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PHEROMONE SIGNALLING IN THE MOUSE: ROLE OF URINARY PROTEINS AND VOMERONASAL ORGAN

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INTRODUCTION

Pheromone signalling in the mouse is characterized by a special chemosensory organ, the vomeronasal organ, and a high concentration of proteins binding and releasing volatile pheromones in male mouse urine. The endocrine effects primed by male mouse urine reported to require both the vomeronasal organ and the urinary proteins are the acceleration of female puberty onset, the pregnancy block and the estrus acceleration/synchronization. This association is challenging and outlines a system which, while sharing some of the properties of the sense of smell, is profoundly different as to the biology, the transduction mechanism, the stimulus/receptor interaction and the adequate stimulus. The study is at an early stage and only rudimentary or suggestive evidence is available today. This presentation intends to highlight some of the problems for investigations to come.

The Lipocalins

In nature, molecules can be used as chemical labels or tags for the signalling and the recognition of individuals, sexes, strains and species. The Major Histocompatibility Group, for example, contributes to single out a haplotype (40) and is recognized by the immune system in order to sort out the alien from the self. Another example are insect pheromones, species-specific molecules that trigger precise reactions in conspecifics (22, 32). In mammals, it is now clearer and clearer that lipocalins are a third class of label molecules used as complex chemosignals (17). In the animal kingdom, lipocalins are a large, disparate and diverse group of small proteins, molecular weight about 20 kDa, many of which are extracellular and display binding with high selectivity and affinity for small hydrophobic molecules. Beta-lactoglobulin is the first lipocalin crystallized from cow's whey in the 1930s (1), and more than thirty proteins are classified as lipocalins today (18). All lipocalins share a common structure. The common structure is a central 8-stranded beta-barrel with simple forth and back repeated topology lining a hydrophobic cavity, an N-terminal helical turn and a long C-terminal helix. The overall sequence homology between different lipocalins is low, typically about 20%, despite the presence of three well-defined and highly conseved motifs. At variance with

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the well defined structure, five lipocalins have been resolved by X-ray crystallography (6, 18) and one recombinant form enriched in ^{13}C and ^{15}N is being resolved by NMR (16), the function remains poorly understood and the data available are rudimentary or suggestive. Possibly the different lipocalins went to cover a variety of functions as they radiated in different phyla and subphyla, crustaceans, insects and vertebrates, and in different species during evolution (45). In mammals some lipocalins have a function within the body, e.g., retinol binding protein of serum carries retinol (5), other lipocalins as beta-lactoglobulin are excreted and a role in body homeostasis can be ruled out. Although we are a long way out from understanding the full extension of the functions covered by this protein family, secreted lipocalins may be used as chemical labels for communication. Several points argue in favour of this hypothesis. Secretory lipocalins are characterized by species-, sex-, strain- and possibly individual-linked polymorphism. For instance, beta-lactoglobulin is excreted in large amounts in the milk of the cow, buffalo, sheep, pig and dolphin but neither guinea pig, camel nor primates; within the species, there is also polymorphism of beta-lactoglobulin among different cow strains, differing but in a few aminoacids (1). The role of secretory lipocalins was highlighted by the observation of Dr. Pelosi that some of them bind odorant molecules, and have been appropriately termed Odorant Binding Protein (OBPs) (39). The odorants are small non charged molecules which are bound in the hydrophobic cavity by short range forces and possibly one hydrogen bond, with affinity of the order of a million per Molar concentration. Most interestingly, the time of dissociation of the odorant-protein complex is very long and lipocalins can store and slowly release in air the odorants for hours and days. In addition, lipocalins are unusually stable to drying and heating and are not likely to be quickly denatured when released out of the body.

The Major Urinary Proteins

The most widely studied family of OBP is the family of the Major Urinary Proteins of rodents (MUPs), whose structure is shown in Fig 1, and more information is available on them than on any OBP. Among the odorants bound by MUPs which have been characterized are a thiazoline derivative, probably produced by bacteria in the gut, and brevicomin, a very complex molecule similar to the bark beetle aggregating pheromone, whose origin in rodents is not known (2, 43). MUPs are synthesized in the liver of adult non castrated mice, are physiologically excreted with urine at the considerable rate of a few tens of mg per day and confer to the male mouse urine a typical odour (26). MUPs, one suspects, must be very important for the biology of mouse species in order to justify the high cost for the nitrogen balance of this proteinuria, the great expansion of the genomic representation, over 35 genes on chromosome 4, the high degree of gene conservation within and the high gene divergence between rat and mouse species which accumulated during about 30 million year of evolution (11). The sexual restriction to adult male sex, moreover, suggests a role in communication about sex and relevant to reproduction.

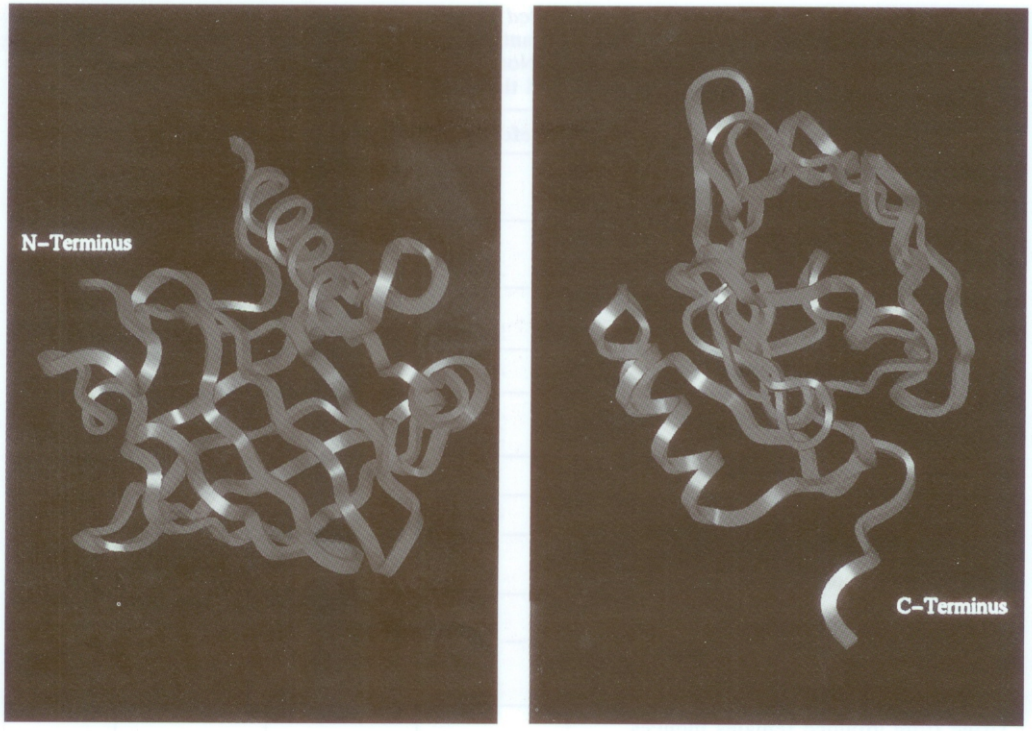

Fig. 1. - *Ribbon representation of the peptide backbone of MUP in two orthogonal projections.*

The projection showing the N-terminus highlights the beta-stranded hairpins forming a barrel-like structure. The projection showing the C-terminus shows the cavity within the MUP as well as the alpha-helix. Data obtained by NMR of isotopically enriched recombinant MUP.

This extraordinary urinary secretion of proteins, makes likely that mice can perform olfactory tasks based on urinary olfactory signals. Chemical cues from conspecifics can release adequate behaviours (releaser pheromones) or modulate the endocrine condition (primer pheromones) (24). Table I reports both eight behavioural effects and six primer effects of chemical cues present in rodents urine and whether protein molecules of urine of the emitter and the vomeronasal organ of the receiver are in cause. At this point a digression is necessary to recall the anatomy of the olfactory system of the mouse.

The Vomerosanal Organ of Jacobson

The olfactory sytem of the mouse as well as nearly all mammals is partitioned into two sections, the Main Olfactory Epithelium (MOE) with its associated central circuits, and the VomeroNasal Organ (VNO) with its central distinct pathways (15). The MOE lines part of the nasal cavity flushed by air, the VNO is remote from air flow and its epithelium lines the vomeronasal canal filled with fluid. Thus, whereas MOE is in position to detect volatile odorants carried by the air stream,

Table I. - *Behavioural and priming effects elicited by mouse urine.*
In the columns it is reported the effect, the relevant reference (Ref.), whether urinary proteins (Prot) and the vomeronasal organ (VNO) are involved. Note that the priming effects of male urine have been reported to require both the urinary proteins and the vomeronasal organ.

Behavioural Effects	Reference	Prot.	VNO	Type
Estrous/pregnant urine more attractive to males	12	ND	ND	F → M
Female urine decreases attacks by aggressive males	35	ND	ND	F → M
Female urine elicits 70 kHz ultrasonic whistle from males	38	ND	Yes (3)	F → M
Male urine more attractive to female	46	ND	ND	M → F
Male urine elicits protective maternal aggression in rats	33	No (36)	No (33)	M → F
Male urine elicits attacks between males	10	No	ND	M → M
Male urine territorial marking	41	ND	Yes (31)	M → M
Urine voided under stress elicits avoidance responses	9	ND	ND	M→ both
Effects primed by urinary cues				
Female grouping in the absence of male suppresses estrus cycle	50	ND	Yes (42)	F → F
Urine from grouped females induces puberty onset delay	13	No (37)	No (37)	F → F
Male urine accelerates puberty onset	48	Yes (49)	Yes (23)	M → F
Urine of strange male blocks pregnancy	8	Yes (28)	Yes (25)	M → F
Male urine induces estrus acceleration/ synchronization	51	Yes (28)	Yes (20)	M → F
Urine from pregnant/lactating females lengthens estrus	19	ND	ND	F → F

the VNO is harnessed to detect non-volatile water-soluble molecules (7). For the behavioural effects, the volatile component are probably an adequate stimulus and eventually MUPs are a storage and releaser system for volatiles. For the third and fourth primer effects of male urine that are listed, the protein is needed (49, 30, 34, 28) as well as the vomeronasal organ (23, 25). Also for the estrus acceleration/ synchronization both urinary proteins and the vomeronasal organ are needed (28, 20). Some detailed information adds evidence to the notion that MUPs are a very special stimulus, e.g., in the case of pregnancy block, MUPs are not only likely to mediate the pregnancy blocking effects of male urine, but they also convey the strain recognition signal of the male pheromone (28, 29). This performance is based, one would think, on the MUPs polymorphism between different mice strains (21). It would not be surprising if wild-type mice were able to recognize

also individuals on the base of MUPs phenotype. On the other hand, the conserved N-terminal aminoacid sequence of MUPs, EEA(R/S)S, is probably a common recognition site for MUPs (34).

Returning now to Table I, it is seen that intraspecific communication is mediated by the VNO in two behavioural effects and in four primer effects. Finally, the joint requirement for a protein component in the urine stimulus and for the VNO in the receiver, in three primer effects, suggests that MUPs and the VNO act in concert as a stimulus-receptor couple.

This observation raises the question as to whether there are distinct receptors in the VNO for the different chemical cues in the urine of conspecifics. Two families of putative Vomeronasal Receptors (VR) have been described in vomeronasal neurons with molecular biology techniques in mice (14) and rats (44). They characterize sensory neurons with different position of the cell body in the neuroepithelium, different central connections and probably different signal transduction pathways (4, 52). The family of the VR1 bears homology to the putative olfactory receptors of the MOE and project to the rostral part of the Accessory Olfactory Bulb (AOB); the family of the VR2, see Fig.2 for an *in situ* hybridiza-

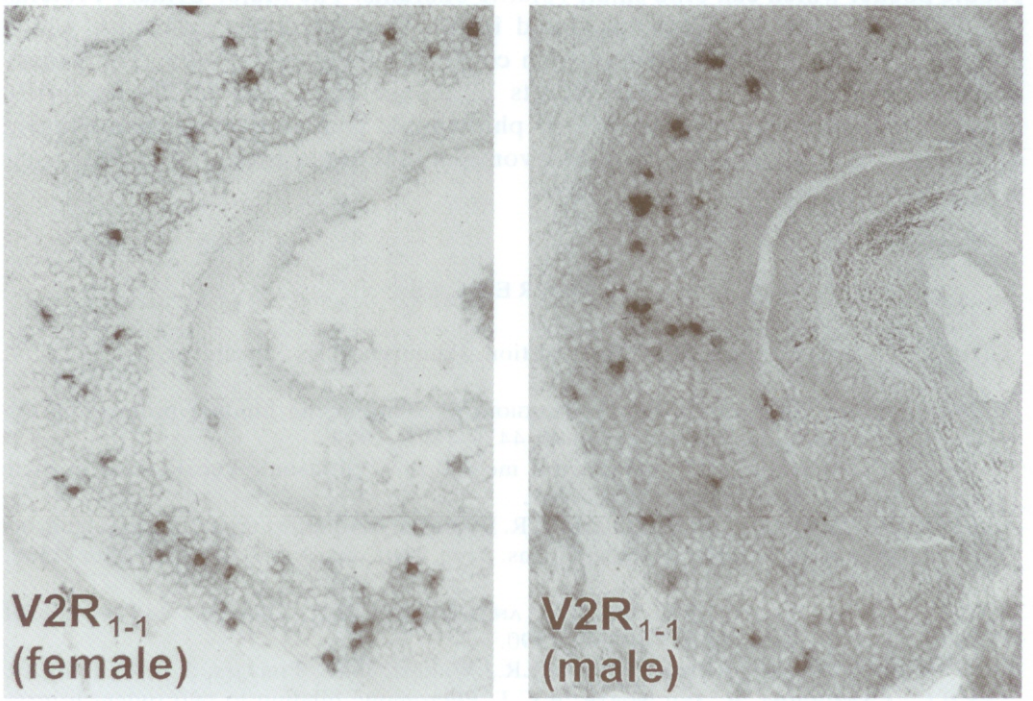

Fig. 2. - *Section of the mouse VNO stained by in situ hybridization with digoxygenin labelled riboprobes hybridizing to the mRNA of a vomeronasal receptor molecule of class V2R.*

Observe the discrete and scattered subpopulation of positive neurons in the crescent of the sensory neuroepithelium of the male and female mouse. magnification: 10x.

tion, bear homology to the calcium sensor receptor and metabotropic glutamate receptors and are characterized by a long extracellular aminoacid domain and project to the posterior part of AOB. It has been suggested that VR1 detect volatile molecules and VR2 proteins or peptides (27, 47).Were the volatiles those carried by MUPs and were the proteins MUPs, this complex signalling system would have a matched sensory counterpart in the VNO.

We do not understand the evolutive pressure which labelled different species, strains, sexes and perhaps individuals with OBPs as olfactory markers in the radiation of mammals. A similar system has not been described, e.g., in birds that rely mainly on visual and acoustic signals for communication. Possibly the pressure arose from the ecological niche occupied by early mammals which was likely to be terrestrial, nocturnal and rich in predators, the evolutionary advantage bearing more on the reproduction than on the survival of the individuals.

SUMMARY

The lipocalin protein family is characterized in structure by a conserved hydrophobic pocket which can bind small volatile odorants. The Major Urinary Proteins (MUPs) are a class of lipocalins found in the urine of adult male mice which concentrate in the urine odorants which confer a characteristic odor. The behavioural as well as the endocrine effects of mouse urine and MUPs are brifly reviewed, suggesting a complex role is pheromonal communication. Some recent data on the molecular receptors of the vomeronasal organ further suggest a complex interaction with the MUP system.

REFERENCES

1. Alais, C. In *Science du lait.* Third Edition, Pontoise, Paris Imprimeur, Pp. 172-174, 1975.
2. Bacchini, A., Gaetani, E. and Cavaggioni, A. Pheromone binding proteins of the mouse *Mus musculus*. *Experientia*, **48**: 4419-4421, 1992.
3. Bean, N.J. Olfactory and vomeronasal mediation of ultrasonic vocalization in male mice. *Physiol. Behav.*, **28**: 31-37, 1982.
4. Berghard, A., Buck, L.B. and Liman, E.R. Evidence for distinct signalling mechanisms in two mammalian olfactory sense organs. *Proc. Natl. Acad. Sci. USA*, **93**: 2365-2369, 1996.
5. Blomhoff, R., Green, M.H., Berg, T. and Norman, N.R. Transport and storage of vitamine A. *Science*, **250**: 399-404, 1990.
6. Böcskei, Zs, Groom, C.R., Flower, D.R., Wright, C.E., Phillips, S.E.V., Findlay, J.B.C., Cavaggioni, A. and North, A.C.T. Pheromone binding to two rodent urinary proteins revealed by X-ray chrystallography. *Nature*, **360**: 186-188, 1992.
7. Broman, I. Das Organon Vomero-nasale Jacobsoni - ein Wassergeruchorgan! *Anat. Hefte*, **174**: 137-191, 1920.
8. Bruce, H.M. An exteroceptive block to pregnancy in the mouse. *Nature*, **184**: 105, 1959.

9. Carr, W.J., Martorano, R.D. and Krames, L. Responses of mice to odors associated with stress. *J. Comp. Physiol. Psychol.*, **71**: 223-228, 1970.
10. Chanel, J. and Vernet-Maury, E. Determination par un test olfactif des inter-attractions chez la souris. *J. Physiol. Paris*, **55**: 121-122, 1963.
11. Clark, A.J., Ghazal, P., Bingham, R.W., Barrett, D. and Bishop, J.O. Sequence structure of a mouse major urinary protein gene and pseudogene compared. *EMBO J.*, **4**: 3159-3165, 1985.
12. Davies, V. J. and Bellamy, D. The olfactory response of mice to urine and effects of gonadectomy. *J. Endocrinol.*, **55**: 11-20, 1972.
13. Drickamer, L.C. Delay of sexual maturation in female house mice by exposure to grouped females or urine from grouped females. *J. Reprod. Fertil.*, **51**: 77-81, 1977.
14. Dulac, C. and Axel, R. A novel family of genes encoding putative pheromone receptors in mammals. *Cell*, **83**: 195-206, 1995.
15. Eisthen, H.L. Evolution of vertebrate olfactory systems. *Brain Behav. Evol.*, **50**: 223-233, 1994.
16. Ferrari, E., Lodi, T., Sorbi, R.T., Tirindelli, R., Cavaggioni, A. and Spisni, A. Expression of a lipocalin in *Pichia pastoris*: secretion, purification and binding activity of a recombinant mouse major urinary protein. *FEBS Letters*, **401**: 73-77, 1977.
17. Flower, D.R. The lipocalin protein family: a role in cell regulation. *FEBS Letters*, **354**: 7-11, 1994.
18. Flower, D.R., North, A.C.T. and Attwood, T.K. Structure and sequence relationships in the lipocalins and related proteins. *Protein Science*, **2**: 753-761, 1993.
19. Hoover, J.E. and Drickamer, L.C. Effects of urine from pregnant and lactating female house mice on oestrus cycles of adult females. *J. Reprod. Fertil.*, **55**: 297-301, 1979.
20. Johns, M.A., Feder, H.H., Komisaruk, B.R. and Mayer, A.D. Urine- induced reflex ovulation in anovulatory rats may be a vomeronasal effect. *Nature*, **272**: 446-448, 1978.
21. Johnson, D., Al-Shawi, R. and Bishop, J.O. Sexual dimorphism amd growth hormone induction of murine pheromone-binding proteins. *J. Mol. Endocrinol.*, **14**: 21-34, 1995.
22. Kaissling, K-E. Chemo-electrical transduction in insect olfactory receptors. *Ann. Rev. Neurosci.*, **9**: 121-145, 1986.
23. Kaneko, N., Debski, E.A., Wilson, M.C. and Whitten, W.K. Puberty acceleration in mice. II. Evidence that the vomeronasal organ is a receptor for the primer pheromone in male mouse urine. *Biol. Reprod.*, **22**: 873-878, 1980.
24. Keverne, E.B. Pheromonal influence on the endocrine regulation of reproduction. *Trends Neurosci.*, **6**: 381-384, 1983.
25. Keverne, E.B. and de la Riva, C. Pheromones in mice: reciprocal interactions between the nose and brain. *Nature*, **296**: 148-150, 1982.
26. Lane, S.E. and Neuhaus, O.W. Further studies on the isolation and characterization of a sex-dependent protein from the urine of male rats. *Biochim. Biophys. Acta*, **257**: 461-470, 1972.
27. Liman, E.R. Pheromone transduction in the vomeronasal organ. *Curr. Opin. Neurobiol.*, **6**: 487-493, 1996.
28. Marchlewska-Koj, A. Pregnancy block elicited by urinary proteins of male mice. *Biol. Reprod.*, **17**: 729-732, 1977.
29. Marchlewska-Koj, A. Pregnancy block elicited by male urinary peptides in mice. *J. Reprod. Fertil.*, **61**: 221-224, 1981.
30. Marchlewska-Koj, A., Pochròn, E. and Sliwowska, A. Salivary glands and preputial glands of males as a source of oestrus-stimulating pheromone in female mice. *J. Chem. Ecol.*, **16**: 2817-2822, 1990.
31. Maruniak, J.A., Wisocki, C.J. and Taylor, J.A. (1986) Mediation of male mouse urine marking and aggression by the vomeronasal organ. *Physiol. Behav.*, **37**: 655-657, 1986.

32. Masson, C. and Mustaparta, H. Chemical information processing in the olfactory system of insects. *Physiol. Rev.*, **70**: 199-245, 1990.
33. Mayer, A.D. and Rosenblatt, J.S. Contributions of olfaction to maternal aggression in laboratory rats (Rattus norvegicus): Effect of peripheral deafferentation of the primary olfactory system. *J. Comp. Psychol.*, **107**: 12-24, 1993.
34. Mucignat-Caretta, C. Caretta, A. and Cavaggioni, A. Acceleration of puberty onset in female mice by male urinary proteins. *J. Physiol., Lond.*, **486**: 517-522, 1995.
35. Mugford, R.A. and Nowell, N.W. The aggression of male mice against androgenized females. *Psychon. Sci.*, **20**: 191-192, 1970.
36. Novotny, M., Harvey, S., Jemiolo, B. and Alberts, J. Synthetic pheromones that promote inter-male aggression in mice. *Proc. Natl. Acad. Sci. USA*, **82**: 2959-2961, 1985.
37. Novotny, M., Jemiolo, B., Harvey, S., Wiesler, D. and Marchlewska-Koj, A. Adrenal-mediated endogenous metabolites inhibit puberty in female mice. *Science*, **231**: 722-725, 1986.
38. Nyby, J., Wysocki, C.J., Whitney, G., Dizinno, G. and Schneider, J. Elicitation of male mouse (Mus musculus) ultrasonic vocalizations: I. Urinary cues. *J. Comp. Physiol. Psychol.*, **93**: 957-975, 1979.
39. Pelosi, P. Odorant-binding proteins. *Rev. Biochem. Mol. Biol.*, **29**: 199-228, 1994.
40. Potts, W.K., Manning, C.J. and Wakeland, E.K.J. Mating patterns in seminatural populations of mice influenced by MHC genotype. *Nature*, **352**: 619-621, 1991.
41. Reynolds, E. Urination as a social response in mice. *Nature*, **234**: 481-483, 1971.
42. Reynolds, J. and Keverne, E.B. The accessory olfactory system and its role in the pheromonally mediated suppression of oestrus in grouped mice. *J. Reprod. Fertil.*, **57**: 31-35, 1979.
43. Robertson, DH.L., Beynon, R.J. and Evershed, R.P. Extraction, characterization, and binding analysis of two pheromonally active ligands associated with the major urinary proteins of the house mouse *Mus musculus*. *J. Chem. Ecol.*, **19**: 1405-1414, 1993.
44. Ryba, N.J.P. and Tirindelli, R. A new multigene family of putative pheromone receptors. *Neuron*, **19**: 371-379, 1997.
45. Sansom, C.E., North, A.C.T. and Sawyer, L. Structure analysis and classification of lipocalins and related proteins using a profile-search method. *Biochim. Biophys. Acta*, **1208**: 247-255, 1994.
46. Scott, J.W. and Pfaff, D.W. Behavioral and electrophysiological responses of female mice to male urine odors. *Physiol. Behav.*, **5**: 407-411, 1970.
47. Tirindelli, R., Mucignat-Caretta, C. and Ryba, N.J.P. Molecular aspects of pheromonal communication via the vomeronasal organ of mammals. *Trends Neurosci.*, **21**: 482-486, 1998.
48. Vanderbergh, J.G. Male odor accelerates female sexual maturation in mice. *Endocrinol.*, **84**: 658-660, 1969.
49. Vandenbergh, J.G., Whitsett, J.M. and Lombardi, J.R. Partial isolation of a pheromone accelerating puberty in female mice. *J. Reprod. Fertil.*, **43**: 515-523, 1975.
50. Van der Lee, S. and Boot, L.M. Spontaneous pseudopregnancy in mice. *Acta Physiol. Pharmacol. Neerl.*, **4**: 442-444, 1955.
51. Whitten, W.K. Modification of the oestrus cycle of the mouse by external stimuli associated with the male. *J. Endocrinol.*, **13**: 399-403, 1956.
52. Wu, Y.M., Ryba, N.J.P. and Tirindelli, R. Evidence for different chemosensory signal transduction pathways in olfactory and vomeronasal neurons. *Biochem. Biophys. Res. Commun.*, **220**: 900-904, 1996.