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Reactions of female bank voles *Clethrionomys glareolus* to male chemosignals

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The behavioural reactions of 110 female bank voles *Clethrionomys glareolus* (Schreber, 1780) to male chemosignals were studied in a 10 min two-choice preference test. Females spent more time investigating odours from the anogenital region of intact males and these contacts were much more frequent than in the case of castrated male. Male urine and homogenate of the salivary glands, kidney and preputial glands were very attractive to females. The extract of the liver, testes and seminal vesicles and coagulating glands had no effect on female behaviour. These findings indicate that the male chemosignals which attract females have a multiple source.

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Introduction

The reproductive physiology and behaviour of the bank vole *Clethrionomys glareolus* (Schreber, 1780) are highly dependent upon olfactory stimulation (Kruczek and Marchlewska-Koj 1986, Kruczek *et al.* 1989, Marchlewska-Koj *et al.* 1989). Chemical cues emitted from conspecifics are important in the recognition of kin, in signalling individual identity, reproductive condition, sex, and status (Doty 1986).

In mice *Mus musculus* these chemosignals are present in the male urine and their production is under androgen control (Bronson and Whitten 1968, Vandenbergh 1983). Despite extensive studies on mice during the last few years, neither the origin of the chemosignals nor the point in the urogenital tract at which active material enters the urine are known.

The present investigations were undertaken to study the behavioural reactions of female bank voles to putative male chemosignals. Since the synthesis of male mouse chemosignals is controlled by testosterone (Bronson and Whitten 1968), bank vole male tissues abundant in testosterone-binding receptors were tested for their ability to attract females.

Material and methods

Animals

The bank voles used in the experiments came from an outbred stock colony reared in the Mammalian Reproduction Group laboratory, the Institute of Zoology of the Jagiellonian University. The animals were kept in metal cages (40 × 20 × 25 cm) at 21 ± 2°C on a photoperiod schedule of 14 h light : 10 h dark. Rabbit chow (Motycz, Poland) and tap water were provided *ad libitum*. The sawdust used as bedding material was changed once a week.

The animals were reared in sexually mixed groups of 3–6 young until weaning at the age of 21 days. The young were then separated by gender and placed in groups of five. At the age of 2 months the females were reared one to a cage for at least 2 weeks before the test. At the time of testing they were from 3–4 months old.

Totally, 110 females were tested and 255 males were used. Twenty nine males were left undisturbed and used in experiments as intact males, 5 intact males were used as urine donors, 30 intact males were killed to collect different tissues, 191 males were castrated.

The males were surgically castrated at 8 weeks of age under pentobarbitone sodium (0.1 ml/10 g body weight, Polfa, Poland) anaesthesia and left undisturbed for 3 weeks.

Tested samples

To collect the tissues, males (3–6 months old, body weight 19.9 ± 0.7 g) were killed and the following organs dissected out and weighted: submandibular salivary glands (136.7 ± 13.0 mg), preputial glands (28.0 ± 2.0 mg), coagulating glands and seminal vesicles dissected together (337.9 ± 3.6 mg), the liver (953.7 ± 50.9 mg), the pairs of testes (722.9 ± 46.6 mg) and kidneys (264.9 ± 9.9 mg). These organs were immediately homogenized with physiological saline (200 mg tissue/0.5 ml), centrifuged for 5 min at 3000 g, and the supernatants were combined from all donors and then divided into 0.5 ml aliquots and stored at −15°C until used.

For urine collection 5 males were kept singly for 24 hours in metabolic cages. The urine from donors males was combined and then divided into 0.5 ml aliquots and stored at −20°C until used. The tissues and urine samples were defrosted just before being used.

Behavioural test

The behavioural reactions (approaches, sniffs, sniff time) of females to male chemosignals were studied in a two-choice preference test. The females were transferred from their home cages into the middle section of a glass vivarium (33 × 19 × 27 cm) divided into three compartments. The floor of the vivarium was covered with clean wood shavings which were changed for each test. At the end of a 4-hour habituation period, anaesthetized with pentobarbitone sodium (Polfa, Poland) two stimulus males were placed in the two end compartments and the partitions removed.

The pairs of stimulus males were:

(1) Intact vs castrated.

(2) Intact vs castrated and treated subcutaneously with 500 μg testosterone propionate (Polfa, Poland) daily for 8 days. The last testosterone injection was 24 hr prior to behavioural tests.

(3) Castrated vs castrated plus intact males' urine or tissue samples. To investigate the presence of chemosignals in the intact male urine or in the intact male tissue, 0.5 ml of urine or 0.5 ml of homogenate of different male tissues was applied onto the anogenital region of castrated males. Other castrated males serving as controls.

Each tested female and stimulus male were used only once.

The female behaviour was taped on video camera PANASONIC M5 for 10 min beginning with her first contact with the test males. If the female did not contact the test males within 5 min that test was terminated and the data from it excluded from the analysis. This occured only once.

The number of approaches, number of sniffs, and the duration of investigative contact of the female towards the mouth or anogenital region of the male were assessed on a TV monitor.

Data were analysed by a Wilcoxon matched-pairs test for dependent samples.

Results

The number of female approaches to intact males (6.9 ± 0.7) and to castrated males (5.1 ± 0.6) did not differ (Z = 1.7, ns). As shown by the results summarized in Table 1, the total number of sniffs and time spent sniffing were significantly higher for intact males that for castrated ones. The body region (mouth or anogenital) influenced this measure. The frequency of contact and the time spent sniffing the anogenital region were higher for intact than for castrated males, but there were no differences in the number of contacts or in the duration of sniffing the mouth region.

The duration of investigative contact was a function of the region of adult intact males only (Table 1). Females spent more time investigating the anogenital region (21.9 ± 2.5) than the mouth (9.3 ± 2.9) (Z = 3.2, $p < 0.01$). This difference did not occur with the castrated males. On the other hand the number of sniffs directed

Table 1. The number and duration of female sniffs with the mouth and anogenital region (A/G) of 14 pairs of anaesthetized stimulus males (mean ± SE). * – $p < 0.05$, ** – $p < 0.01$, ^{ns} – not significant.

	Number of sniffs		Z	Sniff time (sec)		Z
	male	male castrated		male	male castrated	
Total	11.7 ± 1.9	6.9 ± 1.1	2.4*	31.2 ± 5.0	12.3 ± 2.4	3.1**
Mouth	5.8 ± 1.3	3.4 ± 0.8	1.9^{ns}	9.3 ± 2.9	5.6 ± 1.3	0.9^{ns}
A/G	5.9 ± 0.8	3.6 ± 0.6	2.5*	21.9 ± 2.5	6.8 ± 1.6	3.2**
Z	0.2^{ns}	0.0^{ns}		3.2**	0.9^{ns}	

Table 2. The number of female approaches towards anaesthetized stimulus males (mean ± SE).

Tested samples	Tested pairs	Approaches to castrated males		Z	p
	n	without samples	with samples		
Urine	12	4.5 ± 0.7	6.3 ± 0.8	2.8	< 0.01
Kidneys	11	7.2 ± 1.5	9.1 ± 2.1	1.7	ns
Liver	12	5.5 ± 0.8	5.9 ± 1.3	0.7	ns
Salivary glands	11	4.1 ± 0.8	4.7 ± 0.8	1.3	ns
Testes	12	7.8 ± 1.5	7.0 ± 1.1	1.4	ns
Seminal vesicles + coagulating glands	11	8.2 ± 1.0	8.3 ± 0.7	0.2	ns
Preputial glands	12	5.8 ± 1.1	5.7 ± 1.3	0.2	ns

towards the mouth and anogenital region were almost the same for adult intact males (5.8 ± 1.3 and 5.9 ± 0.8, respectively) as for castrated ones (3.4 ± 0.8 and 3.6 ± 0.6, respectively).

In contrast, females (n = 15) did not discriminate between adult intact males and castrated but testosterone treated males. The number of female approaches towards intact males and castrated but testosterone treated males did not differ (7.7 ± 1.1 and 7.6 ± 1.2 respectively, Z = 0.4, ns). There were also no significant differences in the number of female sniffs towards intact males (13.5 ± 1.5) and castrated but testosterone treated males (11.7 ± 1.5) (Z = 1.1, ns) as in the sniffing time (37.7 ± 5.1 and 35.0 ± 5.7 respectively, Z = 0.7, ns).

There were no differences in the number of approaches towards anaesthetized stimulus males in any of the tested groups except for the one in which the male urine was applied onto the castrated males (Table 2). The results presented in Table 3 show that the frequency of female sniffs and time of investigation were

Table 3. Investigative responses of females exhibited towards anogenital region of anaesthetized stimulus males – castrated smeared with male urine or homogenates of male tissue and castrated (control) (mean ± SE).

Tested samples	Tested pairs (n)	Sniffs (n)	Sniff time (sec)
Urine	12	11.2 ± 1.6	70.7 ± 17.7
Control		3.3 ± 0.6	9.0 ± 2.2
Z		3.1, $p < 0.01$	3.1, $p < 0.01$
Kidneys	11	12.5 ± 2.1	44.8 ± 7.7
Control		4.7 ± 0.8	10.4 ± 2.0
Z		2.9, $p < 0.01$	2.8, $p < 0.01$
Liver	12	7.6 ± 1.0	23.4 ± 6.6
Control		5.5 ± 0.7	16.5 ± 3.1
Z		1.6, ns	0.5, ns
Salivary glands	11	7.9 ± 1.5	45.4 ± 15.9
Control		2.6 ± 0.7	4.9 ± 1.7
Z		2.6, $p < 0.01$	2.8, $p < 0.01$
Testes	12	7.4 ± 0.9	16.0 ± 2.8
Control		6.7 ± 1.0	15.6 ± 3.6
Z		0.9, ns	0.7, ns
Seminal vesicles + coagulating glands	11	7.4 ± 0.7	23.2 ± 4.5
Control		7.4 ± 0.8	24.0 ± 5.3
Z		0.1, ns	0.2, ns
Preputial glands	12	7.2 ± 0.9	18.4 ± 4.7
Control		4.2 ± 0.7	7.4 ± 1.9
Z		2.1, $p < 0.05$	2.3, $p < 0.05$

higher in tests with male urine, kidney, salivary glands and preputial glands homogenates than for the control castrated males. Apart from this, other tested organs, i.e. liver, testes, and seminal vesicles and coagulating glands produced no effect on female interest (Table 3).

Discussion

Bank vole females were capable of discriminating the odours of intact and castrated males, the presence or intensity of these odours being testosterone dependent. Females investigated the anogenital region of anaesthetized adult intact males far more frequently and these contacts lasted longer than in the case of a castrated male. Similarly, in the meadow vole the female-attractant cue in male odours is reduced after gonadectomy but is reinstated by testosterone treatment (Ferkin *et al.* 1992). Gonadectomy in other rodent species also reduces the attractiveness of odours to the opposite sex and the extent of olfactory investigation, while replacement with gonadal hormones restores such behaviour (Scott and Pfaff 1970, Johnston 1990, Miernicki *et al.* 1990).

The pattern of female investigative behaviour observed towards intact adult males suggested that mouth area and anogenital odours produce very different scents. It has been suggested that in *Microtus ochrogaster* chemostimuli from the mouth and anogenital regions both appear to contain information about social identity (Smale *et al.* 1990). The mouth odours can influence a vole's decision to either cease or continue investigating other odour sources. Moreover the mouth region of the intact bank vole male tended to be more attractive to the female than that of castrated male, though the differences were not significant (Table 1). Consequently , the odour of the extract of male salivary glands evoked the female's interest for this scent (Table 3). There are a lot of evidence that salivary odours may play an important role in rodent communication. Female prairie voles *M. ochrogaster* are able to differentiate between non-sibling and sibling samples of saliva (Smale *et al.* 1990). Also adult male and female Mongolian gerbils *Meriones unguilatus* are preferentially attracted to saliva from non-siblings of the opposite sex (Smith and Block 1989, 1991). In mice *Mus musculus* the homogenate of male salivary glands induces oestrus in females (Marchlewska-Koj *et al.* 1990).

The increased number of sniffs and sniffing time showed by bank vole females towards the odour of male urine, extract of kidney and preputial glands might reflect the female's interest to male chemosignals which can contain biologically relevant information. A similar preference for male urine has been found in female mice *M. musculus* (Ninomiya and Kimura 1988). Moreover, bank vole females can distinguish between dominant and subordinant samples of male urine (Hoffmeyer 1982). There is some evidence that pheromonal activity in the mouse male urine is bound with a protein fraction (Vandenbergh *et al.* 1975). The major urinary protein fraction has been also described in the bank vole males urine (Kruczek and Marchlewska-Koj 1985).

The results presented above indicate that the increase in female investigation was stimulated by the odour of male kidney homogenate. Clulow and Baddaloo (1987) also found that the sexual maturation of meadow vole females can be accelerated in response to kidney and bladder homogenates.

The preputial gland is believed to play an important role in intraspecific chemical communication in a number of rodent species (Brown and Williams 1972). In mice *M. musculus* the preputial gland contains a biologically active substances that accelerate oestrus (Marchlewska-Koj *et al.* 1990) and is also a source of sex attractant (Bronson and Caroom 1971). Because in the bank vole the preputial glands are larger in dominant than in subordinant males (Christiansen *et al.* 1978, Gustaffson *et al.* 1980) it may be suggested that, at least in this species, the preputial glands participate in signalling the male social status. The results obtained in the experiments described above indicated that chemosignals that attract bank vole females are also present in the preputial glands.

All these findings suggest that the chemical(-s) enters the urine flow at the kidney and, as was suggested for house mice (Bronson 1976), the secretions of preputial glands are added to the urine flow. While the present author's findings do not permit identification of the source of the chemical cues, the activity of the kidney, preputial, and salivary glands suggests that these organs should be studied as candidate sites.

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