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Original Article

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Sensory responses of olfactory and vomeronasal neurons in does are influenced by buck odors and their reproductive status.

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In mammals, olfactory communication between conspecifics is crucial in modulating reproductive function. In anestrous does (i.e. female goats), exposure to hair from sexually active bucks (SAB, i.e. male goats) triggers a luteinizing hormone response that may induce ovulation, the "male effect." However, the chemicals in buck hair responsible for this effect and the sensory pathways used by the females to detect this information remain insufficiently understood. In this study, we investigated whether sensory cells from the main olfactory epithelium (MOE) and the vomeronasal organ (VNO) of does respond differently to olfactory stimuli from SAB versus wethers (CAS, i.e. castrated bucks) and how this response is influenced by the female reproductive status (breeding season, anestrous, or ovariectomized (OVX)). To explore this possibility, we stimulated freshly dissociated cells of MOE and VNO cells with chloroform/methanol and aqueous extractions from buck hair, and we assessed cell activation using calcium imaging. Regardless of the extraction method, we observed more cells activated by SAB hair compounds than by those from CAS males. More MOE cells from anestrous were activated by both chloroform and aqueous extracts from SAB than MOE cells from breeding season, or OVX does. Most of these responses originated from non-mature olfactory neurons. These findings suggest that females can discriminate buck sexual activity through sensory detection by the MOE and VNO. The increased response in the MOE to SAB hair compounds during the anestrous period suggests that the MOE may play a more significant role in the "male effect" during this period.

Key words: goats, sociosexual relationship, male effect, olfactory stimuli, main olfactory epithelium, vomeronasal organ.

1. Introduction

In goats, the reproductive function is controlled by photoperiodic variations. Goats are "short-day" breeders, with a breeding season lasting from early autumn (September/October) to late winter (February/March) under temperate latitudes (Chemineau et al. 1992, 2004). Breeding seasons are separated by a period of sexual rest (i.e. anestrous), during which neither estrus behavior nor ovulation occurs (Chemineau et al. 1992; Fatet et al. 2011). Exposure of does (i.e. female goats) to sexually active bucks (SAB, i.e. male goats) during the anestrous period reactivates ovulatory activity, a phenomenon known as the "male effect" (for review, see Delgadillo et al. 2009). These observations demonstrate that sociosexual sensory cues can override the photoperiodic regulation of the female reproductive status.

Semiochemicals play a pivotal role in mediating intraspecific interactions (Wyatt 2014). Wyatt classified them into 2 categories: "Pheromones" which are evolved signals emitted by an individual that modulate the behavior and physiology (e.g. reproduction) of a conspecific receiver (Wyatt 2010, 2014), and "Signature mixtures" which are variable chemical mixtures learned by conspecifics, enabling the sender to be recognized as an individual by conspecifics (Wyatt 2010, 2014). In the case of the male effect in goats, buck hair but not urine can induce an increase of luteinizing hormone (LH) pulsatility (short-term effect), eventually leading to ovulation within a few days (long-term effect) (Claus et al. 1990; Walkden-Brown et al. 1993). Moreover, multiple-unit activity volley recordings in the arcuate nucleus, a key brain region involved in the regulation of reproductive function (Oakley et al. 2009; Wakabayashi et al. 2010), showed that buck hair induces neuron responses in Shiba does (Murata et al. 2011; Sakamoto et al. 2013). These studies often define the semiochemicals studied as the "male effect pheromone" (Okamura et al. 2010). Their production seems dependent on androgen secretion (Iwata et al. 2000, 2001). Due to the debates surrounding the definition of pheromones in mammals (Wyatt 2014) and the existence of discrepancies in the context of the male effect (for review, see Hawken and Martin 2012), we prefer to use the term "olfactory stimuli" in this manuscript.

The chemical nature of buck hair compounds and the sensory pathways used by does to detect them remain incompletely understood. In the nasal cavity of goats, 2 sensory

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structures are involved in detecting olfactory compounds: the main olfactory epithelium (MOE), the input of the main olfactory system (MOS), and the vomeronasal organ (VNO), the input of the accessory olfactory system (AOS). These structures contain olfactory and vomeronasal sensory neurons (OSNs and VSNs, respectively), which detect olfactory compounds via G protein-coupled receptors: olfactory receptors (ORs) for OSNs (Buck and Axel 1991) and vomeronasal receptors (VRs) for VSNs (Leinders-Zufall et al. 2000). VRs are classified into 2 families: type 1 (V1Rs) and type 2 VRs (V2Rs) (Munger et al. 2009). In total, 11 to 23 genes of the V1Rs family are found in goats, including some pseudogenes (Ohara et al. 2009; Kondoh et al. 2022). However, most V2Rs are pseudogenes and appear non-functional (Wakabayashi 2002; Kondoh et al. 2022).

In contrast to rodents, the MOS of small ruminants appears to play a more critical role than the AOS in detecting, transducing, and integrating social olfactory stimuli, particularly in the context of the male effect (for review, see Keller and Lévy 2012). MOE lesion by zinc sulfate nasal irrigation reduces the response of anestrous females to the introduction of males (goats: Chemineau et al. 1986; sheep: Gelez and Fabre-Nys 2004). In contrast, surgical lesions of ewe VNO did not inhibit the LH response to ram exposure (Cohen-Tannoudji et al. 1989). Moreover, the inactivation of the cortical amygdala, a brain structure involved in the MOS, by lidocaine injection completely blocks LH secretion in response to ram's fleece (Gelez et al. 2004). In contrast, inactivation of 2 regions associated with the AOS, the medial amygdala and the ventromedial nucleus of the hypothalamus, is inefficient in blocking the ewes' endocrine response (Gelez et al. 2004). However, these findings do not exclude the possibility of a role for the AOS in the male effect (Gelez and Fabre-Nys 2004, 2006).

In this study, we used live-cell calcium (Ca^{2+}) imaging on freshly dissociated doe MOE and VNO cells to explore 2 key questions: (i) Does the detection of male olfactory stimuli vary based on the buck's sexual activity (sexually active vs. wether, i.e. castrated buck)? and (ii) Does this detection depend on the doe's reproductive status (breeding season, anestrous, or ovariectomized (OVX))? We also aimed to determine whether responses to buck stimuli stem from mature olfactory neurons. Lastly, we investigated how seasonal hormonal variations affect the turnover of olfactory neurons by examining whether does sampled during the breeding season show a different proportion of mature OSNs in the MOE compared to those in anestrous or OVX states.

2. Material and methods

2.1 Animals

We performed the experiments between January and May 2023 in Nouzilly, France (latitude 47° 32 N and longitude 0° 46 E) on adult Alpine does (*Capra hircus*). A total of 28 nulliparous 1-year-old females maintained indoors were used during the experiments. Animals were fed daily with commercial pellets, hay, and straw. They also had free access to mineral blocks and water. All procedures were carried out in accordance with European Directive 2010/63/EU on the protection of animals used for scientific purposes and also approved by the local ethical committee for animal experimentation (CEEA VdL, Tours, France, n°2022052309503651 and n°2022120511405443).

2.2 Assessment of male and female reproductive status

We used 5 intact SAB and 3 wethers (CAS) of the Alpine breed to collect olfactory stimuli. Males considered as "sexually active" were sampled during the breeding season (November) when they showed high sexual activity (Shelton 1978; Delgadillo et al. 1992). In addition, a weekly plasma testosterone assay was performed to verify their reproductive status (Hochereau-De Reviers et al. 1990). SAB showed testosterone concentrations ≥10 ng/mL compared with <0.1 ng/mL for CAS males.

Female reproductive status was followed by bi-weekly progesterone assay (sensitivity: 0.25 ng/mL; intra- and inter-assay coefficients of variation: <9% and <8.7%) (Canepa et al. 2008). Females were considered cyclic when progesterone concentrations were ≥1 ng/mL in 2 consecutive samples.

2.3 Collection and preparation of olfactory stimuli for Ca^{2+} imaging

2.3.1 Collection of buck olfactory stimuli

All stimuli were recovered using gloves cleaned with ethanol. We collected hair from our 5 intact SAB and 3 CAS males. Hair samples were cut from the neck, the beginning of the back, the head, the beard, and the shoulders, which are regions reported to contain olfactory stimuli associated with the male effect (Wakabayashi et al. 2000). All hair samples from each male were placed in clean glass containers and stored at −20 °C until pooled and used. We also collected urine during urination from 3 SAB and 4 CAS males. Urine samples from SAB and CAS males were pooled in parallel in a clean glass container, then aliquoted into Eppendorf tubes and stored at −20 °C.

2.3.2 Preparation of buck olfactory stimuli

Samples from CAS and SAB were processed simultaneously on the same day. We prepared 3 distinct sources of hair stimuli: (i) buck hair stimuli extracted in an aqueous buffer to isolate hydrophilic molecules; (ii) buck hair extracted in a chloroform solvent to obtain hydrophobic molecules; and (iii) buck hair extracted in a methanol solvent to obtain hydrophilic and hydrophobic molecules. Before each extraction, we pooled an equal mass of hair from the 5 SAB and an equal mass of hair from the 3 CAS males.

For aqueous extraction, 50 mg of finely cut SAB or CAS pooled hair (2 to 4 mm long) was transferred to glass tubes. 2 mL of Hank's balanced salt solution (HBSS, Gibco) supplemented with 5 mM HEPES (2-[4-(2-hydroxyethyl) piperazin-1-yl]ethanesulfonic acid, Merck), named imaging buffer, was added. The tube was placed under constant agitation at room temperature (RT) for 2 h with vigorous vortexing for 10 s every 20 min. Afterward, the solution was filtered through a 0.45 µm filter (Millex-HV) and diluted 1:3 in imaging buffer; the resulting solution is referred to as the "aqueous extract" below. This extract was freshly prepared on the day of Ca^{2+} imaging acquisition.

The chloroform and methanol extraction protocol is based on the method of Bligh and Dyer (Jensen 2008). 100 mg of finely cut pooled hair was transferred into glass tubes. Next, one volume of methanol and one of chloroform, both at −20 °C, were added in a 1:1 ratio. After vigorous vortexing, the mixture was stored for 2 h at −20 °C with vigorous vortexing every 20 min for 10 s. Then, the mixture was incubated in an

ultrasonic bath for 10 min, 4 °C. Hair bits and chloroform/methanol mixture were separated. MilliQ water was added in a 1:1:0.5 ratio to the chloroform/methanol mixture. After mixing, the solution was centrifuged for 10 min at 4,000 × *g* and 4 °C to obtain 3 phases. Methanol and chloroform phases were gently and individually collected into 1 mL extract. Each extract was completely dried using a SpeedVac (Eppendorf) at RT. Dry pellets were stored at −20 °C and used within 3 to 4 days. On the day of Ca^{2+} imaging acquisition, 1 dry chloroform pellet was resolubilized in 30 µL of dimethyl sulfoxide (DMSO) using 5 to 10 pulses of an immersion sonicator (Bioblock Scientific) at maximum intensity. The solution was then diluted 1:500 in freshly prepared imaging buffer, resulting in what is referred to as the "chloroform extract" below. A control solution of this solvent was also prepared by diluting only DMSO at 1:500 in the imaging buffer. The dry methanol pellet was resolubilized in 30 µL of 50% ethanol using a pipette. The resulting solution was then diluted 1:500 in freshly prepared imaging buffer and referred to as the "methanol extract" below. A control solution of this solvent was also prepared by diluting 50% ethanol at 1:500 in the imaging buffer.

An aliquot of urine from CAS males and SAB was thawed on each acquisition day and then diluted 1:100 in the imaging buffer in glass tubes.

2.4 Ca^{2+} imaging on dissociated MOE/VNO cells from does

2.4.1 Collection and preparation of freshly dissociated cells for acquisition

The Ca^{2+} imaging approach using freshly dissociated MOE and VNO cells has been previously described in does (Meunier et al. 2023, 2024). We have standardized this method to minimize inter-individual variation during cell collection. Briefly, on the day of acquisition, 1 animal was euthanized by intravenous injection of 5 mL ketamine (Ketamidor, Axience) and decapitation by a licensed butcher. The skull was cut in half, 2 cm from the midline in the sagittal plane. The MOE and VNO were recovered after the removal of the nasal bones. We dissected both structures in parallel in Petri dishes before detaching and dissociating the epithelia for 20 min at 37 °C in Phosphate Buffer Sodium (PBS) solution containing papain (2.2 units/mL, Merck) and 40 mM urea (Merck) for the MOE. Next, we added DNase to the solution (25 units/mL, Thermo Scientific), and the cell suspension was centrifuged for 5 min at 1,000 rpm after the addition of Dulbecco's modified Eagle medium.

(DMEM) 1× high glucose + GlutaMAX (61965026, Gibco) supplemented with 10% of fetal bovine serum (FBS, 17974731, Gibco) and penicillin/streptomycin (11548876, Gibco). Recovered dissociated cells were plated on coverslips with 0.5 µg/µL concanavalin-A (Merck) and incubated for 45 min at 37 °C with 5% CO_2. After washing with imaging buffer, cells were loaded with 5 µM fura-2AM (Thermo Scientific) for at least 30 min at RT.

2.4.2 Ca^{2+} imaging acquisition and data analysis

The coverslip with the cells was placed in a perfusion chamber on an Olympus IX71 microscope for image acquisition and connected to a perfusion system. Perfusion alternated between 30 s of a given stimulus solution and 3 min of imaging buffer. Images were acquired at a frequency of 0.25 Hz by illuminating the cells at 340 nm and 380 nm. The emitted light between 500 nm and 560 nm was recorded by a Hamamatsu C10600-10B camera fitted to the microscope (with 10× magnification). The selected cell fields contained at least 100 well-dissociated cells. We opted for fields that generally contained between 500 and 2,500 cells. The number of cells recorded for each experiment is detailed in Supplementary Table S1.

Image stacks were analyzed using ImageJ software (Fiji), which included background subtraction, detection of regions of interest, and calculation of mean pixel values. We analyzed Ca^{2+} transients using the open-source software calipR (Trives 2024). The analysis involved removing low-quality cells (with 0 or missing values more than 10% of the time). Then, the software estimated and corrected background fluctuations that did not match the kinetics of cell responses with a Generalized Additive Model fit around the potential signal (based on Balkenius et al. 2015) detected with the derivative passing accumulation (DPA) method (Liu and Lin 2019). Denoised traces were then standardized (*z*-score) using the baseline period as a reference (time during imaging buffer perfusion at the start of acquisition before stimulation). Peaks were finally identified by combining deconvolution (Jewell et al. 2020) and *z*-score thresholding. A cell is considered as "responding" to a stimulus when the signal values exceed 2.5 standard deviations from the baseline with our deconvolution parameters (Lambda = 2500, Gamma = 0.95). All cells showing any response to the negative controls (initial imaging buffer application and solvent control solution) were removed from the analysis.

2.4.3 Experimental design of Ca^{2+} imaging

Does were divided into 3 distinct groups (Fig. 1A). Littermates were separated to avoid genetic effects. Only a few females had prior exposure to a male before this experiment. These were evenly distributed across the 3 groups (2 to 3 females per group). The first group of females, corresponding to the breeding season, was euthanized between January 17 and February 2 (Season, $n = 10$). The second group of females was euthanized between March 8 and May 5 (Anestrous, $n = 10$). The third group of females was OVX on February 13 to remove the vast majority of steroid production. Does were OVX by laparotomy under anesthesia. Before the operation, females received an antibiotic (clamoxyl LA, 5 mL/day for 3 days, Zoetis). Anesthesia was induced by intravenous injection of ketamine (ketamidor 100, 10 mg/kg, Axience) and xylazine (rompun, 0.05 mg/kg, Dechra) and maintained with 3% isoflurane. Before the incision, a local anesthetic was administered subcutaneously (procamidor, 3 mL, Axience) at various injection points. At the end of the surgery, females received an intramuscular injection of a non-steroidal anti-inflammatory drug (antalzen, 2 mL at 50 mg/mL, Virbac). Subcutaneous analgesia infusion of buprenorphine was given at the beginning and end of surgery (buprecare, 0.3 mg/mL, 1 ampoule of 1 mL in 2 doses, Axience). Females were euthanized between March 14 and April 13 (OVX, $n = 8$).

Four experiments per female were conducted, each experiment using 1 MOE coverslip and 1 VNO coverslip sequentially perfused with different stimuli (Fig. 1B). The first experiment tested chloroform extracts of male hair. MOE and VNO cells were perfused with the control solution of the solvent (Ctrl Chloro), followed by the chloroform extracts from CAS (Chloro CAS) and SAB (Chloro SAB). The second

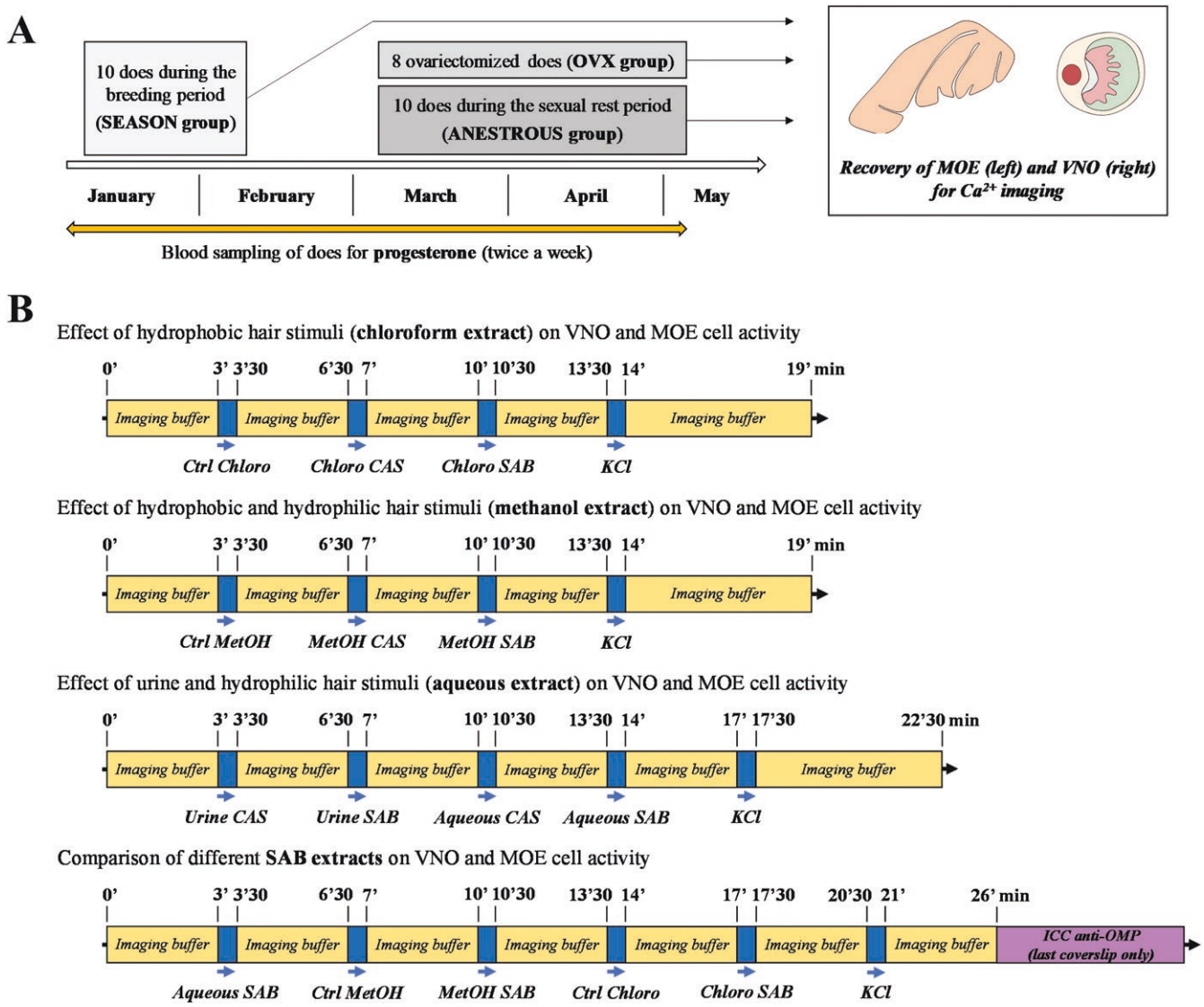

Fig. 1. Experimental design. (**A**) In January, adult does were assigned to 3 groups. The first group was euthanized during the breeding season between January 17 and February 2 (SEASON, $n = 10$). The second group was euthanized during the anestrous period between March 8 and May 5 (ANESTROUS, $n = 10$). The third group was ovariectomized and then euthanized between March 14 and April 13 (OVX, $n = 8$). MOE and VNO were recovered from each individual for Ca^{2+} imaging. (**B**) For each animal, 4 experiments were conducted on MOE and VNO coverslips. Experiment 1 tested the chloroform extracts of hair from wethers (CAS) and sexually active bucks (SAB), while experiment 2 tested methanol extracts of hair. Experiment 3 tested urine and the aqueous hair extracts from CAS and SAB. Experiment 4 tested all hair extracts from SAB. Each experiment concluded with perfusion of a high (90 mM) KCl solution. A box and a blue arrow indicate the timing of stimulus perfusion. Abbreviations: Chloro, chloroform extracts; MetOH, methanol extracts.

experiment followed the same principle as the first, using the methanol extracts of male hair. Dissociated cells were perfused with the control solution of the solvent (Ctrl MetOH), then with the methanol extracts of CAS (MetOH CAS) and SAB (MetOH SAB). The third experiment tested the aqueous extracts of male hair and urine. MOE and VNO cells were perfused with diluted urine from CAS (Urine CAS) and SAB (Urine SAB), followed by aqueous hair extracts (Aqueous CAS and Aqueous SAB, respectively). Finally, the fourth experiment tested all the hair extracts from SAB (Chloro SAB, MetOH SAB, and Aqueous SAB) and their respective controls (Ctrl Chloro, Ctrl MetOH, and imaging buffer). Each experiment concluded with the perfusion of a high KCl depolarizing solution (90 mM) to estimate the proportion of excitable cells.

2.5 Immunocytochemistry of olfactory marker protein (OMP) after Ca^{2+} imaging

We performed anti-OMP immunocytochemistry on the last coverslip directly on the acquisition field for each animal to identify specific responses from mature OSNs (MOE coverslips, $n = 4–5$ per group) or VSNs (VNO coverslips, $n = 4–5$ per group). We followed a previously described protocol (Meunier et al. 2023, 2024). Briefly, we fixed cells dissociated from the acquisition field directly in the perfusion chamber for 20 min with 4% paraformaldehyde (Merck). Cells were then washed with imaging buffer for 5 min and pre-incubated for 30 min in saturation solution. Tris-buffered saline (TBS) supplemented with 0.1% Triton and 2% normal donkey serum (Merck). Cells were incubated for 30 min with a goat anti-OMP primary antibody (1:2,000, FUJIFILM Wako

Chemicals, RRID: AB_664696) diluted in saturation solution. After a further 5 min wash with imaging buffer, we incubated the cells for 30 min with donkey anti-goat Alexa Fluor 647 secondary antibody (1:500, Thermo Scientific, RRID: AB_2535864) diluted in TBS-Triton 0.1. After a further 5 min wash with imaging buffer, we added 2 min of Hoechst solution (1:5,000, Thermo Scientific) diluted in TBS and washed one last time with imaging buffer for 5 min. Fluorescent images were acquired using a Hamamatsu C10600-10B camera fitted to an Olympus IX71 microscope (x10 magnification). The number of OMP-positive cells was counted using ImageJ software (Fiji).

2.6 Statistical analysis

Ca^{2+} imaging and immunocytochemistry data are expressed as a box and whiskers plot showing the median and mean (and minimum and maximum values). Individual values are also represented in each graph. We performed statistical analyses with GraphPad software (GraphPad Prism v10.3.0). To select the appropriate statistical test, we tested assumptions of normality (one-way analyses: Shapiro–Wilk test, mixed-effects analyses: Q–Q plot) and homogeneity of variances (one-way analyses: Bartlett test, mixed-effects analyses: sphericity appreciated by a homoscedasticity plot). We used a one-way ANOVA with Welch's correction followed by Dunnett's T3 multiple comparisons test to compare the mean percentages of cells responding to KCl. A Kruskal–Wallis test was performed to compare progesterone concentrations on the day of euthanasia. We used a mixed-effects analysis on repeated measures to compare multiple variables (Stimulus, Reproductive status, and Interaction) followed by Tukey's multiple comparison tests as post hoc analyses. We used a Kruskal–Wallis test and a one-way ANOVA to compare the mean percentages of on-field total OMP-positive cells in our MOE and VNO preparations, respectively. We used R software (version 4.3.0) to evaluate the ratio between the percentage of responding OMP-positive cells among responding cells and the total percentage of OMP-positive cells. This ratio was studied using a Spearman correlation ("nparcomp" package) followed by a permutation test for linear models ("lmPerm" package). The α risk chosen for these statistical tests was 0.05.

3. Results

We conducted a Ca^{2+} imaging approach to explore how MOE and VNO cells respond to olfactory stimuli from CAS males vs. SAB according to the female reproductive status. Details on the number of screened and responding cells for each experiment can be found in Supplementary Table S1. We also used a high KCl depolarizing solution (90 mM) to estimate the proportion of excitable cells in our preparations, as illustrated in Supplementary Fig. S1. We observed variations between groups in the percentage of cells stimulated by KCl. In the MOE, this percentage was significantly higher in Anestrous females than in Season females ($W(2, 12.49) = 6.522$, $P < 0.05$). In the VNO, this percentage was higher in Anestrous and Season females than in OVX females ($W(2, 12.53) = 39.78$, $P < 0.001$).

3.1 Assessment of female reproductive status

Female reproductive status was determined by bi-weekly measurement of plasma progesterone concentrations (Fig. 2A). On the day of cell preparation (euthanasia), progesterone concentrations were significantly higher in SEASON (cyclic ewes) does than Anestrous and OVX does (Fig. 2B, Kruskal–Wallis test, $P < 0.001$; Dunn test, $P < 0.01$). All Season females were cyclic and euthanized during the luteal phase (progesterone concentrations ≥1 ng/mL). All Anestrous females were euthanized at least 2 weeks after cyclicity stops (progesterone concentrations <1 ng/mL in 2 consecutive samples). All OVX females were euthanized several weeks after the removal of the ovaries (progesterone concentrations <1 ng/mL). Therefore, all the females were euthanized in the expected reproductive status.

3.2 Impact of hair stimuli sources and reproduction status on MOE cell activity

We first examined Ca^{2+} responses to hydrophobic molecules from hair extracted with chloroform in dissociated MOE cells from anestrous ($n = 10$), season ($n = 9$–10), and OVX does ($n = 8$) (Fig. 3A). As expected, the Chloro SAB extract significantly increased MOE cell activity compared to Chloro CAS extract (Fig. 3B, mixed-effects analysis, Stimulus: $F(1, 24) = 67.73$, $P < 0.001$; Tukey test, $P < 0.01$). The reproductive status did not show a statistically significant main effect ($F(2, 24) = 3.155$, $P = 0.061$), but a significant interaction between stimulus type and reproductive status was observed ($F(2, 24) = 3.405$, $P < 0.05$), suggesting that the response to the Chloro SAB varies depending on the reproductive state of the does. Thus, we explored group comparisons and observed that MOE cells from Anestrous does responded more to Chloro SAB extract than cells from Season (Tukey test, $P < 0.05$) and OVX does (Tukey test, $P < 0.01$).

Next, we analyzed Ca^{2+} responses to the mixture of lipophilic and hydrophilic molecules extracted with methanol in MOE cells from does across the 3 different reproductive statuses (Fig. 3C). MetOH SAB extract stimulated more MOE-responding cells compared to MetOH CAS extract, without significant interaction with the reproductive status factor (Fig. 3D, mixed-effects analysis, Stimulus: $F(1, 25) = 30.67$, $P < 0.001$; Interaction: $F(2, 25) = 1.119$, $P = 0.342$; Tukey test, $P < 0.05$). Unlike the Chloro SAB extract, there was no significant difference in response to MetOH SAB extract based on reproductive status (Reproductive status: $F(2, 25) = 1.118$, $P = 0.343$).

We next compared the effect of buck urine and aqueous hair extracts on MOE cell activity in the same experiment (Fig. 3E). We observed a significant overall effect of the "Stimulus" factor on the percentage of MOE-responding cells (Fig. 3F, mixed-effects analysis, Stimulus: $F(1.541, 38.52) = 88.08$, $P < 0.001$). The Aqueous SAB extract triggered more MOE cell responses across all groups compared to urine from both SAB and CAS males (Tukey test, $P < 0.05$). Additionally, aqueous SAB extract induced significantly more cell responses than Aqueous CAS extract, but only in Anestrous does (Tukey test, $P < 0.001$). The Aqueous CAS extract stimulated more MOE-responding cells than Urine SAB in Season and OVX does (Tukey test, $P < 0.05$), and more than Urine CAS in Season does (Tukey test, $P < 0.05$). The interaction between "Stimulus" and "Reproductive status" factors was highly significant (Interaction: $F(6, 75) = 9.648$, $P < 0.001$). In addition, the "Reproductive status" factor had an overall effect on MOE-responding cells (Reproductive status: $F(2, 25) = 5.088$, $P < 0.05$). Similar to Chloro SAB

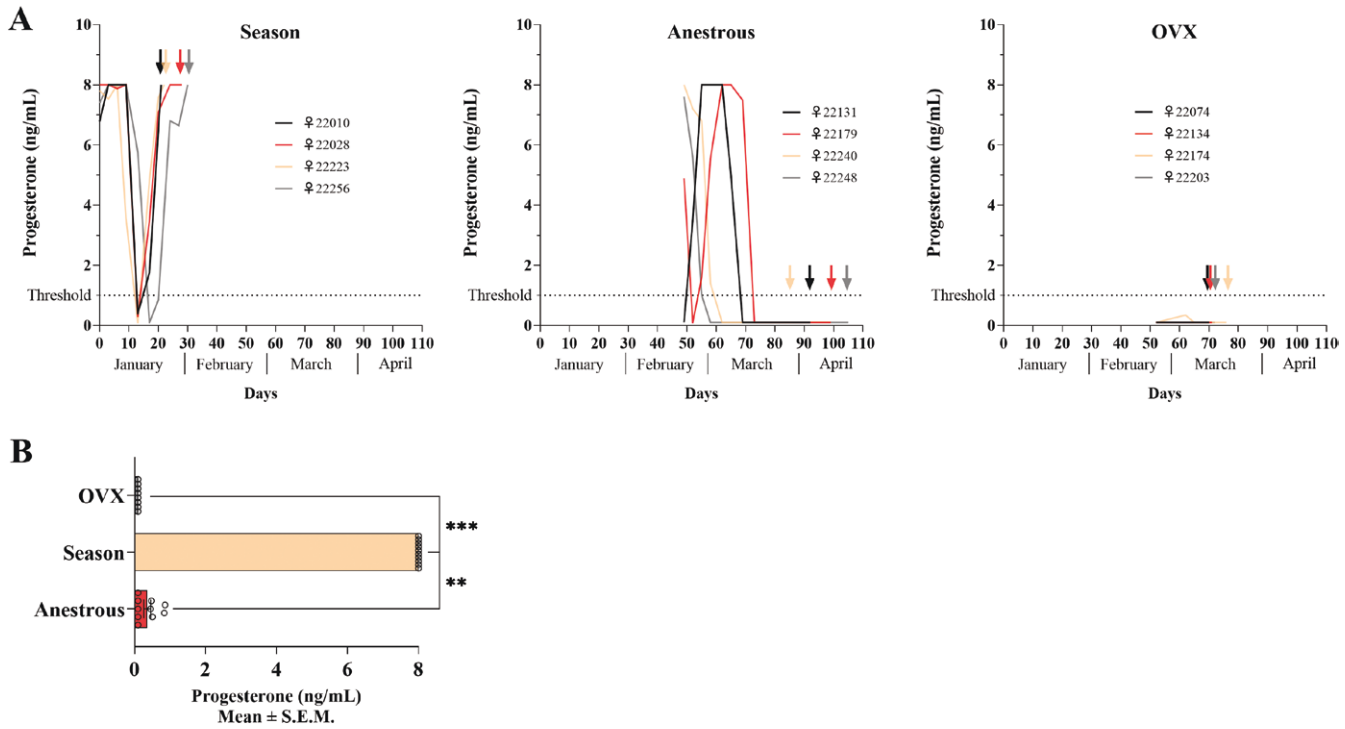

Fig. 2. Variations of progesterone secretion levels in does. (**A**) Representative profiles of plasma progesterone secretion. Does were divided into 3 distinct groups. Blood samples were collected twice a week. The first group of females was euthanized between January 17 and February 2 (SEASON, $n = 10$). The second group of females was euthanized between March 8 and May 5 (ANESTROUS, $n = 10$). The third group of females was ovariectomized on February 13 and euthanized between March 14 and April 13 (OVX, $n = 8$). Females were considered cyclic if 2 consecutive progesterone values were ≥1 ng/mL. Colored arrows indicate the day of euthanasia. (**B**) Assessment of progesterone concentration on the day of euthanasia. Data are expressed as mean ± SEM. Comparisons were made using the Kruskal–Wallis test ($P < 0.001$) followed by multiple comparisons using Dunn tests (**$P < 0.01$, ***$P < 0.001$).

extract, Aqueous SAB extract triggered more MOE cell responses with Anestrous does compared to Season (Tukey test, $P < 0.01$) and OVX does (Tukey test, $P < 0.001$).

3.3 Impact of hair stimuli sources and reproduction status on VNO cell activity

Following the results obtained in MOE cells, we next explored whether VNO cells would respond differently to buck stimuli based on the reproductive status of does. Similar to the MOE cells, Chloro SAB extract induced significantly more VNO-responding cells than Chloro CAS extract (Fig. 4A,B, mixed-effects analysis, Stimulus: $F(1, 24) = 228.9$, $P < 0.001$; Tukey test, $P < 0.001$). The interaction between "Stimulus" and "Reproductive status" factors was highly significant (Interaction: $F(2, 24) = 10.18$, $P < 0.001$). VNO cells from OVX does responded less to Chloro SAB extract compared to cells from Anestrous and Season does (Reproductive status: $F(2, 24) = 11.44$, $P < 0.001$; Tukey test, $P < 0.001$). Additionally, VNO cell responses showed a tendency to be higher in Anestrous than Season does (Tukey test, $^{\#}P = 0.0695$).

For the MetOH SAB extract (Fig. 4C), VNO cells showed more responses than for MetOH CAS extract with no significant interaction with the "Reproductive status" factor (Fig. 4D, mixed-effects analysis, Stimulus: $F(1, 25) = 107.4$, $P < 0.001$, Interaction: $F(2, 25) = 0.962$, $P = 0.396$; Tukey test, $P < 0.01$). As with Chloro SAB extract, no significant difference was observed between reproductive statuses in response to MetOH SAB extract (Reproductive status: $F(2, 25) = 0.683$, $P = 0.515$).

Urine and aqueous hair extracts were also tested in the same experiment (Fig. 4E). As with MOE cells, there was an overall effect of the stimulus on the percentage of VNO-responding cells (Fig. 4F, mixed-effects analysis, Stimulus: $F(1.097, 27.43) = 171.1$, $P < 0.001$). Aqueous SAB extract triggered more VNO cell responses across all groups compared to Aqueous CAS, Urine CAS, and Urine SAB extracts (Tukey test, $P < 0.05$). Furthermore, Aqueous CAS induced more VNO-responding cells than Urine SAB (in Anestrous and OVX does, Tukey test, $P < 0.05$) and more than Urine CAS (Anestrous and Season does, Tukey test, $P < 0.05$). Once again, the interaction between "Stimulus" and "Reproductive status" factors was highly significant (Interaction: $F(6, 75) = 7.764$, $P < 0.001$), and an overall effect of the reproductive status was also observed (Reproductive status: $F(2, 25) = 3.752$, $P < 0.05$). VNO cells from OVX does showed fewer responses to Aqueous SAB extract compared to cells from Anestrous (Tukey test, $P < 0.01$) and Season does (Tukey test, $P < 0.05$).

3.4 Comparison of the effect of SAB hair extracts on VNO and MOE cells

To further understand how cells respond to various molecules in 3 distinct SAB extracts based on the reproductive status of does, we compared intracellular Ca^{2+} transients of dissociated MOE and VNO cells in response to each SAB hair extract (chloroform, methanol, and aqueous, Supplementary Fig. S2).

In MOE cells, we found that both "Stimulus" and "Reproductive status" factors had significant effects, with

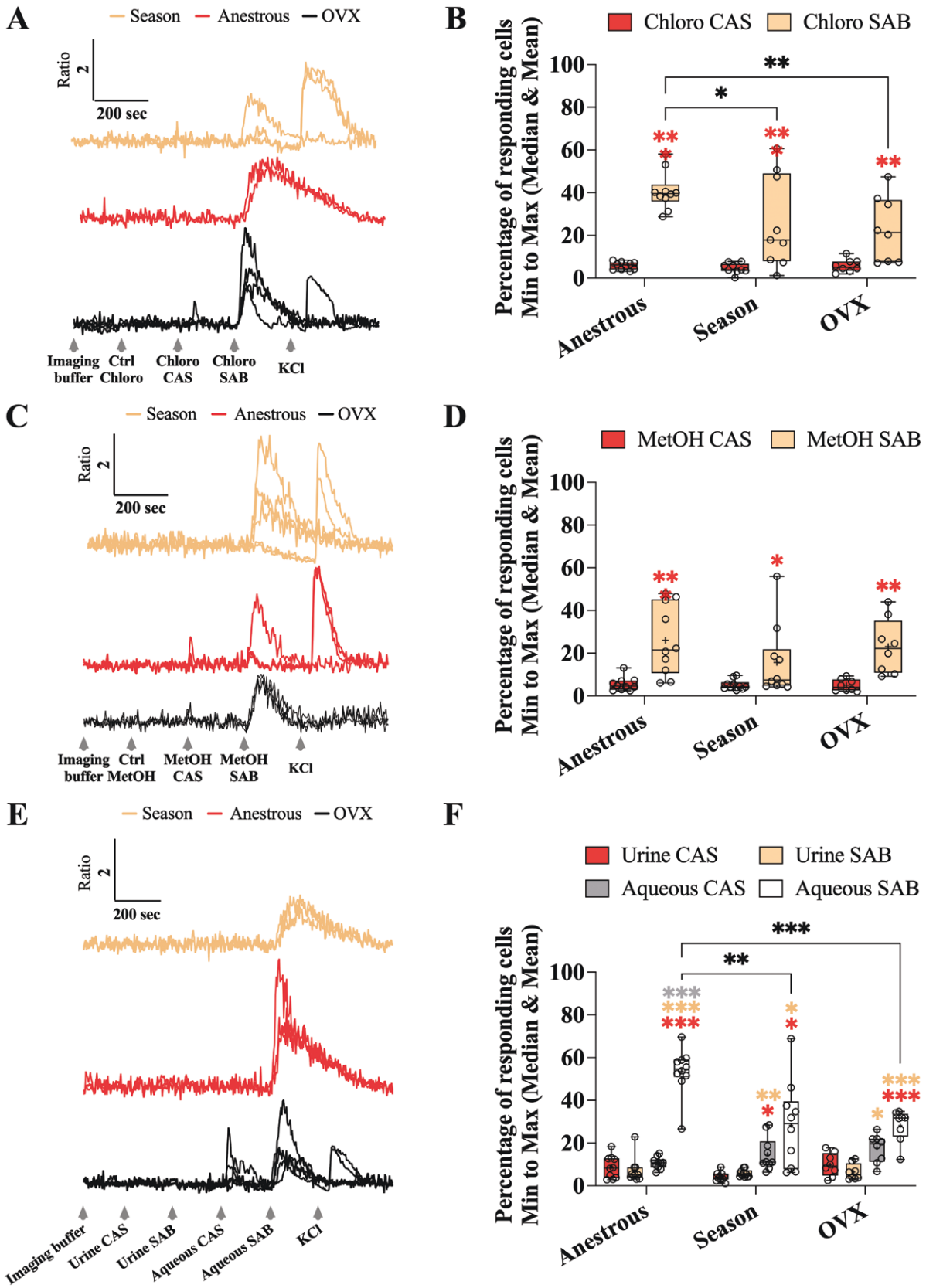

Fig. 3. Impact of hair stimuli sources and reproduction status on MOE cell activity. (**A**, **C**, **E**) Examples of Ca^{2+} transients imaged on dissociated MOE cells from Anestrous, Season, and OVX does stimulated with chloroform hair extracts (**A**), methanol hair extracts (**C**), aqueous hair extracts and urine (**E**) from SAB and CAS males. (**B**, **D**, **F**) Analysis of Ca^{2+} responses of dissociated MOE cells from Anestrous (n = 10), Season (n = 9–10), and OVX does (n = 8) induced by (**B**) Chloro CAS and Chloro SAB extracts (mixed-effects analysis, Stimulus: $F(1, 24) = 67.73$, $P < 0.001$, Reproductive status: $F(2, 24) = 3.155$, $P = 0.061$, Interaction: $F(2, 24) = 3.405$, $P < 0.05$), (**D**) MetOH CAS and MetOH SAB extracts (mixed-effects analysis, Stimulus: $F(1,$

a notable interaction between them (Fig. 5A, mixed-effects analysis, Stimulus: $F(1.880, 45.13) = 21.75$, $P < 0.001$, Reproductive status: $F(2, 24) = 5.779$, $P < 0.01$, Interaction: $F(4, 48) = 3.222$, $P < 0.05$). For the "Stimulus" factor, Aqueous SAB extract induced more MOE-responding cells than Chloro SAB and MetOH SAB extracts, but only in Anestrous does (Tukey test, $P < 0.01$). Additionally, cells responded more to Chloro SAB extract than MetOH SAB extract (Tukey test, $P < 0.05$). Regarding the "Reproductive status" factor, the percentage of MOE cells responding to Aqueous SAB extract was significantly higher in Anestrous does compared to Season (Tukey test, $P < 0.001$) and OVX does (Tukey test, $P < 0.05$).

For VNO cells, there was also an overall effect of the stimulus along with a significant interaction (Fig. 5B, mixed-effects analysis, Stimulus: $F(1.863, 46.57) = 14.79$, $P < 0.001$, Reproductive status: $F(2, 25) = 0.4439$, $P = 0.647$, Interaction: $F(4, 50) = 2.716$, $P < 0.05$). In Anestrous does only, VNO cells responded more to Aqueous SAB extract than Chloro SAB and MetOH SAB extracts (Tukey test, $P < 0.01$). Moreover, the percentage of VNO-responding cells tended to be higher in Anestrous does than in Season does (Tukey test, $^{\#}P = 0.060$).

Many MOE and VNO cells exhibited multiple responses to different SAB hair extracts (MOE: Supplementary Fig. S3A; VNO: Supplementary Fig. S3B). In MOE cells, most responses to Aqueous SAB extract were single, regardless of the doe reproductive status (51.86% to 61.72%, Fig. 5C). In contrast, single responses to Chloro SAB and MetOH SAB extracts were minor compared to dual or triple responses (Chloro SAB: 25.53% to 47.97%, MetOH SAB: 18.96% to 43.27%). In VNO cells, the majority of responses to Aqueous SAB extract were single for Anestrous does, but not in Season and OVX does (Anestrous: 51.96%, Season: 45.55%, OVX: 46.14%, Fig. 5D). As MOE cells, VNO mono-responding cells to Chloro SAB and MetOH SAB extracts were minor compared to dual- and triple-responding cells, whatever the reproductive status (Chloro SAB: 21.13% to 40.77%, MetOH SAB: 17.00% to 39.51%).

3.5 Activation of mature OSNs and VSNs by SAB hair extracts

To determine whether intracellular Ca^{2+} transients observed in response to SAB hair extracts originated from mature neurons, we performed OMP-immunocytochemistry directly on the Ca^{2+} imaging acquisition field (Fig. 6A).

First, we calculated the percentage of total OMP-positive cells (Fig. 6B). In the MOE, Season does tend to have more OMP + OSNs compared to Anestrous does (Kruskal–Wallis test, $P = 0.05$; Dunn test, $P = 0.07$). No significant differences were observed in the percentage of OMP + VSNs in the VNO of does based on their reproductive status (one-way ANOVA, $F(2, 11) = 0.614$, $P = 0.559$).

Next, we investigated the percentage of OMP-positive responding cells over the total of responding cells. In the MOE, we observed only a trend for an overall effect of the "Reproductive status" factor without any significant interaction with the "Stimulus" factor (Fig. 6C, mixed-effects analysis, Reproductive status: $F(2, 11) = 3.621$, $P = 0.062$, Stimulus: $F(1.737, 19.10) = 2.616$, $P = 0.105$, Interaction: $F(4, 22) = 0.274$, $P = 0.892$). By exploring group comparisons, we found that Season does tended to have more cell responses to Chloro SAB ($^{\#}P = 0.085$) and Aqueous SAB ($^{\#}P = 0.083$) extracts from OMP + OSNs compared to Anestrous does. However, this trend was strongly influenced by the percentage of total OMP + OSNs. For each stimulus, we observed a strong positive correlation between the percentage of OMP + responding OSNs among the responding cells and the overall percentage of total OMP + OSNs (Supplementary Table S2, Spearman correlation: $0.7011 < r_s < 0.7890$, $P < 0.01$). After building a linear model with permutations (lmPerm package from R), we found that the group did not significantly predict the percentage of OMP + responding OSNs (Supplementary Table S2, lmPerm: $-0.666 < \beta < 0.261$, $P > 0.05$). In contrast, the percentage of total OMP + OSNs explained a substantial and significant amount of the variance (Supplementary Table S2, lmPerm: $0.826 < \beta < 0.847$, $P < 0.01$). Concerning the VNO, no significant difference was observed in the percentage of OMP + responding VSNs (Fig. 6D, mixed-effects analysis, Reproductive status: $F(2, 11) = 0.621$, $P = 0.555$; Stimulus: $F(1.680, 18.48) = 0.084$, $P = 0.891$; Interaction: $F(4, 22) = 0.273$, $P = 0.892$).

We also investigated the percentage of OMP-positive cells among single, dual, and triple-responding cells to SAB hair extracts (Supplementary Fig. S4). In both olfactory inputs of Season does, the vast majority of mono-responding cells were OMP + OSNs (62.46% to 71.68%, Supplementary Fig. S4A) and VSNs (68.36% to 73.19%, Supplementary Fig. S4B). This percentage was less critical in both MOE and VNO of OVX does (MOE: 42.72% to 52.37%, VNO: 32.14% to 53.43%), and in the MOE of Anestrous does (30.57% to 39.95%). However, most mono-responding VNO cells in Anestrous does were OMP + VSNs (54.96% to 63.04%). Interestingly, a majority of dual- and triple-responding cells in both MOE and VNO, regardless of doe reproductive status, were OMP + OSNs (Anestrous, dual: 45.99% to 52.70%, triple: 52.45%; Season, dual: 74.75% to 76.59%, triple: 80.77%; OVX, dual: 49.53% to 60.00%, triple: 58.15%) and VSNs (Anestrous, dual: 62.20% to 84.09%, triple: 78.36%; Season, dual: 75.14% to 84.87%, triple: 84.01%; OVX, dual: 59.75% to 72.99%, triple: 68.93%).

We also calculated the percentage of OMP-positive responding cells over the total population of OMP-positive cells. In the MOE, we detected only an overall effect of the stimulus (Fig. 6E, mixed-effects analysis, Reproductive status: $F(2, 11) = 2.474$, $P = 0.130$, Stimulus: $F(1.830, 20.13) = 8.923$, $P < 0.01$, Interaction: $F(4, 22) = 0.857$, $P = 0.505$). Multiple comparisons revealed no significant differences (Tukey test, $P > 0.05$). No significant difference was detected in the VNO (Fig. 6F, mixed-effects analysis, Reproductive status: $F(2, 11) = 0.202$, $P = 0.820$, Stimulus: $F(1.617, 17.79) = 1.527$, $P = 0.243$, Interaction: $F(4, 22) = 0.575$, $P = 0.684$).

25) = 30.67, $P < 0.001$, Reproductive status: $F(2, 25) = 1.118$, $P = 0.343$, Interaction: $F(2, 25) = 1.119$, $P = 0.342$), (**F**) Urine CAS, Urine SAB, Aqueous CAS, and Aqueous SAB extracts (mixed-effects analysis, Stimulus: $F(1.541, 38.52) = 88.08$, $P < 0.001$, Reproductive status: $F(2, 25) = 5.088$, $P < 0.05$, Interaction: $F(6, 75) = 9.648$, $P < 0.001$). We performed multiple comparisons using Tukey tests. Significance between stimuli is indicated by colored stars and between reproductive status by black stars (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$). Data are expressed as a box and whiskers plot Min to Max (median: line, mean: +). Abbreviations: Aqueous, aqueous hair extracts; CAS, wethers; Chloro, chloroform hair extracts; MetOH, methanol hair extracts; MOE, main olfactory epithelium; SAB, sexually active bucks.

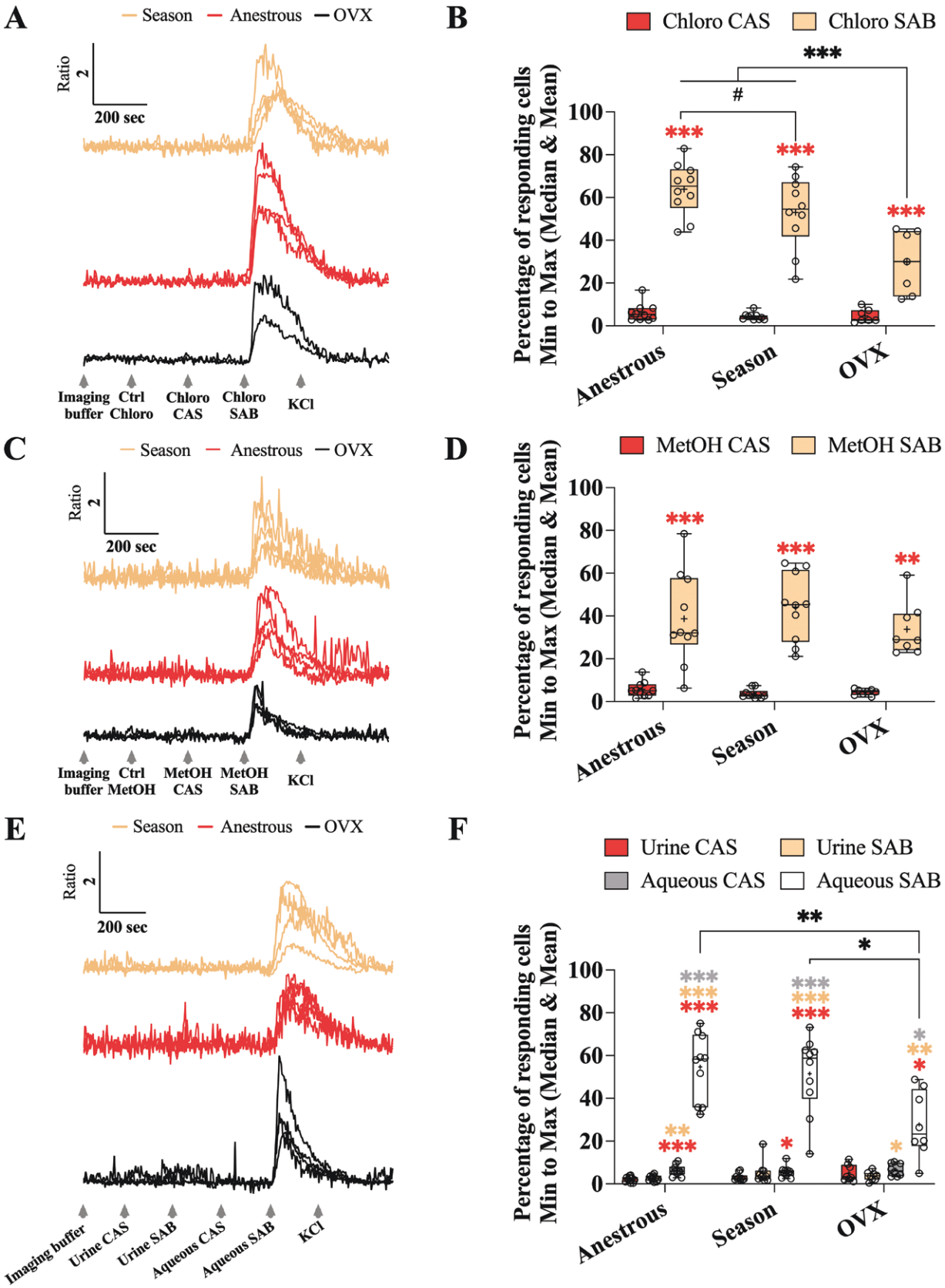

Fig. 4. Impact of hair stimuli sources and reproduction status on VNO cell activity. (**A**, **C**, **E**) Examples of Ca^{2+} transients imaged on dissociated VNO cells from Anestrous, Season, and OVX does stimulated with chloroform hair extracts (**A**), methanol hair extracts (**C**), aqueous hair extracts and urine (**E**) from SAB and CAS males. (**B**, **D**, **F**) Analysis of Ca^{2+} responses of dissociated VNO cells from Anestrous (n = 10), Season (n = 10), and OVX does (n = 7–8) induced by (**B**) Chloro CAS and Chloro SAB extracts (mixed-effects analysis, Stimulus: $F(1, 24) = 228.9$, $P < 0.001$, Reproductive status: $F(2, 24) = 11.44$, $P < 0.001$, Interaction: $F(2, 24) = 10.18$, $P < 0.001$), (**D**) MetOH CAS and MetOH SAB extracts (mixed-effects analysis, Stimulus: $F(1,$

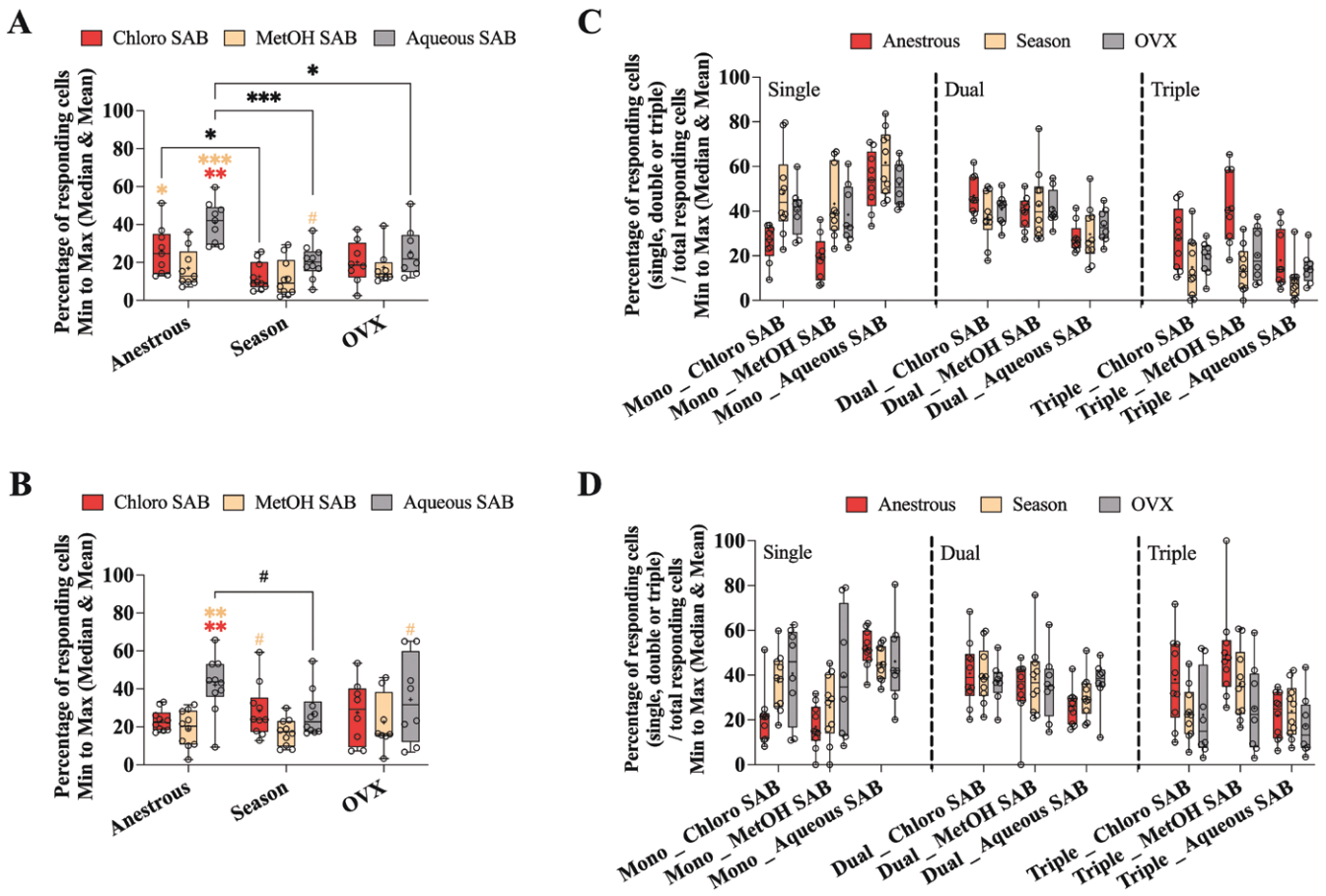

Fig. 5. Comparison of the effect of SAB hair extracts on VNO and MOE cells. (**A, B**) Analysis of Ca^{2+} responses of dissociated MOE (**A**) and VNO cells (**B**) from Anestrous (n = 9–10), Season (n = 10), and OVX does (n = 8) induced by Chloro SAB, MetOH SAB and Aqueous SAB extracts. Data are expressed as mean ± SEM. Comparisons were performed using a mixed-effects analysis (MOE, Stimulus: $F(1.880, 45.13) = 21.75$, $P < 0.001$, Reproductive status: $F(2, 24) = 5.779$, $P < 0.01$, Interaction: $F(4, 48) = 3.222$, $P < 0.05$; VNO, Stimulus: $F(1.863, 46.57) = 14.79$, $P < 0.001$, Reproductive status: $F(2, 25) = 0.4439$, $P = 0.647$, Interaction: $F(4, 50) = 2.716$, $P < 0.05$) followed by multiple comparisons using Tukey tests. Significance between stimuli is indicated by colored stars and between reproductive status by black stars ($^{\#}P = 0.060 - 0.053 - 0.098$, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$). (**C, D**) Graphical representation of the percentage of single, dual, and triple responses per stimulus with MOE (**C**) and VNO cells (**D**). Data are expressed as a box and whiskers plot Min to Max (median: line, mean: +). Abbreviations: Aqueous, aqueous hair extracts; CAS, wethers; Chloro, chloroform hair extracts; MetOH, methanol hair extracts; MOE, main olfactory epithelium; SAB, sexually active bucks; VNO, vomeronasal organ.

4. Discussion

Our study was established in the context of the male effect, with the potential involvement of an olfactory stimulus in its triggering. We shed light on the dynamics of how MOE and VNO cells in does respond differently to buck hair compounds, influenced by male sexual activity (CAS males vs. SAB) and the does' reproductive status (Season vs. Anestrous vs. OVX).

4.1 Variations of cell responses according to buck sexual activity and doe reproductive status

As we expected, SAB hair compounds induced more cell responses than CAS males in both MOE and VNO of does. This occurred regardless of the reproductive status of females. These results support a difference in the olfactory bouquet of Alpine bucks according to their sexual activity. Such differences have already been demonstrated in the Shiba breed between intact bucks and wethers (Murata et al. 2014). In that study, the authors identified various compounds specific to intact bucks, such as ethyl-branched aldehydes, ketones, and diketones (Murata et al. 2014). However, the non-seasonal reproduction of the Shiba breed does not allow us to generalize the compositions observed in this previous study to the seasonal Alpine breed. Chemical analyses of Alpine bucks are necessary to determine whether seasonal variations in olfactory bouquets exist and how they correlate with male reproductive status. Nonetheless, we can hypothesize that different molecules can activate MOE and

25) = 107.4, $P < 0.001$, Reproductive status: $F(2, 25) = 0.683$, $P = 0.515$, Interaction: $F(2, 25) = 0.962$, $P = 0.396$), (**F**) Urine CAS, Urine SAB, Aqueous CAS, and Aqueous SAB extracts (mixed-effects analysis, Stimulus: $F(1.097, 27.43) = 171.1$, $P < 0.001$, Reproductive status: $F(2, 25) = 3.752$, $P < 0.05$, Interaction: $F(6, 75) = 7.764$, $P < 0.001$). We performed multiple comparisons using Tukey tests. Significance between stimuli is indicated by colored stars and between reproductive status by black stars ($^{\#}P = 0.0695$, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$). Data are expressed as a box and whiskers plot Min to Max (median: line, mean: +). Abbreviations: Aqueous, aqueous hair extracts; CAS, wethers; Chloro, chloroform hair extracts; MetOH, methanol hair extracts; SAB, sexually active bucks; VNO, vomeronasal organ.

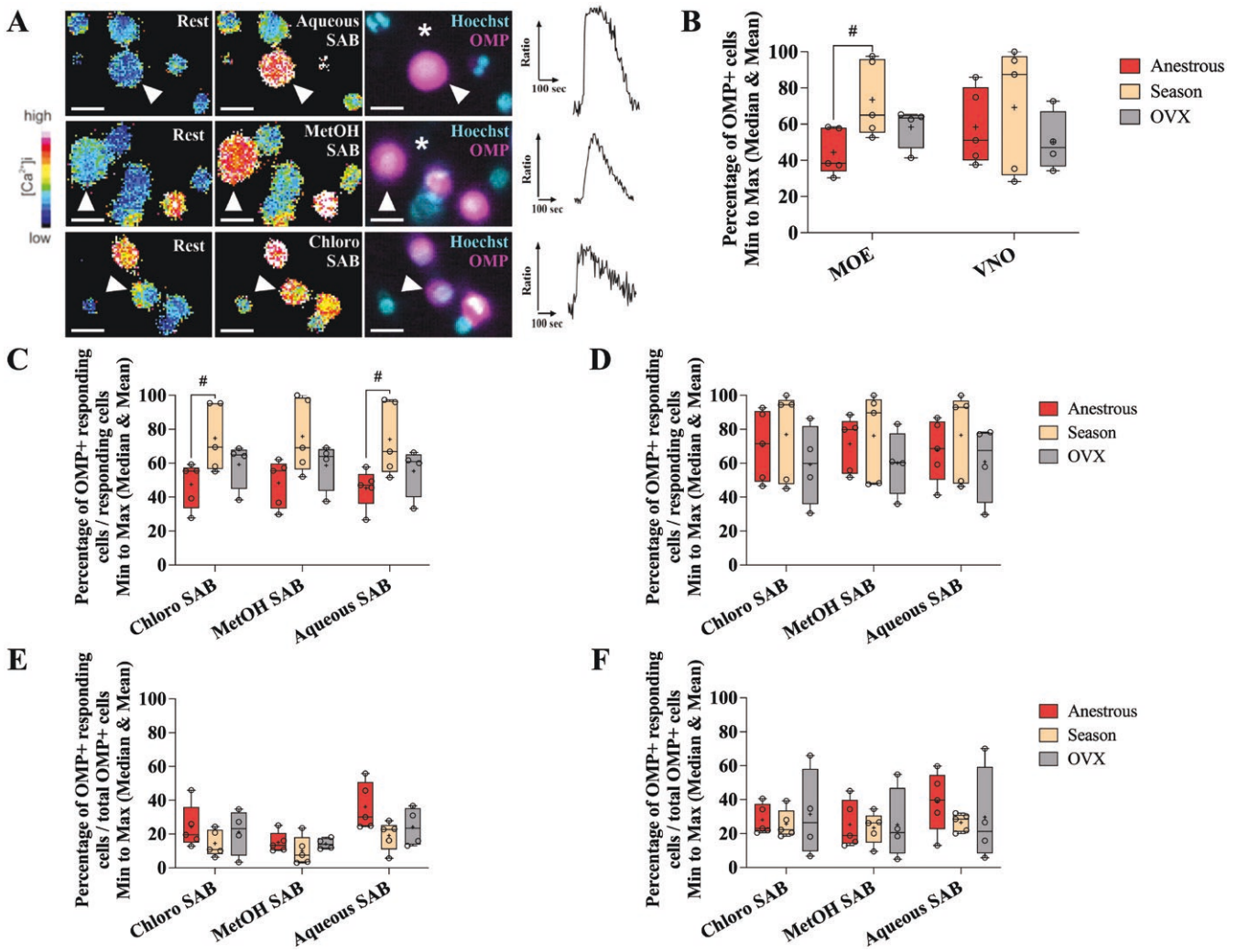

Fig. 6. Activation of mature OSNs and VSNs by SAB hair extracts. MOE and VNO cells were obtained from Anestrous (n = 5), Season (n = 5), and OVX (n = 4) does. Data are expressed as a box and whiskers plot Min to Max (median: line, mean: +). (**A**) Examples of Fura-2 ratio and post-acquisition immunocytochemistry images of Anestrous doe VNO cells indicating that cells activated by Aqueous SAB, MetOH SAB, and Chloro SAB extracts were OMP-positive (magenta). We also performed nuclear labeling (Hoechst, cyan). Representative Ca^{2+} transients for imaged cells (white arrowheads) are indicated for each condition (scale bars, 10 µm). White asterisks indicate cells detached from the coverslip during the acquisition process. (**B**) Percentage of total OMP-positive cells/total MOE and VNO cells. Comparisons were performed using a Kruskal–Wallis test (P = 0.05) followed by multiple comparisons using Dunn tests ($^{\#}P$ = 0.07). (**C**, **D**) Percentage of responding OMP-positive cells/total responding MOE (**C**) and VNO (**D**) cells for a given stimulus. Comparisons were performed using a mixed-effects analysis (MOE, Reproductive status: F(2, 11) = 3.621, P = 0.062, Stimulus: F(1.737, 19.10) = 2.616, P = 0.105, Interaction: F(4, 22) = 0.274, P = 0.892; VNO, Reproductive status: F(2, 11) = 0.621, P = 0.555, Stimulus: F(1.680, 18.48) = 0.084, P = 0.891, Interaction: F(4, 22) = 0.273, P = 0.892), followed by multiple comparisons using Tukey tests ($^{\#}P$ = 0.085 – 0.083). (**E**, **F**) Percentage of responding OMP-positive cells/total OMP-positive MOE (**E**) and VNO (**F**) cells for a given stimulus. Comparisons were performed using a mixed-effects analysis (MOE, Reproductive status: F(2, 11) = 2.474, P = 0.130, Stimulus: F(1.830, 20.13) = 8.923, P < 0.01, Interaction: F(4, 22) = 0.857, P = 0.505; VNO, Reproductive status: F(2, 11) = 0.202, P = 0.820, Stimulus: F(1.617, 17.79) = 1.527, P = 0.243, Interaction: F(4, 22) = 0.575, P = 0.684). Abbreviations: Aqueous, aqueous hair extracts; Chloro, chloroform hair extracts; MetOH, methanol hair extracts; MOE, main olfactory epithelium; OMP, olfactory marker protein; OSN, olfactory sensory neuron; SAB, sexually active bucks; VNO, vomeronasal organ; VSN, vomeronasal sensory neuron.

VNO cells that allow females to discriminate between SAB and CAS males.

Our study shows that the response difference detected between SAB and CAS male samples was observed with each type of extract tested (chloroform, methanol, and aqueous). The exact composition of our 3 extracts of buck hair remains unknown, but it is most likely to vary between them. Chloroform extracts mostly contain apolar hydrophobic molecules like lipids, while the methanol and aqueous extracts essentially contain polar hydrophilic molecules like aldehydes and proteins. Several classes of molecules have been described to show potential implications in the male effect. Apolar hydrophobic compounds such as fatty acids and other categories of lipids induce a rapid LH response in anestrous does (Claus et al. 1990). On the other hand, a relatively polar hydrophilic compound such as 4-ethyloctanal aldehyde induces electrical activity in neurons of the ARC of Shiba females (Murata et al. 2014). This compound appears to be the most promising of the olfactory stimuli provided by the buck (Murata et al. 2014). We intend to test it in the future, but obtaining it—whether through purification, synthesis, or commercial suppliers—has proven extremely difficult. Interestingly, despite the expected difference in composition between extracts, we observed a large proportion of dual- and

triple-responding cells, except for Aqueous SAB in the MOE, represented by a majority of single-responding cells. These data suggest 2 non-mutually exclusive hypotheses. The first is that a given compound could be found in several extracts by being both hydrophilic and hydrophobic (i.e. amphiphilic). The second is that chloroform/methanol extraction, which is longer and more fractionating, could deplete the active molecules, unlike aqueous extraction, which is shorter and less stringent. A detailed analysis of the chemical composition of each SAB hair extract is required to confirm these hypotheses.

As expected, no response differences between urine from SAB and CAS males were observed, and urine induced significantly fewer cell responses than hair extracts. This result is consistent with previous reports showing that buck urine is ineffective in inducing ovulation in females in the context of the male effect (Walkden-Brown et al. 1993). Thus, our results support the hypothesis that the olfactory stimulus involved in the male effect originates predominantly from buck hair (Claus et al. 1990; Hamada et al. 1996; Ichimaru et al. 1999; Murata et al. 2009, 2011).

As we expected, SAB hair compounds, at least the ones contained in chloroform and aqueous extracts, induced more cell responses in Anestrous does than Season does. Interestingly, this result was only observed with MOE cells. In sheep and goats, the MOS seems to play a more critical role than the AOS for detecting, transducing, and integrating social olfactory information, at least in the context of the male effect (Gelez and Fabre-Nys 2004, 2006; Keller and Lévy 2012). Therefore, our data reinforces this hypothesis. Moreover, one V1R receptor gene (*gV1ra1*) is expressed in the goat MOE, which may further contribute to detecting and transmitting male olfactory stimuli to the olfactory bulb (Wakabayashi 2002; Wakabayashi et al. 2007). Our study does not distinguish whether MOE-responding cells express an OR coupled to a G_{olf} protein or the gV1ra1 receptor coupled to a $G\alpha_{i2}$ protein (Wakabayashi et al. 2007). These two G protein-coupled receptors activate distinct signaling pathways: ORs-G_{olf} are associated with the adenylate cyclase pathway, while V1Rs-$G\alpha_{i2}$ are linked to the phospholipase C pathway (Tirindelli et al. 2009). Inhibiting these pathways in Ca^{2+} imaging experiments could provide insights into the receptor types involved in detecting compounds in SAB hair extracts. Regarding the VNO, given the high number of responding cells, we cannot exclude a significant role for this sensory organ in the detection of male-derived compounds.

Contrary to our initial hypothesis, OVX does showed fewer responses than Anestrous does in MOE and VNO cells, and less than Season does only in VNO cells. Based on their response to the male effect (Rincón et al. 2011), we expected OVX does to respond as much as Anestrous does and more than Season does. Steroid hormones may play a role in these observations. In females, estradiol and progesterone are central regulators of reproductive function, enabling both breeding seasons and ovarian cycles to be established (Goodman and Karsch 1980; Goodman et al. 1982, 2010; Martin et al. 1983). However, olfactory stimulation seems able to bypass this central regulation, inducing LH pulsatility during the anestrous period (Claus et al. 1990; Walkden-Brown et al. 1993; Vielma et al. 2009) when estradiol appears to repress the gonadotropic axis strongly (Goodman et al. 1982, 2010; Martin et al. 1983). Steroid hormones may also act locally on sensory neurons, affecting the olfactory sensory perception of does. In the mouse VNO, progesterone has been shown to provide a down-regulating effect by silencing the sensory responses of some VSN populations (Dey et al. 2015). Estradiol has also been shown to stimulate vomeronasal progenitor cell proliferation in the mouse VNO epithelium (Oboti et al. 2015). In goats, this regulation could be more critical in the MOE than in the VNO. As our Season does were euthanized during the luteal phase (i.e. with high progesterone concentrations), so that the down-regulatory effect could be more significant and lead to a reduction in olfactory sensory perception. We also cannot exclude a substantial increase in olfactory sensory perception during the estrus period, which is a mating-seeking phase characterized by high concentrations of estradiol (Chemineau et al. 1982). The absence of steroid hormones in OVX does could be thus considered as a basal state of olfactory sensory perception.

4.2 Activation of mature OSNs and VSNs by SAB hair compounds

We used anti-OMP immunocytochemistry in the final microscope acquisition field to identify mature OSNs and VSNs. Season does tend to have more mature OSNs in the MOE compared to Anestrous does. However, the significant difference between the 2 groups was diminished when the OVX group was included in the statistical analysis. These findings are consistent with our previous study on pubertal transition in does, which showed that cyclic sexually naïve or male-exposed pubescent does displayed more mature OSNs than non-cyclic prepubescent does (Meunier et al. 2024). This supports our hypothesis that hormonal changes associated with pubertal transition and seasonal reproduction may drive sensory plasticity mechanisms in the MOE. Similar mechanisms have been observed in mice (MOE and VNO: van der Linden et al. 2018) and rabbits (VNO only: Villamayor et al. 2022) depending on olfactory experience. Addressing the expression of specific goat ORs (Octura et al. 2018) and VRs (Wakabayashi et al. 2007; Kondoh et al. 2022) could shed light on these sensory plasticity mechanisms.

We also analyzed the percentage of mature responding OSNs and VSNs per stimulus. We only observed that cell responses to chloroform and aqueous extracts of SAB hair tended to come more from mature OSNs in Season does (>74%) than Anestrous does (<48%). This difference was less pronounced when including the OVX group in the statistical analysis, highlighting a correlation with the total percentage of mature OSNs. Moreover, most single-, dual-, and triple-responding cells in the MOE of SEASON does were mature OSNs (>62%). This was not the case for ANESTROUS does (<40%). These observations lead us to ask 2 new questions: (1) Where do most of the responses observed in ANESTROUS does come from? While the identity of OMP-negative cells remains unknown, we speculate that these cells could be immature OSNs. In mice, immature OSNs have been shown to be involved in detecting and transmitting odorants (Huang et al. 2022). Further molecular characterization is necessary to explore this hypothesis in goats. (2) How can most dual- and triple-responding cells be mature OSNs when they respond to different extracts? Two hypotheses arise: first, compounds may be present in all SAB hair extracts and be amphiphilic (see Section 4.1); second, ORs that recognize multiple compounds (Munger et al. 2009). These 2 hypotheses are not mutually exclusive, and chemical analyses are necessary to determine the composition of our SAB hair extracts.

We also examined the percentage of mature responding OSNs/VSNs within the total population of mature OSNs and VSNs. In contrast to the pubertal transition (Meunier et al. 2024), we found no significant differences among the physiological conditions studied. Therefore, these data do not support any modification of cellular identity and response properties within the overall population of mature OSNs and VSNs during the transition between breeding season and anestrous.

4.3 Limitations of elevated external KCl as an excitability indicator for goat MOE and VNO cells

As in our previous study on young females (Meunier et al. 2024), the percentage of cells responding to KCl is greater in MOE (10% to 50%) than in VNO (5% to 25%). OMP-positive cells (i.e. mature OSNs and VSNs) were relatively abundant in our preparations (MOE: 30% to 100%, VNO: 35% to 100%). The percentage of OMP-positive cells responding to KCl (Supplementary Fig. S5) was relatively high in MOE (25% to 85%) but much lower in VNO (2% to 30%). Based on these findings, along with the strong responses observed to SAB extracts, we propose that KCl serves as a good indicator of excitability for MOE cells but not for VNO cells in goats.

In addition, our study also revealed variations in KCl-induced excitability based on reproductive status. Such differences have not been previously reported for olfactory neurons, likely because most studies focus on non-seasonal models and often use KCl solely for normalization. Given the hormonal fluctuations across our experimental groups, particularly in estradiol and progesterone, we suggest that these variations may influence the excitability or baseline reactivity of OSNs and VSNs to elevated external KCl.

Supplementary material

Supplementary material can be found at http://www.chemse.oxfordjournals.org/

Acknowledgments

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Author contributions

M.A.M.: conceptualization, formal analysis, investigation, writing—original draft, and writing—review and editing. E.T.: investigation, software, and writing—review and editing. E.M.: investigation and writing—review and editing. C.P.: investigation and writing—review and editing. A.A.: Investigation and writing—review and editing. P.Chem.: conceptualization and writing—review and editing. P.Cham: conceptualization and writing—review and editing. H.V.: conceptualization, investigation, supervision, and writing—review and editing. M.K.: conceptualization, funding acquisition, supervision, and writing—review and editing.

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Conflict of interest

The authors declare no competing interests.

Data availability

All relevant data and supplementary information can be found within the article.

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