

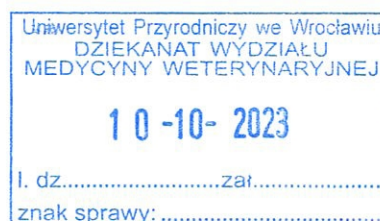
Conclusion form for Doctoral Dissertation Evaluation

Name of examiner : Prof. Dr Alain Fontbonne, DVM, MSc, PhD, Habil, Dip.ECAR

Department : UMES – Alfort National Veterinary College - Maisons Alfort – France

Email address : alain.fontbonne@vet-alfort.fr

Name of the candidate : Martyna Woszczylo, DVM



First part : General comments.

1. DVM Martyna Woszczylo's dissertation represents a considerable amount of work and a significant contribution to the veterinary scientific community. The major part of this dissertation is composed of four co-authored published articles in three peer-reviewed international scientific journals : Animals (impact factor 2023 : 3.231), Veterinary Science (impact factor 2023 : 2.518), Plos One (impact factor 2023 : 3.7). According to my own experience as having been in the jury of several PhDs, the number of international publications in the dissertation of DVM Woszczylo is higher than what is found in many PhD dissertations in Europe. Furthermore, she has been previously co-author of several articles on the same topic. I therefore consider that Martyna Woszczylo has presented in her doctoral dissertation a high level of knowledge on the topic of semiochemical communication in dogs.
2. The dissertation of DVM Martyna Woszczylo focuses on « selected aspects of semiochemical communication in dogs in the context of sexual behavior ». It was important to see whether the four articles presented have a thematic continuity. It is indeed the case. Among her four published papers, the author tries to understand what are the semiochemical factors influencing male dogs attractiveness towards females. It is especially important because, in the context of reproduction, sexual communication provoking arousal and the mating decision in dog is not fully understood. The first two articles focus on the role of urine in semiochemical communication in the canine species, trying to analyse the differences in the composition of urine observed between different phases of the cycle. The third article aims to study the factors that influence the libido of male dogs during semen collection and the fourth article presents a clinical case of a castrated male stimulating sexual interest in other males and presenting some characteristics in the composition of urine closed to what is found in estrus females. Along all these studies, Martyna Woszczylo tries to go deeper in the understanding of the topic,

showing an obstinacy which is a very good sign for a young scientist. I especially fully appreciated, in the four articles, the long and very complete discussions, analysing all aspects of the methodology and the results obtained, comparing them with the findings of other authors or previous studies – even in other species - therefore demonstrating therefore a high degree of understanding of her subject and a real ability of independent reasoning and scientific maturity. She has with no doubt the creativity to develop protocols, to write articles and the ability to conduct scientific research.

3. From her studies, DVM Martyna Woszczylo demonstrates that there are with no doubts some differences in the composition of urine in estrus bitches (article 2) and in sexually attractive male dogs (article 4). Furthermore, she and her co-authors demonstrate that it is non-volatile compounds that seem more important to increase the interest of males (article 1). These are original findings that are important to understand how to sexually stimulate male dogs, and may be to develop products intended to facilitate semen collection in dogs, which is demonstrated as being a non-stressful procedure, which is important to know for the veterinary community (article 3).
4. For all these reasons, Martyna Woszczylo's dissertation fully merits acceptance as a doctoral dissertation. Due to her involvement in the same field of research for several years and her high degree of competence within this field, I think that she should be awarded a doctorate scholarship.

Second part : detailed analysis of the chapters/articles of the dissertation.

5.1. General information about the candidate.

Name : Martyna Woszczylo

Date of the master degree : 10.02.2016

Institution: Faculty of Veterinary Medicine, Wrocław University of Environmental and Life Science.

5.2. The title of the dissertation is : « Selected aspects of semiochemical communication in dogs in the context of sexual behavior ».

Being a specialist in Animal Reproduction and not in semiochemical communication, I have examined the dissertation from the point of view of the quality of the manuscript, the clarity of the writing, the creativity of the experimental designs, the capacity to present and to discuss the results, and the scientific input, which should facilitate further studies to be carried out on the same theme.

The layout is appropriate. However, I think that at the end of the « introduction », it would be appreciated if the specific references that are cited in this preliminary chapter were added at the end of the « introduction », even if they are also cited in the different published articles that follow. Also, I would appreciate a final « conclusion » (and not conclusions) after the four articles, and not at the end of the introductory chapter. I think this conclusion should be extended and it should describe in more details what could be studied in the future to better understand the semio-chemical communication in dogs in the context of sexual attraction. In this way, it will demonstrate that the author is fully capable to continue and lead her research.

The introduction is a 11 pages chapter which is a complete summary about the « state of the art » of our knowledge of canine pheromones, especially in the context of sexual behavior. The author first reminds the readers about what we know about interindividual communication in the different phases of the reproductive cycle in the bitch. Then, the role of pheromones in canine communication is described, followed by a description of the canine olfactory system and its role in canine reproductive behavior. The author describes in the differences between the main olfactory system and the vomeronasal organ. *Note that there is a slight mistake in the penultimate line page 10 : the concentration of sulphide compounds is increased and not reduced during diestrus.* A definition and classification of the pheromones is then given, followed by the description of the different compounds that have been speculated to be acting as canine sex pheromones. At the end of this introduction, I think that the reader is able to fully understand the aims pursued in the dissertation and to read the four articles presented.

In the first article entitled « The role of urine in semiochemical communication between females and males of domestic dogs during estrus », the authors investigate whether the volatile compounds present in the female's urine are sufficient to trigger a robust sexual response in male dogs. The main conclusion of this paper is that urine odor is not used for long-distance semiochemical communication, but rather for close distance signaling. In experiment 1, the experimental design is inventive and rather complex, with two steel chambers linked by a pipeline. Of course, we can wonder if the dogs are not stressed to be placed inside such a closed and metallic chamber, but in the discussion, the authors state that the dogs were used to this equipment. The protocol is well prepared, avoiding the biases : trials always practised in the same order, food at the end, the person reviewing the filmed sequences in a totally blind manner. In experiment 2, the position of the different boxes containing urine from different sources (human, females in anestrus or estrus, male dogs) is at random and unknown by the person who evaluated the video recordings. This latter experiment clearly demonstrates the role of urine for close distance communication, as all 25 male dogs demonstrated licking behavior for the estrous urine sample, and 12 male dogs showed a specific facial muscle reaction in this situation. The demonstration seems achieved, even if, in experiment 1, we don't know why the dogs are more interested by the smell of real estrous females than only urine. The authors state that it could be the body, the

paws or even the vulval region. This paves the way for future investigations. For example, it could be interesting to study if urine collected in estrous bitches by cystocentesis, without any vaginal contact, bears the same attractivity than urine collected during a normal miction. According to me, the first article is of good scientific quality, with a good experimental design and an interesting finding concerning the role of urine in close-contact communication, which has not been described in the canine species so far.

The second article entitled « Urinary proteins of female domestic dog during ovarian cycle » is in the continuity of the preceding one. As specific urinary biomarkers are lacking in the dog, the authors, using Mass Spectrometry make a proteomic analysis aiming to compare the composition of urine samples taken from estrus, anestrus or immature bitches. They clearly demonstrate that there is a clear separation between the estrus and anestrus samples. Once again, the experimental design is well established, detailed and accurate. The results show the presence of several proteins which seem to be characteristic of the estrus female. Among them, Major Urinary Proteins such as lipocalins, already identified in the urine of mice and rats, are found. As these molecules are involved in the transport of small molecules, they could play a role in chemical signaling. As beta-lactoglobulins were absent in the anestrus samples, it is speculated that they could be carriers of semiochemicals in the urine of estrous bitches. The potential role of other proteins identified in the estrus samples, such as clusterin, proenkephalin, liver-expressed antimicrobial peptide 2, odorant-binding proteins or even the potential role of vaginal microflora is discussed, reminding what we know from other species. Once again, this interesting article paves the way to further studies, and the authors, in the conclusion, write that « it is prudent to conduct follow-up studies ».

The third article is entitled « The influence of manual semen collection in male trained dogs, in the presence or absence of a female in estrus, on the concentrations of cortisol, oxytocin, prolactin and testosterone ». After a long introduction justifying the choice of assaying these four hormones, the experimental design is well presented, with a nice figure 1. When assaying hormonal status, it is important to mention that the experiments were always performed at the same time of the day, as there is a circadian pattern of many hormones. Even if no significant differences are found between males whose semen was collected without the presence of an estrus bitch and males whose semen was collected with the presence of an estrus bitch, the fact that manual collection of semen does not sufficiently stimulate the endocrine system to induce an increase in the level of hormones above what is considered normal, is in itself an interesting finding, as it tends to show that this procedure respects the well-being of the dogs. The cautionable aspect is that these dogs were used to manual collection of semen, therefore probably not stressed by the procedure. This article may appear a little bit different in its aims than the three others, more focused on urine, but it fully has its place in this dissertation on communication in dogs in the context of sexual behavior. Maybe it could be better placed in the fourth position, after the three

articles focused on the role of urine in semiochemical communication : two experimental studies and one case report.

The fourth article is a case report entitled : « The case of atypical sexual attractiveness in a male domestic dog – a case study ». This article is a nice illustration about the role that urine may have in sexual attractiveness among dogs. The authors carefully rule out the hypothesis of any estrogenic impregnation of the organism of this dog. The discussion is long and complete, describing the potential different hypotheses that may explain that the urinary profile of volatile scent chemicals in the castrated dog, which was sexually attractive for male dogs, was quite similar to estrus females. The role of alpha-melanocyte-stimulating hormone could be studied in a future experiment, and also the role of diet.

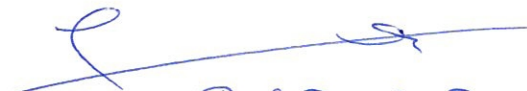
In the dissertation of Martyna Woszczylo, the choice of the bibliography appears relevant. In the four articles, the discussion is extended and very complete, analysing all the potential weaknesses and strenghts of the experimental design and of the results. Each of the articles provides new insights and advances our knowledge of semiochemical communication in dogs. For all these reasons, the dissertation deserves to be accepted.

5.3. According to Pubmed database, Martyna Woszczylo has been involved in research on semiochemical signaling in dogs since 2018 and has probably acquired a strong scientific comptence in this topic. The introduction and the four articles are well designed, well written, and demonstrate the candidate's broad knowledge and her ability of independent scientific reasoning.

5.4. In my opinion Martyna Woszczylo's dissertation is outstanding (number of articles published, originality of the protocols, clear leadership in this field, research leading to prospects for the future). Therefore she deserves a special recognition for this excellent dissertation.

In conclusion, the doctoral dissertation meets the requirements of Article 13, paragraph 1 of the Act of March 14th 2003, on scientific degrees and titles and on degrees and titles in the field of art (Journal of Laws of 2017, item 1789, as amended).

Maisons-Alfort, September 19th 2023


Prof. Dr. A. FONTBONNE