E-A ns
Signif. of differences	Test 3 Dog models (Dm)	6.25 ± 4.83 S-Dm, n.s.	5.05 ± 3.40 E-A ns S-Dm, n.s.
Total duration of sniffing (sec)	Test 2 Spots on grass (S)	29.8 ± 23.4	21.9 ± 14.9 E-A ns
Signif. of differences	Test 3 Dog models (Dm)	70.4 ± 59.9 S-Dm, $P = 0.004$	35.1 ± 24.8 E-A, $P < 0.02$ S-Dm, $P = 0.004$
Total number of licking and salivation	Test 2 Spots on grass (S)	0	0
Signif. of differences	Test 3 Dog models (Dm)	6 S-Dm, $P < 0.05$	1 E-A, n.s. S-Dm, n.s.
Total number of urinations on the urine sample	Test 2 Spots on grass (S)	28	20 E-A, n.s.
Signif of differences	Test 3 Dog models (Dm)	31 S-Dm, n.s.	9 E-A, $P < 0.001$ S-Dm, $P < 0.05$

gradually decreased, and from the fourth-fifth trials on, no reaction, even in the form of a short sniffing, was observed as the dogs would go away from the urine samples.

In test 3, the male dogs were more interested in urine samples placed in the artificial vagina of dog models than in urine spilled in the grass, which was demonstrated by a significantly longer sniffing and more frequent licking of the artificial vagina (Table 1). Dogs did differentiate estrus versus anestrus samples placed in the models because the mean sniffing duration for estrus samples was two times longer than nonestrus ones ($P < 0.02$, Table 1), and this was accompanied by licking and salivation. However, licking urine samples occurred only in some trials. The licking frequency did not differ significantly between estrus and anestrus samples; however, it was observed only for estrus urine samples placed in the models (Table 1).

Some dogs marked the models with their own urine. Urination on or near the dog models did not occur in every test. Only one male dog urinated on the models in every trial. The number of urinations in response to estrus versus anestrus urine samples did not differ significantly in spots on the grass but was significantly more frequent on anestrus samples spilled in the grass than on anestrus urine samples placed in dog models ($P < 0.05$, Table 1). Estrus samples placed in the models provoked more frequent urinations than anestrus samples ($P < 0.001$, Table 1). One of the dogs (Border collie) during the repeated trials vigorously pawed the dog model after sniffing it and began to play with. The pawing may be regarded as the first phase to mount the model, and this behavior was a bit problematic for the test because the model was mostly thrown aside or displaced and had to be fixed firmly to the ground.

Similarly to test 2, the dogs' interest in models decreased in consecutive trials, so that after conducting more than five trials practically no results could be obtained, as dogs either went away from the models or, in the case of one dog, played with the models as if they were objects to be retrieved and/or to be bitten.

In test 4, the trained dogs correctly indicated the estrus urine in 98.6% of trials with a 50% probability of being correct by chance (Figure 1) and in 74.6% of trials with a 20% probability of correct indication by chance. The percentage of false negatives/misses and hesitations was low (0.29% and 0.07%, respectively). In 0.78% of trials, the dogs ignored the estrus urine sample in the lineup. Longer sniffing of estrus urine samples in the lineup was observed only during the first trial of the test ($P < 0.001$, Figure 2).

In contrast to tests 2 and 3, no decrease in the motivation of trained dogs to respond to the lineup was observed in consecutive trials within a day. Apart from a shorter sniffing duration compared to the first trial in the lineup, in consecutive trials no change in the rate of correct indication of estrus urine was ascertained.

No erections of the penis were observed in any of the tests.

Discussion

According to the classification of tests given by Fendt et al. (2017), tests 1, 2, and 3 can be regarded as nonassociative tests, whereas test 4 is a typical associative test.

The lack of spontaneous and regular sniffing of containers in test 1 was surprising, especially as it was observed in all 12 males, among them in those that usually showed strongly expressed interest in females in heat. This observation makes the test 1 impracticable.

The inability to localize precisely the estrus urine sample spilled in the grass in test 2 suggests that the pheromones in bitch urine are either poorly volatile or must be accompanied by other volatile odor molecules, for example, from the anal glands or by some visual stimuli such as those related to the dog models. In test 4, the dogs were previously trained to sniff the containers in the lineup (i.e., to put the nose closely to the container); therefore, it played a minor role if the pheromones in the urine were poorly volatile.

Comparing tests 2, 3, and 4, a clear effect of odor novelty was observed. As a result of the novelty effect, tests 2 and 3 could be performed only 2-3 times with the same individual. Thus, the practical usefulness of tests 2 and 3 for pheromone detection or identification is problematic. Also, the other characteristic behavioral reactions toward female pheromones such as licking and salivation were not observed toward female urine in the grass and were not always observed for the dog models.

Scent lineup using trained dogs (Test 4) can be considered suitable for detection and discrimination of estrus odors when large numbers of urine samples have to be tested. However, no full behavioral repertoire of the sniffing dogs, which is a weak point of this method. It should be mentioned that the dogs used for the test 4 were successfully used in consecutive experiment to discriminate particular chemical compounds present in female urine during estrus (paper in preparation).

It could be expected that in accordance with the classification by Fendt et al. (2017), our tests 2 and 3 as nonassociative tests might provide evidence not only for pheromone detection but potentially also give a rough estimate of odor preference and discrimination. As

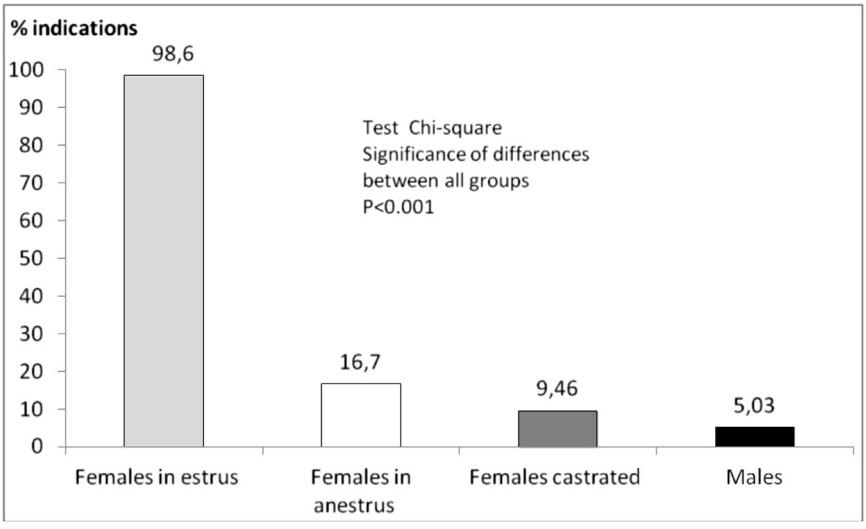

Figure 1. Percent of male dogs' indications of urine samples placed in the lineup, at 50% of correct indications by chance.

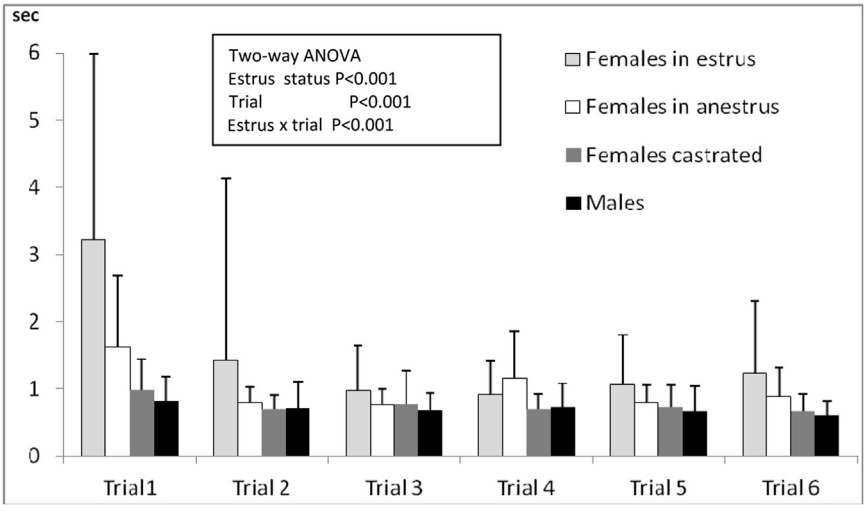

Figure 2. Mean sniffing time and standard deviation of estrus versus other urine samples in consecutive trials in the lineup.

symptoms of pheromone detection and attractiveness, approaches to the odor source, the sniffing duration, and the intensity of characteristic male behavior toward female urine such as licking, salivation, and squelching with the tongue could be considered. These behaviors were, however, observed only sporadically and only in some dogs. On the other hand, the dogs we used for the testing, when being walked, on seeing other dogs urinating, were obviously interested in the spot where the other dog urinated and constantly sniffed and licked the urine left on the ground.

Associative tests based on operant conditioning involve motivation for earning a reward obtained after performing a learned behavior, indicating that a dog has found the target odor.

In dogs, operant conditioning is widely used for many kinds of odor detection or discrimination (Helton, 2009). One of the best known associative tests is human identification by dogs based on individual scent in the lineup for forensic purposes (Schoon, 1996; Jezierski et al., 2010) or for detection of cancer odor markers (Jezierski et al., 2015). Dogs have also been successfully trained to discriminate estrus odor in cows (Jezierski 1992; Fischer-Tenhagen et al., 2011).

In many forms of odor detection or discrimination by canines, both in the scent lineup and in a free search, the target odor as such is not rewarding for dogs because it has no biological meaning. The reward is often a piece of food or a favorite object thrown by the handler to be retrieved by the dog and to play with. Training using operant conditioning is more time-consuming than nonassociative tests; however, it may be applied for discrimination of a variety of odors. As stated by Fendt et al. (2017), the associative conditioning tests have some advantages because they deliver easily scored measures of the response accuracy. The other advantage is that associative methods, like the lineup used in the present study, allow multiple trials to be run on individual subjects and to calculate the detection sensitivity and specificity. In the lineup method, the containers differ only with regard to the test odors contained. The position of particular odor samples in the lineup is randomized to avoid learning effects that could occur if the position of the target sample would be unchanged. Using the lineup, the same subjects can be tested over a large number of conditions, for example, to assess effects of odor mixtures, odor thresholds, or odor generalization, as listed by Fendt et al. (2017) as advantages of associative methods. Because the dogs during the trials in the lineup are mainly interested in odor samples, and not for example in odors on the floor and were never observed urinating on or near odor samples, there is no minor inconvenience of washing the floor after a dog has completed the test. The glass lids on the container with holes were mostly not touched by dogs with their nose, neither were the lids licked, at least by our dogs. Although it is advisable to use clean lids for each trial, there is no confirmed evidence that contamination of glass lids with the dogs' own odors plays a significant role. Last but not least, the test duration in the lineup method is very short, because for one trial consisting of sniffing five odor samples, the dogs need only 10-15 seconds, and there is no problem of waiting until the dog begins to be interested in the odors tested. Thus, multiple trials can be conducted within short time.

A question may arise on why the dogs made false positive indications, alerts by indicating samples of urine from females in anestrus, castrated females, and even males. The odor of urine of females in estrus is believed to be so specific, different from other odors, and is attractive to males that confusing it with, for example, male urine was surprising. Our previous studies on discrimination of different odors by specially trained dogs in the lineup (individual human scent—Jezierski et al., 2010, drugs—Jezierski et al., 2014, human cancer odor—Walczak et al., 2012) showed that dogs are usually not able to reach average discrimination accuracy of 100%, except for some short test series in which the accuracy may be actually 100%. A cause for mistakes, even in well-trained dogs, may be related to the fact that while working for a reward, they may change their strategies for solving the problem, for example, by using the "trial and error" strategy. Our studies also showed that reducing the rate of false alerts by dogs is very difficult (Jezierski et al., 2010). Another cause of mistakes could be related to the fact that animals respond not only to species-wide pheromones but also to individual signature mixtures. Animals use signature mixtures to recognize individual conspecifics or members of particular social groups such as family or colony (Wyatt, 2017). The odor signature of such individuals may be differentially attractive or aversive to the recipient of the odor.

It should be mentioned that there was no problem with retraining of dogs that had previously been trained to detect cancer odor.

Another kind of objective measure of the dogs' responses in the lineup is the duration of sniffing. However, because of very short sniffing bouts (mostly less than 1 sec), a precise estimation of the sniffing time was technically difficult and required analyzing the video recording in slow motion mode. Although the sniffing time was significantly longer for estrus samples than for all other non-estrus samples, the significant effect of consecutive trials, in terms of decreasing sniffing time, makes this parameter problematic in multiple testing.

Sniffing time was used in pheromone studies in different species, for example, in horses (Bűda et al., 2012) or rodents (e.g., Mayeaux and Johnston, 2002; Nielsen et al., 2016) and in dogs (Dzięcioł et al., 2013). For example (Bűda et al., 2012), tested responses of stallions to cresols using two-choice tests presenting o-cresol versus m-cresol, o-cresol versus p-cresol, and m-cresol versus p-cresol. The two stimuli were presented simultaneously for 1 minute, and the time spent by each stallion sniffing the samples was recorded. The authors found that the stallions spent significantly more time sniffing p-cresol compared with m- and o-cresols. No preferences were observed between o- and m-cresols or between the two water samples. No novelty effect was mentioned in the studies of Bűda et al. (2012). Although it is well established that individuals of many species habituate to the presence of familiar conspecifics or their cues, according to Wyatt (2017) experiments conducted so far have not been designed to separate male novelty and male pheromones. The same could concern females as shown in our experiment. Mayeaux and Johnston (2002) demonstrated that in hamsters the higher level of investigation of a novel odor than of a familiar one was observed primarily when the novel odor was placed in a novel location. To avoid the issue of odor habituation or novelty, the trials were mostly conducted only once and the time of the first investigation was assessed. The intervals between consecutive tests were 6-9 days (e.g., Nielsen et al., 2016). Using this approach, Nielsen et al. (2016) concluded that even sexually naive rats are able to detect the smell of estrus; however, this ability is more pronounced in male rats that had sexual experience with females.

In our study, the trained discrimination lineup (Test 4) offers a useful way to distinguish urine samples from estrus versus anestrus females. However, a biologically relevant bioassay for female dog sex pheromones, which reproduces the natural male response, remains undiscovered. This is a focus of ongoing research.

Conclusion

The lineup method using trained dogs (Test 4) seems to be the most useful of all the tested methods to discriminate estrus odor, especially if multiple testing is required. Though our lineup method cannot be regarded as a true bioassay of pheromones, because not all typical sexual behaviors could be observed in this method, a quantification of dogs' responses is one of the advantages for subsequent studies on the chemical structure of urinary compounds.

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Authors' contributions: The experiments were designed by Tadeusz Jezierski, Michał Dzięcioł, Antoni Szumny, Wojciech Niżański, and Ewa J. Godzińska. The experiments were performed by Tadeusz Jezierski, Michał Dzięcioł, Martyna Woszczylo, and Barbara Pieczewska. The data were analyzed by Tadeusz Jezierski. The article was written by Tadeusz Jezierski and Michał Dzięcioł.

Ethical considerations

Local Ethical Commission for Animal Experimentation (resolution no. 67/2014) stated that no special permission for the use of dogs in such noninvasive studies was required.

Conflict of interests

The authors declare no conflict of interests.

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