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RESEARCH

Sexual discrimination and attraction through scents in the water vole, *Arvicola terrestris*

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Abstract

In mammals, especially rodents, social behaviours, such as parenting, territoriality or mate attraction, are largely based on olfactory communication through chemosignals. These behaviours are mediated by species-specific chemosignals, including small organic molecules and proteins that are secreted in the urine or in various fluids from exocrine glands. Chemosignal detection is mainly ensured by olfactory neurons in two specific sensory organs, the vomeronasal organ (VNO) and the main olfactory epithelium (MOE). This study aimed to characterise the olfactory communication in the fossorial ecotype of the water voles, *Arvicola terrestris*. We first measured the olfactory investigation of urine and lateral scent gland secretions from conspecifics. Our results showed that water voles can discriminate the sex of conspecifics based on the smell of urine, and that urinary male odour is attractive for female voles. Then, we demonstrated the ability of the VNO and MOE to detect volatile organic compounds (VOCs) found in water vole secretions using live-cell calcium imaging in dissociated cells. Finally, we evaluated the attractiveness of two mixtures of VOCs from urine or lateral scent glands in the field during a cyclical outbreak of vole populations.

Keywords Olfactory behaviour · Volatile organic compounds (VOC) · Calcium imaging · Vomeronasal organ · Main olfactory epithelium

Introduction

In rodents, olfaction is largely involved in social communication between conspecifics (Wyatt 2014). This olfactory communication occurs via chemosignals of diverse chemical nature, including small volatile organic compounds (VOCs), steroids, peptides and proteins such as lipocalins (Novotny et al. 1985; Leinders-Zufall et al. 2004; Kimoto et al. 2005; Chamero et al. 2007; Liberles 2014). These chemosignals are usually secreted in urine, saliva, tears or fluids from exocrine glands (Johnston 2003). Chemosignals are mainly detected by olfactory neurons in two olfactory sensory organs, the main olfactory epithelium (MOE) and the vomeronasal organ (VNO) (Margolis 1980; Mombaerts et al. 1996). The detection of chemosignals induces specific socio-sexual behaviours, such as aggression towards an intruder, maternal care, sexual discrimination and attraction (Tirindelli et al. 2009; Trouillet et al. 2019, 2021).

For example, male mouse urine contains two VOCs, 2-sec-butyl-dihydrothiazol and 3,4-dehydro-exo-brevicomin, which elicit different behaviours including aggression or sexual attraction, depending on the sex of the receiver (Novotny et al. 1985; Jemiolo et al. 1991). These behaviours can also be triggered by protein chemosignals present in male urine. Indeed, some major urinary proteins (MUPs), such as darcin, promote male–male aggression, and mediate

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sexual attraction of females (Chamero et al. 2007; Roberts et al. 2010; Kaur et al. 2014; Dey et al. 2015). MUPs can act independently, but they also bind volatile compounds to release them gradually and to increase their diffusion (Hurst et al. 1998). Whether similar olfactory communication mechanisms also exist in water voles is an issue poorly explored.

Water voles are small semi-aquatic rodents widespread in Eurasia. They have androgen-dependent sebaceous glands on their flanks, the lateral scent glands (LSG), similar to what has been described in the hamster (Vandenbergh 1973; Lucky et al. 1986). Those lateral glands are thought to be involved, together with urine, in individual recognition and mate attraction. (Stoddart 1972; Frost et al. 1973; Lai et al. 1996; DelBarco-Trillo et al. 2009; Liu et al. 2011). In water voles, LSG show seasonal changes in their morphology and sebaceous activity (Stoddart 1972; Poissenot et al. 2021a, b) and may be involved in territoriality and mate attraction (Stoddart et al. 1975; Saucy 1988). LSG may represent a major olfactory source in addition to urine. In a previous study on the fossorial ecotype of the water vole, we identified VOCs released in urine and LSG (Nagnan-Le Meillour et al. 2019), mainly found in males. How these VOCs are involved in mate attraction and discrimination in water voles is currently unknown.

In this context, we studied olfactory communication in the fossorial water vole. To this end, we first used two-choice olfactory tests to measure the olfactory investigation of urine and secretions from the lateral olfactory glands of conspecifics and assess the vole sexual discrimination on the basis of these secretions and whether they could represent an attractive signal. Then, we explored the anatomy of the MOE and VNO in this species and analysed the response of MOE and VNO dissociated cells to chemostimulation with specific VOCs found in urine and LSG secretions from males. Finally, we tested the on-field attractiveness of some of these VOCs using a trapping approach.

Materials and methods

Species and trapping

The fossorial ecotype of water vole (*Arvicola terrestris*/*Arvicola amphibius*, Linnaeus, 1758) belongs to the *Cricetidae* family. Voles were caught in fields with traps (Topcat®, Andermatt, France) placed into their galleries. Captures were performed during the day and the traps were inspected every ~ 15 min. Captures were carried out on permanent meadows on Valbeleix (45° 28′ 17″ N, 3° 0′ 26″ E, alt. 1130 m, department of Puy-de-Dôme, France) and Charmensac (45° 13′ 28″ N, 3° 4′ 6″ E, alt. 1050 m, department of Cantal, France). Animals were then used either directly on the field or transferred to housing facilities for laboratory tests.

Behavioural tests

Adult males and females water voles were tested for olfactory investigation of urine or LSG secretions from conspecifics in olfactory two-choice tests. Urine was collected from adult males and females trapped in the field during the breeding period and stored at − 80 °C until use. LSG were freshly dissected on trapped animals just before the behavioural test session. The behaviour expressed by animals was video recorded and analysed with Behavioural Observation Research Interactive Software (BORIS, University of Torino). The duration of olfactory investigation was defined by sniffing movement near to the urine or LSG samples.

Investigation of urine of conspecifics in animal facilities

Twenty-two males and 21 females water voles were used for behavioural tests in an animal facility. Once captured, voles were housed individually at a controlled temperature of 20 °C under 12 h light/dark cycle with food and water *ad libitum* at least for 2 weeks before being tested. Tests were performed in the home cage of each animal (43 (L) × 27 (W) × 16 (H) cm). The metal grid was removed and replaced by a plexiglass pierced plate. After 2 min of habituation, 10 µl of odour solutions was pipetted on filter papers that were presented simultaneously on the two opposite sides of a pierced plate, to prevent direct contact with the olfactory source. The duration of olfactory investigation was recorded for 10 min. Sex discrimination was assessed using urine pooled from 4 females and from 6 males. Urine attractiveness or repulsiveness was assessed in the two-choice test by presenting urine pooled from either females or males next to a neutral stimulus (water). The voles were tested once per day over three consecutive days in random order for each animal.

Investigation of urine or lateral scent gland secretion of conspecifics in the field

In the field, 7 males and 9 females water voles were tested for olfactory investigation of conspecifics urine, and 8 males and 12 females were tested for olfactory investigation of LSG secretions. Chemostimuli from males or females were simultaneously placed in the arm's ends of a Y-maze (arms: 35 (L) x 9 (W) × 15 (H) cm) on filter papers behind a perforated wall to prevent direct contact with the olfactory source. Urine was deposited on a filter paper as previously described and LSG secretions were deposited by rubbing glands from at least two animals for each sex. The time of olfactory investigation was recorded for 5 min after 2 min of habituation to the maze. Before being tested, animals were placed

in a box containing soil during 20 to 30 min to recover from the trapping stress.

VNO and MOE anatomy: histological staining and immunolabeling

Male and female water voles were anesthetized with isoflurane and then euthanized by cervical dislocation. The entire snouts were collected and placed into 4% paraformaldehyde (PFA) in phosphate buffer (PB) for 48 h, and then into decalcification solution consisting on 0.045 mM EDTA in PB buffer for 8 weeks at 4 °C. The decalcified snouts were dehydrated in successive baths of 50%, 70%, and 90% ethanol, followed by three baths of absolute ethanol for 30 min each. After one night at 4 °C and 2 h at Room Temperature (RT) in butanol, snouts were impregnated in paraffin twice at 60 °C before final embedding. Once dried, the samples were sliced into 10 µm sections and mounted on gelatine-coated slides. Before staining or immunolabeling, sections were deparaffined in xylene, two baths, 5 min each. Then, sections were rehydrated with successive baths of absolute, 90%, 70% ethanol, and a final bath of water, twice for 5 min each.

For histological staining, sections were placed in hematoxylin for 30 s and cleared under tap water. After a quick bath in 70% ethanol, sections were stained again in eosin for 5 s. Finally, sections were dehydrated and cover slipped in DPX (Sigma) mounting medium.

For immunolabeling, sections were washed three times for 5 min in Tris buffer saline (TBS), and then incubated for 2 h at RT in TBS containing 2% normal donkey serum with 0.3% Triton X-100 to block non-specific binding. Sections were then incubated overnight at 4 °C in TBS, 0,1% Triton, 2% normal donkey serum (TBS-T-NDS) with a goat-anti-OMP antibody (1:2000, Wako #019-22291 (RRID: AB_664696)). After three washes of 5 min with TBS, cells were incubated for 2 h at RT in TBS-T-NDS with a Cy3 donkey anti-goat antibody (1:500, Jackson ImmunoResearch (RRID: AB_2307351)). Sections were washed three times with TBS and incubated for 5 min with Hoechst (1:5000, ThermoFisher) and washed before being mounted in Fluoromount-G (Invitrogen).

Calcium imaging

Cell preparation for calcium imaging

Eight male voles were used for Ca^{2+} imaging experiments. Each Ca^{2+} imaging experiment was performed on pools of VNO or MOE from two animals. Cell preparation was performed as previously described (Trouillet et al. 2019). Animals were euthanized with CO_2 and the MOE and VNO were collected. The vomer bone and the cartilage surrounding the VNO were manually removed under a binocular microscope. VNOs or MOEs were minced in PBS solution at 4 °C. Then, the tissue was incubated 20 min at 37 °C in PBS solution supplemented with papain (0.22 U/ml, Worthington biochemical corp.), 1.1 mM EDTA and 5.5 mM cysteine-HCl, followed by treatment with DNase I (10 U/ml, ThermoFisher). The papain was inactivated with DMEM (Invitrogen) supplemented with 10% Foetal Bovine Serum (FBS). After 5 min of centrifugation at 100 × g, the dissociated cells were plated on a coverslip previously coated overnight with concanavalin-A type IV (0.5 mg/ml, Sigma). For MOE, the same protocol as described above for VNO was used, except for the PBS solution with papain that was supplemented with 40 mM urea.

Calcium activity recording

Immediately after preparation, cells were used for calcium imaging. Cells were incubated at room temperature with fura-2/AM (5 µM, Invitrogen) in Hanks' balanced salt solution (HBSS, Invitrogen, $CaCl_2$ 1.26 mM, ($MgCl_2$–$6H_2O$ 0.407 mM, $MgSO_4$–$7H_2O$ 5.33 mM, KCl 5.33, KH_2PO_4 0.441 mM, NaCl 137.93 mM, Na_2HPO_4 0.338 mM, and D-Glucose 5.56 mM) supplemented with 10 mM hydroxyethyl-piperazine ethane–sulfonic acid buffer (HEPES, Sigma) for at least 30 min. Coverslips containing VNO or MOE cells were placed in a laminar-flow chamber (Warner Instruments) and constantly perfused at 22 °C with HEPES-HBSS solution. Cells were alternately illuminated at 340 and 380 nm, and light emitted above 510 nm was collected by using a Hamamatsu C10600-10B camera installed on an Olympus IX71 microscope. Images were acquired at 0.25 Hz and analysed by using ImageJ (NIH), including background subtraction, ROI detection, and signal analyses. ROIs were selected manually and always included the whole cell body. The image ratio/fluorescent values were analysed with the R package calipR (developed in our lab) followed by automatic validation of response curves. Briefly, the software removes low quality cells with missing or 0 values more than 10% of the time. Then, it estimates and corrects noise background, defined as the long-term baseline fluorescence fluctuations during resting conditions (non-stimulation). This step is implemented through a Generalized Additive Model (GAM) fit adapted from previously described method (Balkenius et al. 2015). Next, a standard normalisation step is performed by converting the denoised fluorescence traces to z scores, using the baseline mean fluorescence as a reference. Peaks of interests of at least 3 standard deviations of the baseline are then identified by deconvolution (Jewell and Witten 2018) coupled with thresholding.

Chemostimulation

Chemostimuli used for Ca^{2+} imaging were VOCs mainly found in urine and LSG of male water voles (Nagnan-Le Meillour et al. 2019). For each Ca^{2+} imaging experiment, chemostimuli were freshly prepared from a stock solution (1 M, in DMSO stored at − 20 °C) and diluted in extracellular HEPES–HBSS buffer to a final concentration of 1 µM. The urinary VOCs are 3-methyl-1-nitrobutane (Enamine), trimethyl-pyrazine (Sigma) and 2,5-dimethyl,3-ethyl-pyrazine (Sigma). The VOCs from LSG are isovaleric acid (Sigma), undecylenic aldehyde (Sigma) and dec-2-enal (Sigma). Urine of male water voles was diluted to 1:100 in HEPES–HBSS buffer. Chemostimuli were individually tested in two stimulation paradigms consisting on sequential application of: (1) isovaleric acid, trimethyl-pyrazine, 2,5-dimethyl,3-ethyl-pyrazine and male urine and (2) dec-2-enal, undecylenic aldehyde, 3-methyl-1-nitrobutane and male urine. The stimulation by each chemostimulus lasted 30 s and was 3 min apart. To avoid counting nonspecific responses, cells that responded to control stimulation with HEPES–HBSS buffer were excluded from the analysis (0.1–1% of all cells analysed). The total number of dissociated cells analysed was 4007 from VNO and 2153 from MOE in stim paradigm 1 and 5005 from VNO and 2037 from MOE in paradigm 2. A solution containing KCl 50 mM was used as a positive control of viable excitable cells. Responses to urine and synthetic odors were analysed on these KCl-activated cells only.

Field capture test with mixtures of volatile compounds sprayed on the traps

Two mixtures of VOCs diluted at 1 µM in mineral oil (also used as control, Sigma) were used: a urinary VOCs mixture composed of 1-nitro-3-methylbutane, trimethyl-pyrazine and 2,5-dimethyl,3-ethyl-pyrazine, and an LSG VOCs mixture composed of isovaleric acid, undecylenic aldehyde and dec-2-enal. For each colony of water voles, three traps with the two different VOCs mixtures or control were distributed in the gallery around a mound corresponding to a nest. Each trap was separated at least 1.5 m. Mixtures of VOCs or control were vaporized into the trap just before being placed into the gallery. Capture tests were performed in three sessions of 1.5 days during the breeding season in 2021 and in 2022 on a total of 108 water vole colonies. Traps were re-sprayed after each capture or after 2 h without capture.

Statistics

GraphPad Prism 9.1.2 (GraphPad Software Inc, San Diego, California, USA) was used to perform statistical analyses and plots. Normality of data distribution was verified with the Shapiro–Wilk test. According to the normality, data of two-choice olfactory test were analysed either with paired Student t-test or Wilcoxon test. Field capture test was analysed using Chi^2 for comparison of the observed values to the expected values.

Results

Olfactory investigation of conspecific secretions

We assessed the sex discrimination capability of water voles based on urine and LSG secretions using two-choice olfactory tests carried out in laboratory and in the field (Fig. 1a and c). Male and female water voles spent significantly more time investigating male urine than female urine in the two-choice olfactory test performed under laboratory conditions (Fig. 1b, Wilcoxon test, $p = 0.015$ for males and $p = 0.001$ for females). A similar result was obtained when the tests were performed in the field using Y-maze (Fig. 1c and d, Student t-test, $p = 0.045$ for males; Wilcoxon test, $p = 0.039$ for females). We also tested males and females under field conditions using a Y-maze for preference of the secretion of LSG of conspecifics. However, statistical analysis did not reveal any differences in olfactory investigation times between LSG secretion of males and those of females in both sexes (Fig. 1e; Wilcoxon test, $p = 0.109$ for male, $p = 0.850$ for females).

We also assessed the attractiveness of conspecific urine compared with water as a neutral odour using the two-choice olfactory tests in the laboratory (Fig. 2a and b). Male voles did not spend significantly more time investigating the male or female urine of conspecifics when compared with water (Fig. 2a). By contrast, female voles spent significantly more time investigating urine of male conspecifics (Fig. 2b), Wilcoxon test, $p = 0.015$), but not female urine.

Morphological organisation of the VNO and MOE in water vole

The MOE and the VNO of water voles show a similar morphological organisation to the one observed in other rodents, such as mice and rats. Indeed, the VNO is located above the palate in nasal cavity between the upper incisors and the nasal septum. The MOE lines the roof and the caudal part of the nasal cavity on turbinates (Fig. 3a). An additional ethmoturbinate compared to what is described in mice and rats is present in water voles (Supplementary figure). The MOE is organised in a pseudostratified epithelium (Fig. 3b, c, d) containing basal cells, olfactory sensory neurons and supporting cells. The basal cells separate the sensory epithelium from the lamina propria where blood vessels and olfactory nerve bundles are located. The vomeronasal cartilage encapsulates

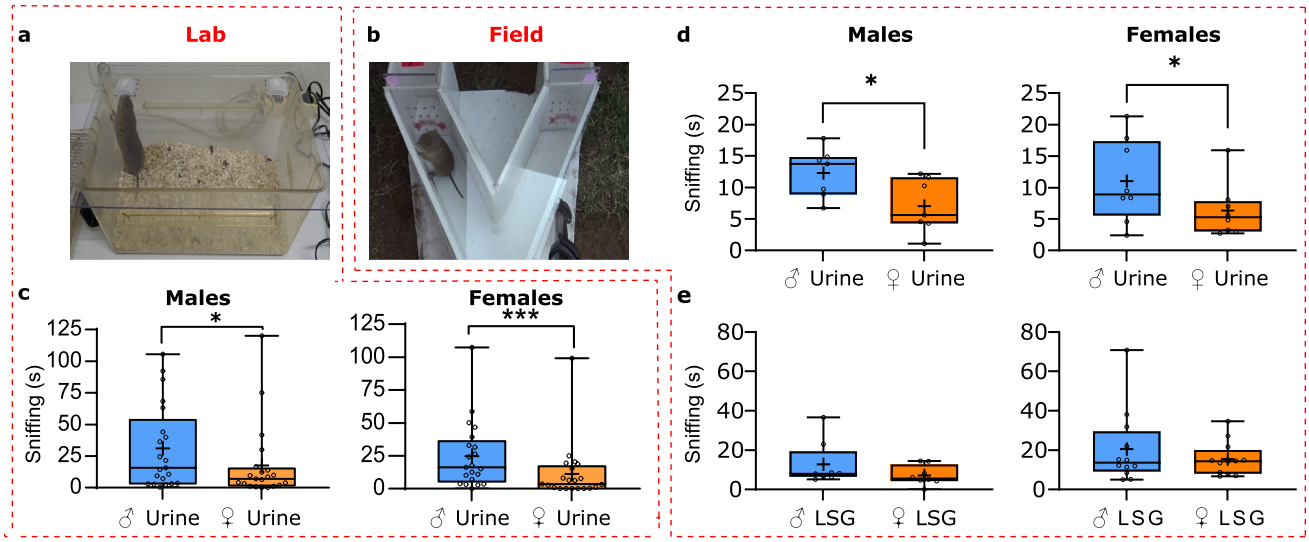

Fig. 1 Sex discrimination capability of water voles by olfactory investigation in a two-choice tests. **a**, **b** Voles were tested in their own cage in the animal facility and using a Y-maze in the field. Males and females were tested for their interest for, **c** male and female urine in animal facility, and **d** in the field. **e** time of investigation for male and female secretions of lateral scent glands in the field. Results are shown as boxplot with each individual represented by circles and means indicated by a cross. *p<0,05; ***p<0,005

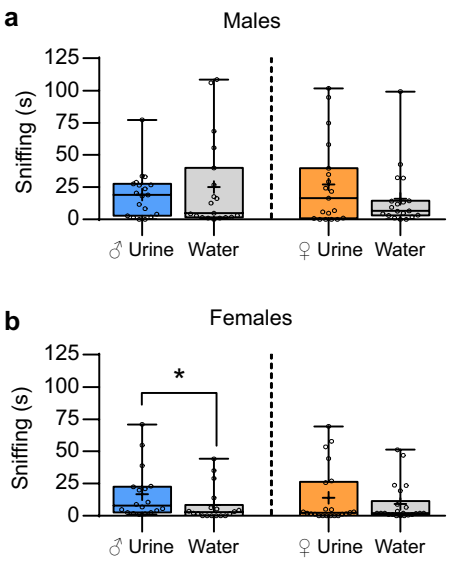

Fig. 2 Evaluation of the attractiveness of the urine of conspecifics compared with a neutral stimulus (water) in olfactory two-choice test. **a** male and **b** female water voles were tested for the time spend to investigate urine of male or female conspecifics compared to water. Results are shown as boxplot with each individual represented by circles and means indicated by a cross. *p<0,05

the parenchyma which contains the vomeronasal sensory epithelium (VSE) arranged in a crescent shape around the lumen (Fig. 3e and f). The nonsensory epithelium resides at the opposite side of the lumen, next to a large blood vessel. In the MOE and the VSE, the presence of sensory neurons is evidenced by the expression of OMP (Fig. 3c and f). Sensory neurons project their dendrites into the lumen through the supporting cell layer (Fig. 3d and g).

Responses of dissociated cells from VNO and MOE to chemostimulation with VOCs found in urine and LSG secretions

Next, we collected VNOs and MOEs from water voles and performed calcium imaging in dissociated cells to evaluate their responses to chemostimulation with six VOCs previously found in urine and LSG secretions (Nagnan-Le Meillour et al. 2019). We validated the presence of sensory neurons among the dissociated cells by immunolabeling against OMP (Fig. 4a). On average, 7,7% and 25,7% of VNO and MOE cells, respectively, were activated by high potassium (Fig. 4b and c), indicating that viable excitable cells, likely sensory neurons, are present in our cell preparation. Of these high potassium-activated cells 29.5% in VNO and 11,1% in MOE also responded to urine stimulation (Fig. 4b and c). We also observed robust responses for all the six volatile compounds tested (Fig. 4c and d) of 41–90% of VNO and 49–96% of the MOE cells when normalised to male urine responses. Among single VOCs, stimulation with isovaleric acid induced the greatest number of responses in VNO and MOE cells (> 90% of urine responses). In the VNO, a large number of responses was also observed with the two pyrazines (75,8% for DMP and 79,3% for TMP of urine responses) (Fig. 4d). Together, these results indicate that vole VNO and MOE cells can be activated by urine and synthetic compounds present in urine.

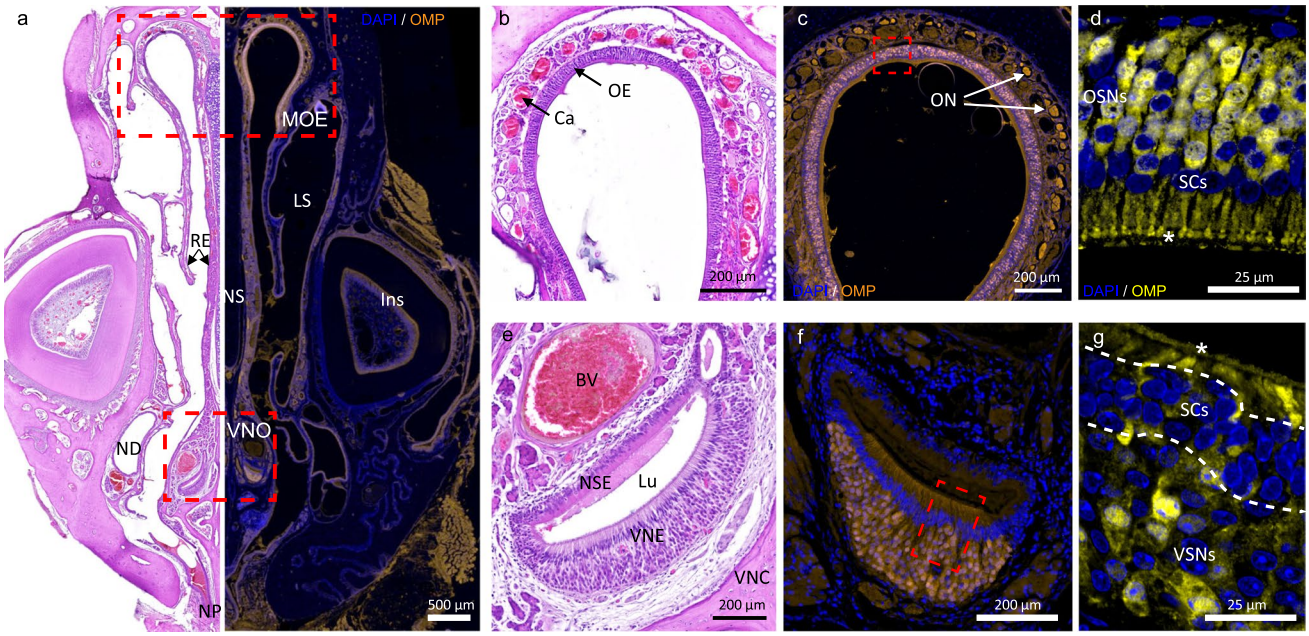

Fig. 3 Histological sections of the water vole snout. **a** Coronal sections of half snout stained with hematoxylin and eosin (left) or labelled for the olfactory marker protein (OMP, in orange/yellow, right) showing the location of the main olfactory epithelium (MOE) and the vomeronasal organ (VNO). *NS* nasal septum, *NP* nasal palate, *RE* respiratory epithelium, *ND* nasolacrimal duct,*Ins* incisor teeth, *LS* lateral septum. **b**, **c** Magnification of MOE. *OE* olfactory epithelium, *Ca* cavernous tissues, *ON* Olfactory nerves. **d** Image showing the olfactory sensory neurons (OSNs) labelled with the OMP antibody and the non-labelled supporting cells (SCs) and basal cells (BCs) in the MOE. Asterisk (*) indicate the dendritic knob layer. **e**, **f** Magnification of the VNO.*VNE* vomeronasal sensory epithelium, *Lu* lumen, *NSE* nonsensory epithelium, *BV* Blood vessels, *VNC* vomeronasal cartilage. **g** Image showing the vomeronasal neurons (VSNs) labelled with the OMP antibody in the sensory epithelium and the non-labelled SCs in the VNO

Trapping test with mixtures of volatile compounds

The attractiveness of the VOCs tested in calcium imaging experiments was evaluated in the field (Fig. 5a and b) using two mixtures: one with the volatiles identified in urine and a second mixture with the volatiles identified in LSG secretions. A total of 100 male and female voles were trapped in 68 of the 108 colonies tested. Thirty-four animals were caught in the traps sprayed with the neutral odour (34%, mineral oil), 25 with the LSG compounds mixture (25%) and 41 with the urinary mixture (41%) (Fig. 5c). In males, the number of individuals trapped in urinary mixture (15) and control (14) was similar, and slightly lower with LSG mixture (11). A higher number of females (26) were trapped using the urinary mixture rather than with mineral oil (20), while the number of females trapped with LSG mixture (14) was lower. However, no statistically significant differences in the number of females caught were found (Fig. 5c, chi-square test, $p = 0.1653$). Even though the urinary mixture might seem to be more attractive for females (26 [63%] females vs. 15 [37%] males trapped), more research is needed to confirm these results.

Discussion

In this study, we first assessed sexual discrimination ability of fossorial water voles based on conspecific urine and LSG secretions using olfactory tests without direct contact to the odorant sources. Male and female water voles sniffed the urine and LSG secretions of both sexes. When male water voles were exposed to male and female urine simultaneously, they spent significantly more time investigating male urine than female urine. Similarly, female voles also investigated significantly more male urine than female urine. These results indicate that water voles can distinguish male and female urine by olfactory investigation. This behaviour was consistently displayed under both laboratory and field conditions.

The preference of males for male urine was somehow unexpected because male mice show a strong preference for female urine over male urine (Nyby et al. 1985; Keller et al. 2009). In addition, male voles showed no preference for male urine when female urine was replaced by neutral odour, indicating that female urine is not repulsive to males. In contrast to the mouse, monogamy is the main mating system reported in the fossorial water vole that lives in pairs in their

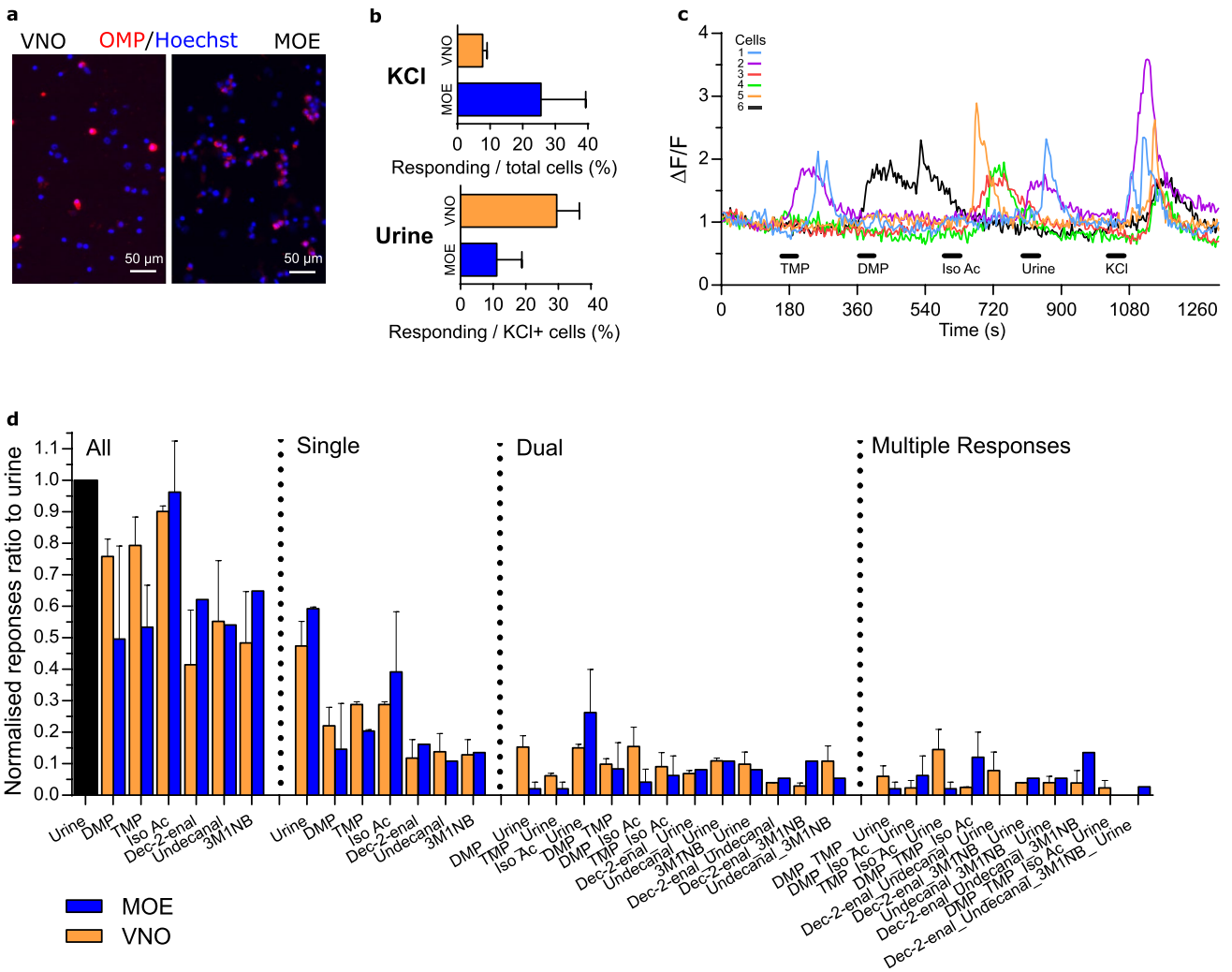

Fig. 4 Sensory function of water vole olfactory neurons. **a** Representative images of immunochemistry against the olfactory marker protein (OMP) in dissociated cells from VNO and main olfactory epithelium (MOE) in the coverslip used for Ca^{2+} imaging. **b** Average percentage of dissociated cells responding to KCl stimulation and average percentage of KCl positive cells responding to ♂ urine. **c** Representative Ca^{2+} responses imaged in different neuronal cells from vomeronasal organ (VNO) of male water voles, F/F represent the change in fluorescence normalized to the background. **d** Ratio of responses of neuronal cells (KCl^{+}) to compounds normalised by responses to male urine as indication of the level of response triggered by the different compounds in VNO and MOE. All combined responses include responses from cells that showed a single, dual, and multiple responses to stimulations. Compounds identified in urine or LSG of water voles were tested at 1µM in two stimulation paradigms in separate experiments: 1) isovaleric acid (Iso Ac), Trimethyl-pyrazine (TMP), 2,5-dimethyl,3-ethyl-pyrazine (DMP), urine and KCl and 2) Dec-2-enal, undecylenic aldehyde (Undecanal), and 3-methyl-1- nitrobutane (3M1NB), urine and KCl. Results are expressed as mean ± S.E.M

burrow (Airoldi 1976, 1978; Somoano et al. 2017). Thus, the male vole's preference for male rather than female urine may reflect stronger aggressive or/and territorial behaviour, especially in outbreak conditions, where resources are scarce. Indeed, monogamous rodents as prairie voles exhibit strong aggression toward intruders (Winslow et al. 1993; Lee et al. 2019). In addition, we could also hypothesis that the presence of the female odor in the discrimination test could enhance the response of the male in comparison to the situation when male urine is opposed to water. Female water voles prefer to investigate male rather than female urine or neutral odour, showing that they can discriminate these odours and that male urine is attractive, which is consistent with the behaviour of other female rodents, such as hamsters, mice, prairie and meadow voles (Johnston 1979; Hurst 1990; Ferkin and Zucker 1991; Ferkin et al. 1994). Furthermore, female water voles appear to show a preference for the scent of dominant males over subordinates, as in meadow voles (Shapiro and Dewsbury 1986; Evsikov et al. 1994).

In contrast to the urine investigation, we did not find significant differences in the time spent investigating LSG secretions from male and female conspecifics. Therefore,

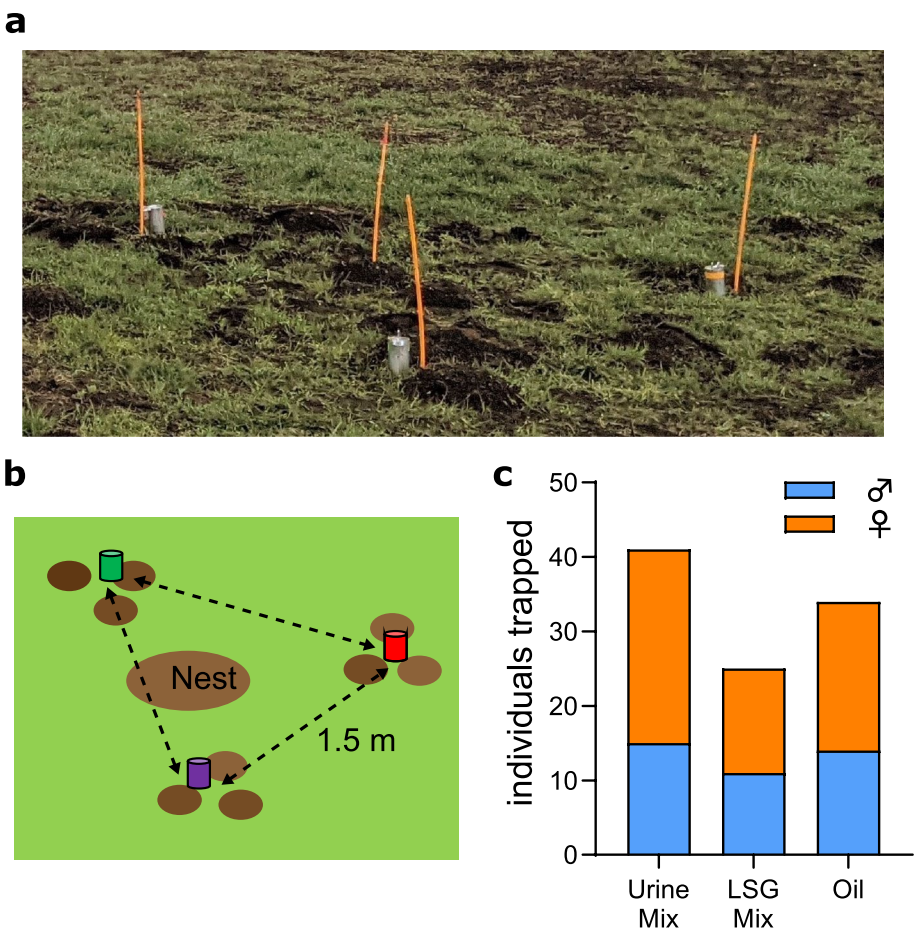

Fig. 5 Experimental setup and results of field capture tests with mixtures of urinary or lateral scent glands volatiles sprayed into the traps.**a** Representative photo of traps placed into the gallery of a colony of water voles. **b** Each trap was placed around the largest mound corresponding to the nest at least 1.5 m from the other traps. **c** Number of male and female water voles caught in the traps sprayed with the urine volatiles mixture of (3-methyl-1-nitrobutane, trimethyl-pyrazine, 2,5-dimethyl,3-ethyl-pyrazine), the lateral scent glands volatiles (isovaleric acid, undecylenic aldehyde, dec-2-enal), or with mineral oil

we cannot conclude that fossorial water voles are able to discriminate the sex from the volatile odours of LSG secretions. The lack of sex-specific variations during spring and summer in term of number of olfactory compounds identified in these secretions may explain this result (Nagnan-Le Meillour et al. 2019). However, in other rodent with similar sebaceous glands like hamster or Alston's brown mouse, animals can discriminate the sex of conspecifics from these specific glands (Johnston 2003; Fernández-Vargas et al. 2008). In our study, olfactory tests were performed without direct access to the odour source. Water vole LSG secretions are oily and contain several low-volatile long-chain fatty acid esters, that could limit VOC diffusion (Nagnan-Le Meillour et al. 2019). Therefore, sexual discrimination from sebaceous glands might require direct contact investigation to be detected. The potentially attractive or repulsive nature of these secretions from LSG remains also to be determined.

In rodents, detection of the VOCs emitted by conspecifics was mainly ensured by olfactory neurons located in VNO and MOE. An anatomical analysis of these sensory organs showed that they are similar to those observed in other rodents, with the exception of an additional MOE ethmoturbinate that is present in water voles (Barrios et al. 2014; Zancanaro 2014). OMP immunoreactivity reveals the presence of mature sensory neurons in the vomeronasal sensory epithelium and in the main olfactory epithelium (Margolis 1980). Due to the absence of species-specific antibodies, the expression of Gαi2 or Gαo proteins (markers of type 1 [V1Rs] and type 2 [V2Rs] vomeronasal receptor-expressing neurons, respectively) by vomeronasal neurons could be not characterized. While V1R genes are expressed in most of mammals, V2R genes are largely absent in most mammalian species with the exception of rodents, lagomorphs, marsupials, platypus, and lower primates (Tirindelli 2021). Based on this literature, fossorial water voles are expected to express both VR families, but this remains to be demonstrated.

The sensory function of VNO and MOE neurons was tested in calcium imaging experiments. A fraction of VNO and MOE dissociated cells were positive for OMP immunolabeling and showed calcium transients in response to a depolarizing stimulus (high K^+), indicating that viable sensory neurons were present in the cell preparation. Among these high K^+-activated cells, both VNO and MOE cells showed responses to chemostimulation with male urine, but also to single VOCs identified in urine and LSG secretions of conspecifics (Nagnan-Le Meillour et al.

2019). The proportion of cells responding to these different chemostimulations was similar to what is observed in mice in response to other chemosignals (Chamero et al. 2007; Trouillet et al. 2019). Unexpectedly, the proportion of cells activated by single compounds (i.e. isovaleric acid) was nearly as high as those activated by whole urine, which consist of a complex mixture of many hundreds of molecules (Fig. 4d). The reason for this high sensitivity to the synthetic compounds is unknown, but the fact that these molecules were used at a relatively high concentration (1 µM) is likely playing an important role.

We next wanted to verify whether the compounds tested in the cell imaging preparation could elicit a behavioural response in vivo. Field trapping tests were performed to evaluate the attractiveness of urinary and LSG VOCs mixtures. The LSG VOCs mixture did not improve vole trapping. Similarly, no significant statistic difference was found between the number of voles trapped with urinary VOCs mixture and mineral oil. However, the number of females trapped with the urinary VOCs mixture mainly found in males (Nagnan-Le Meillour et al. 2019) was increased by 30% compared to mineral oil. This observation is consistent with the observed female vole preference for male urine, although more tests are needed to establish the attractiveness of the urinary VOCs mixture in the field. Thus, the use of VOCs may represent a new sustainable method of controlling the population control of fossorial water vole populations. In house mice and brown rats, a similar approach with the use of mixture of VOCs identified in these species can significantly improve their field trapping (Varner et al. 2019, 2020). As reported in mice, responses to chemosignals may be enhanced by association between VOCs and other low volatile compounds found in the urine as sex steroids or proteins of the lipocalin family (Novotny et al. 1985; Roberts et al. 2010). We have previously shown that the urine of males contain a higher concentration of a lipocalin called arvicolin when compared to females (Nagnan-Le Meillour et al. 2019). This protein may allow gradual release of VOCs and may thus increase the duration of the signal (Hurst et al. 1998; Pelosi et al. 2014), and/or may be part of the signal itself similar to MUPs in mice. In this context, it would be interesting to test whether arvicolin alone, and the combination of arvicolin and urine VOCs mixture may have an effect on the trapping efficiency in female voles

In conclusion, our results show that water voles can discriminate male and female urine from conspecifics, and male urine represents an attractive signal for female water voles. Specific VOCs found in male urine activated dissociated cells of the MOE and the VNO in calcium imaging experiments. Finally, the use of VOCs as a new sustainable method of controlling the population control of fossorial water vole populations still needs to be confirmed with further field investigations, which could involve the combined use of the VOCs and the arvicolin.

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Author contributions KP, A-CT, MN, DC, ET, and CM performed experiments; KP and A-CT analysed the data; KP wrote the first draft of the manuscript and designed the figures; PC, VL, A-CT, ET, AC, FS, JD, CLD, PNLM provided equipment, animal facility acces and helped conceiving the study; All authors reviewed the manuscript; MK supervised the study and acquired the funding.

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Data availability Relevant data are available from the corresponding author.

Declarations

Conflict of interest The authors declare no competing interests.

Ethical statement This study was carried out in accordance with European directive 2010/60/UE and was approved by ethical committees for animal experimentation (C2EA-02, project 21994-109 201907510411944 and C2EA-18, project 1857-2018110717044913). Trapping of water voles was allowed in the French department of Puy-de-Dôme by prefectural authorization 19–00100.

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