Hair from sexually active bucks strongly activates olfactory sensory inputs but fails to trigger early first ovulation in prepubescent does

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Abstract

Early exposure of does to sexually active bucks triggers early puberty onset correlating with neuroendocrine changes. However, the sensory pathways that are stimulated by the male are still unknown. Here, we assessed whether responses to olfactory stimuli is modulated by social experience (exposure to males or not) and/or endocrine status (prepubescent or pubescent). We used a calcium imaging approach on goat sensory cells from the main olfactory epithelium (MOE) and the vomeronasal organ (VNO). For both cell types, we observed robust responses to active male hair in females under three physiological conditions: prepubescent females isolated from males (ISOL PrePub), pubescent females exposed to males (INT Pub) and isolated females (ISOL Pub). Response analysis showed overall greater proportion of responses to buck hair in ISOL PrePub. We hypothesized that females would be more responsive to active buck hair during the prepubertal period, with numerous responses perhaps originating from immature neurons. We also observed a greater proportion of mature olfactory neurons in the MOE and VNO of INT Pub females suggesting that sexual experience can induce plastic changes on olfactory cell function and organization. To determine whether stimulation by male odor can advance puberty, we exposed prepubescent

does to active buck hair (ODOR). In both ODOR and females isolated from males (ISOL) groups, puberty was reached one month after females exposed to intact bucks (INT), suggesting that olfactory stimulation is not sufficient to trigger puberty.

Keywords

Puberty, Sociosexual relationship, Goats, Calcium Imaging, Olfactory stimuli

1. Introduction

Puberty marks the transition from a sexually immature juvenile stage to a sexually mature adult stage. This complex process encompasses morphological, physiological and behavioral changes [1]. In seasonally reproducing species such as sheep and goats, the reactivation of the hypothalamic-pituitary-gonadal axis marks the onset of puberty and can be modulated by a variety of internal and external factors [2,3]. Photoperiod appears to be the main environmental factor driving puberty onset, since puberty only occurs during the breeding season [4,5]. However, the relative weight of another key factor, namely social environment, has recently been reconsidered in this context [6].

Sociosexual relationships commonly modulate the reproductive function of many species including female small ruminants. Exposure to a sexually active male has been shown to induce ovulation in females during the period of sexual rest (known as anestrous), the so-called "male effect" [7]. More recently, exposure to a sexually active buck has also been shown to induce an early puberty in does about 1.5 months earlier than females exposed to wethers or isolated from males [8]. Two main observations support the involvement of male sexual activity in this phenomenon. First, wethers (sexually inactive males) have no effect on the timing of first ovulation in females [8,9]. Second, females exposed to intact bucks reach puberty only when the latter become sexually active during the breeding season [8,9]. Somatosensory stimuli seem to be dispensable to induce early puberty onset, since the removal of direct contact with bucks can still induce this precocious puberty [8]. Yet, active male sensory stimuli responsible for the early neuroendocrine changes surrounding female puberty remain unknown [9].

In rodents, a similar effect of male-induced early puberty has been described for many decades: the “Vandenbergh effect” [10,11]. The special feature of this effect is that female puberty can be triggered by a male or his odor [12,13]. The “Vandenbergh effect” is one of the many effects caused by olfactory stimuli [14]. These olfactory stimuli are mainly detected by two structures: the main olfactory epithelium (MOE) and the vomeronasal organ (VNO). These two structures contain bipolar neurons called olfactory sensory neurons (OSNs) and vomeronasal sensory neurons (VSNs) respectively. Olfactory stimuli are detected by G protein-coupled receptors: olfactory receptors (ORs) for OSNs and vomeronasal receptors (VRs) for VSNs [15,16]. Interestingly, surgical lesion of the VNO [17–19] and genetic inactivation of VSNs [20,21] prevents the onset of the “Vandenbergh effect” in mice, indicating a major role for this olfactory sensory input.

In small ruminants, olfactory stimuli have been reported to play a role on reproductive function in the context of the "male effect". In does, exposure to buck hair induces electrical activity in neurons of the hypothalamic arcuate nucleus region (ARC), known to contain kisspeptin neurons that are essential for reproductive function [22,23]. This exposure leads to the pulsatile secretion of luteinizing hormone (LH), which can induce ovulation [24,25]. Interestingly, destruction of the MOE of anestrous females with zinc sulfate does not fully suppress the response to the male effect [26]. These data, together with other studies point to a major role of the main olfactory system (MOS), including the MOE, on the detection and integration of male olfactory stimuli [27–29]. However, these studies do not exclude the involvement of the accessory olfactory system (AOS), and hence of the VNO, on this effect [26,28]. Thus, the contribution of the MOE and VNO to the detection of male-derived olfactory signals remains to be elucidated.

In this study, we tested the hypothesis that olfactory stimuli from sexually active bucks could trigger early puberty onset in does. First, we investigated whether olfactory stimuli detection could be modulated by social experience (exposure to males or not) and/or endocrine status (prepubescent or pubescent). To answer this question, we used a calcium (Ca^{2+}) imaging approach previously developed in our laboratory on freshly dissociated adult doe MOE and VNO cells [30, in press]. This approach allows the measurement of intracellular Ca^{2+} transients in response to a given stimulus. This method has been previously used to study the role of VNO and MOE-specific neuronal populations in the emergence of social behaviors in mice [31–33]. Then, we exposed prepubescent females to hair from sexually active bucks to test whether such olfactory stimulation can trigger male-induced early puberty.

2. Materials and methods

2.1 Experiment 1: Is the detection of olfactory stimuli from sexually active males by females dependent on social experience and/or endocrine status?

2.1.1 Animals

Experiments were conducted between August 2021 and December 2022 in Nouzilly, France (latitude 47° 32N and longitude 0° 46E) on Alpine goats (*Capra hircus*). We used 13 does for this experiment. A first batch of does was born between January 15th and January 25th, 2021,

and a second batch between January 11th and January 31st, 2022. All females were weaned at 2.5 months of age and then randomly allocated to a group during their year of birth:

- ISOL PrePub (n = 4): females housed in a barn with no males and euthanized during the prepubertal period (in August, at about 8 months old)
- ISOL Pub (n = 4): females housed in a barn with no males and euthanized when they were pubescent, during the luteal phase of the reproductive cycle (between November and December, at about 11-12 months of age). Females reached puberty at 11 months of age
- INT Pub (n = 5): females were exposed to intact bucks from mid-August to induce early puberty in September [9]. They were euthanized during the luteal phase (between November and December, at about 11-12 months of age). Females reached puberty at 11 months of age

To prevent odor contamination, doe groups were housed in different barns (distance > 300 m). Animals were fed daily using commercial pellets, hay and straw, with free access to water and mineral blocks. Blood samples were collected twice a week in 5 ml heparinized tubes by jugular venipuncture to measure progesterone levels. We considered that two consecutive progesterone concentration values above 1 ng ml^{-1} reflected the first ovulation and therefore the onset of puberty [9,34]. All procedures were performed following European directive 2010/63/EU on the protection of animals used for scientific purposes and were approved by the local ethics committee for animal experimentation (CEEA VdL, Tours, France, n°2021040111221610 and n°2022052309503651).

2.1.2 Collection of male stimuli for Ca^{2+} imaging

We collected beard hair, back hair, and urine from five sexually experienced Alpine bucks during the breeding season (November). We considered these males as “sexually active” because intact bucks exhibit high sexual activity from late summer to late winter [35,36]. As for urine, hair from all males was pooled and stored in clean glass containers at -20°C before use.

2.1.3 Ca^{2+} imaging on female VNO/MOE cells

We developed a Ca^{2+} imaging approach on freshly dissociated goat VNO/MOE cells [30, in press]. Does were euthanized by intravenous injection of 5 ml ketamine (Ketamidor®, Axience) followed by decapitation by a licensed butcher. Each skull was cut in half in the

sagittal plane, 2 cm from the midline. Nasal bones were removed to gain access to the MOE and VNO (**Fig. 1**). The two olfactory structures were dissected in parallel in two different Petri dishes. The epithelia were detached from the cartilage and dissociated in a PBS solution containing papain (2.2 units ml^{-1}, Merck) and supplemented with 40 mM urea (Merck) for the MOE. Tissue pieces were then incubated 20 min at 37°C. After dissociation, a DNase I solution (25 units ml^{-1}, Thermo Scientific) was added followed by DMEM supplemented with 10% FBS and penicillin/streptomycin (Gibco), centrifugation was done for 5 min at 1000 rpm. Dissociated cells were resuspended in DMEM-FBS medium and plated on coverslips previously coated with concanavalin-A (0.5 $\mu g\ \mu l^{-1}$, overnight at 4°C, Merck). Cells were then incubated for 45 min at 37°C with 5% CO_2 and washed in imaging buffer containing HBSS medium (Gibco) supplemented with 5 mM HEPES (2-[4-(2-hydroxyethyl) piperazin-1-yl]ethanesulfonic acid, Merck). Cells were used immediately for imaging after loading with fura-2/AM (Thermo Scientific) for at least 30 min at room temperature. Each coverslip containing cells was placed in a perfusion chamber (Warner Instruments) on an Olympus IX71 microscope and connected to a system allowing constant perfusion of each stimulus. During acquisition, cells were alternately illuminated at 340 and 380 nm, and the light emitted (500-560 nm) was recorded by a Hamamatsu C10600-10B camera fitted to the microscope (x10 magnification). Perfusion was alternated between 3 min of imaging buffer and 30 s of stimulus solution. Images were acquired at 0.25 Hz.

Each stimulus was prepared in imaging buffer. Hair extracts (back and beard) were prepared by mixing 0.2 g sample in 15 ml imaging buffer for 2 h followed by filtration. Urine pool of sexually active bucks was diluted 1:100. The sequence of stimuli was randomly designated for each coverslip and always ended with application of a high KCl solution (150 mM) as a viability control. Three to four coverslips per organ and animal were used.

2.1.4 Immunocytochemistry for OMP after Ca^{2+} imaging

We performed immunocytochemistry directly on the same imaged MOE cell field on one coverslip of each animal studied. This immunocytochemistry was directed against the Olfactory Marker Protein (OMP) to determine whether the observed responses to stimuli were specific to mature OSNs. The protocol is the same as described [30, in press]. In brief, we fixed the dissociated MOE cells with 4% paraformaldehyde (Merck) for 20 min directly in the perfusion chamber on the microscope after imaging acquisition. After 5 min washing with imaging buffer, cells were pre-incubated for 30 min with a saturation solution containing Tris-buffered saline (TBS), 0.1 Triton and 2 % normal donkey serum (Merck). Cells were then

incubated for 30 min with a goat anti-OMP primary antibody (1:2000, FUJIFILM Wako Chemicals, RRID: AB_664696) diluted in the saturation solution. After a further 5 min wash in imaging buffer, cells were incubated for 30 min with Alexa Fluor 647 donkey anti-goat secondary antibody (1:500, Thermo Scientific, RRID: AB_2535864) diluted in TBS-Triton 0.1. After 5 min wash, a Hoechst solution (1:5000, Thermo Scientific) diluted in TBS was added for 2 min, and then the cells were washed again for 5 min. Fluorescence images were acquired using a Hamamatsu C10600-10B camera fitted to an Olympus IX71 microscope (x10 magnification). OMP-positive cells (mature OSNs) were counted using ImageJ software (FIJI).

The same immunocytochemistry protocol was used on a VNO coverslip for each animal. The secondary antibody used was donkey anti-goat Cy3 (1:500, Jackson ImmunoResearch, RRID: AB_2307351). Although this step did not allow us to match this labelling with cell responses, it did enable us to establish a percentage of mature VSNs in our preparations. Fluorescence images were acquired using a Zeiss LSM 780 confocal microscope (x20 magnification, z-stack followed by the maximum intensity projection feature). OMP-positive cells (considered as mature VSNs) were counted using QuPath software (version 0.3.1).

2.1.5 Data analysis

Ca^{2+} imaging image stacks were analyzed using ImageJ software (FIJI), including background subtraction, detection of regions of interest (ROIs) and measurement of mean pixel values. Semi-automated analysis of ligand-induced responses was performed using an open source software dedicated to Ca^{2+} data analysis: calipR. The analysis consists in first clearing low quality cells (with missing or 0 values more than 10% of the time). Then, estimation and correction for long-term fluorescence fluctuations that do not match the kinetic of cell responses. Inspired by [37], this step is achieved by identifying the potential cell signal (with the Derivative Passing Accumulation (DPA) method [38]) and then fitting a Generalized Additive Model (GAM) on the remaining background trace. Data are then normalized by converting the denoised fluorescence values to z scores using the baseline period as reference (negative control represented by the imaging buffer at the start of acquisition previous to stimulation). Peaks of interest are then identified by deconvolution [39] coupled with thresholding. A response is considered positive when signal values exceed 2.5 standard deviations of the baseline. All cells showing any response to the initial imaging buffer application (negative control) were removed from the analysis.

2.2 Experiment 2: Can olfactory stimuli from sexually active bucks trigger precocious puberty in does?

2.2.1 Animals

We conducted experiment 2 between August 22nd and November 22nd in Nouzilly, France (latitude 47° 32N and longitude 0° 46E) on Alpine goats (*Capra hircus*). We used 42 animals for this study: 38 spring-born prepubescent does and 4 sexually experienced intact bucks. Does were born in 2022, between January 11th and February 2nd then weaned at 2.5 months of age. Females were allocated into three groups balanced for bodyweight one week before the start of the experiment. Littermates were also separated to avoid genetic effects. Animals were fed daily using commercial pellets, hay and straw, with free access to water and mineral blocks. To ensure that the average body weight between groups was balanced and to detect any potential metabolic effects, does were weighed once a month.

All procedures were performed following European directive 2010/63/EU on the protection of animals used for scientific purposes and were approved by the local ethics committee for animal experimentation (CEEA VdL, Tours, France, n°2022052309503651).

2.2.2 Exposure to bucks and their odors

To prevent odor contamination, doe groups were housed in different barns (distance > 300 m). From August 22nd until November 22nd, one group of does was isolated from bucks (ISOL, n = 11) and a second group was continuously exposed to intact bucks (INT, n = 13; **Fig. 2A**). INT group was exposed to males in the adjacent enclosure, separated by a barrier allowing direct contact between sexes (4 males for 13 females). Twice a week, one buck fitted with an apron was introduced into the female pen for 1 h to allow direct contact and behavioral teasing. Each week, bucks were switched to prevent any possible habituation and interindividual effects.

A third group of does was continuously exposed to sexually active buck hair (ODOR, n = 14). Hair was collected from 78 sexually active bucks (during the breeding season or after photoperiodic treatment) on the neck, head, shoulders and beard. Hair collected from these regions was shown to contain olfactory stimuli capable of triggering electrical activity in the neurons of the hypothalamus of exposed females and the secretion of LH [40]. Females were exposed to the stimulus using a perforated polyvinyl chloride device suspended with a rope in the enclosure (**Fig. 2B**). This device was available in triplicate for each group of does (empty

for ISOL and INT groups). For ODOR group, two cloth bags containing about 50 g of hair were placed inside the device. Twice a week, we added around 20 g of hair to each device. The contents of the bags were changed every 4 weeks. To amplify the exposure to the olfactory stimulus, we stimulated the females 5 times a week by placing a hair-filled gauze on the muzzle for 15 s and rubbing a handful of hair on the palate.

2.2.3 Assessment of male sexual activity

To assess male sexual activity, we collected blood samples twice a month by jugular venipuncture in order to measure plasma testosterone levels. We considered a buck to be "sexually active" when testosterone concentrations exceeded 10 ng ml^{-1} [8,9]. We also used testicular weight and odor intensity as markers of male sexual status. Testicular weight was estimated by assessing testicular volume with an orchidometer every two weeks [35]. We also subjectively assessed odor intensity using the Walkden-Brown *et al.* method [25]. Once a week, an operator sniffed each buck at about 10 cm from the neck and assigned a score of 0 (neutral odor), 1 (weak buck odor), 2 (moderate buck odor) or 3 (strong buck odor).

We also assessed buck sexual behavior during bi-weekly introduction into the female pen. We measured the number of buck/doe physical contacts, flehmens, ano-genital sniffs, lateral approaches and mounts [41,42].

2.2.4 Evaluation of puberty onset

We determined the timing of puberty onset by assessing ovulatory activity. Twice a week, we collected blood samples by jugular venipuncture in 5 ml heparinized tubes to measure plasma progesterone concentrations. All females were sampled from August 22nd. We considered progesterone levels to reflect ovulation when they exceeded 1 ng ml^{-1} on two consecutive values [9,34]. An ovulating female was therefore designated as pubescent.

2.3 Hormone assays

To collect the plasma, blood samples were centrifuged for 30 min at 4000 g. Plasma testosterone levels of bucks were determined by radioimmunoassay (sensitivity of 0.1 ng ml^{-1}) [43]. Plasma progesterone levels of does were measured by enzyme immunoassay (sensitivity of 0.25 ng ml^{-1}) [44]. The intra-and inter- assay coefficients of variation were < 9 % and < 8.7 %, respectively.

2.4 Statistical analysis

Ca^{2+} imaging and immunocytochemistry data (experiment 1) are expressed as proportions. For each stimulus in each experimental group, the total number of responding cells was extracted and expressed as a proportion of the total cell number in this group. Group proportions were statistically compared in R (version 4.3.0) with the Fisher's exact test as implemented in the function « fisher.multcomp » from the « nparcomp » package. The false discovery rate was controlled by the Benjamini & Hochberg method [45]. We used a Spearman correlation test followed by a permutation test for linear models ("lmPerm" package) to assess the relationship between the proportion of OMP+ MOE cells among responding cells and the total proportion of OMP+ cells.

Data from experiment 2 are reported as mean ± SEM (standard error of the mean). Statistical tests were performed using GraphPad software (GraphPad Prism 10.0).

We performed parametric tests when data were both normally distributed (Shapiro-Wilk test: $p > 0.05$) and showed homogenous variances between groups. Otherwise, we performed non-parametric equivalent tests. The number of occurrences of each buck sexual behavior was compared as a function of time by pairwise comparisons using Wilcoxon rank sum test with Bonferroni correction. The area under the curve (AUC) was evaluated to compare the cumulative plasma progesterone secretion with a one-way ANOVA test followed by a Tukey post hoc test. Age at puberty was assessed using Kaplan–Meier survival analysis and was compared using the Log-rank test.

3. Results

3.1 Experiment 1: Is the detection of olfactory stimuli from sexually active males by females dependent on social experience and/or endocrine status?

3.1.1 Dissociated doe MOE and VNO cells respond to olfactory stimuli from sexually active bucks

We used a live-cell Ca^{2+} imaging approach to investigate doe olfactory sensory organ cells ability to respond to different sexually active buck compounds and identify the most potent stimuli. Freshly dissociated MOE and VNO cells loaded with fura-2 responded to diluted urine and aqueous hair extracts from sexually active males. Ca^{2+} transients were observed in response to these stimuli regardless of the reproductive status and social environment of the sampled females (**Fig. 3A**).

We observed a significantly higher proportion of MOE-responding cells to urine from active males in ISOL Pub females (979 of 14727, 6.65 %) when compared to ISOL PrePub females (*vs* 1086 of 20447, 5.31 %; Fisher's exact test: $p < 0.001$) and INT Pub females (*vs* 668 of 14756, 4.53 %; Fisher's exact test: $p < 0.001$) (**Fig. 3B**). The proportion of responses was also significantly higher in ISOL PrePub does than in INT Pub does (Fisher's exact test: $p < 0.01$). No significant difference was found between groups in response to urine from active bucks in VNO cells (Fisher's exact test: $p > 0.05$). A significantly higher proportion of MOE cells were activated by male hair in ISOL PrePub does (1586 of 20447, 7.76 %) compared to ISOL Pub does (*vs* 869 of 14727, 5.90 %; Fisher's exact test: $p < 0.001$) and INT Pub does (*vs* 784 of 14756, 5.31 %; Fisher's exact test: $p < 0.001$). Moreover, the proportion of responses was significantly higher in ISOL Pub females than in INT Pub females (Fisher's exact test: $p < 0.05$). We also observed a significantly higher proportion of responses to active male hair in VNO cells from ISOL PrePub (1035 of 16187, 6.39 %) females than from ISOL Pub (*vs* 590 of 15895, 3.71 %; Fisher's exact test: $p < 0.001$) and INT Pub (*vs* 397 of 9369, 5.29 %; Fisher's exact test: $p < 0.001$) females. By contrast, the proportion of responses was significantly higher in INT Pub females than in ISOL Pub females (Fisher's exact test: $p < 0.001$) (**Fig. 3B**).

We also analyzed responses to high KCl depolarizing solution (150 mM) to estimate the proportion of excitable cells in our preparation (**Fig. 3C**). The proportion of MOE responses to high KCl ranged between 30-50 %, and was significantly lower in the INT Pub (4641 of 14759, 31.45 %) group than in the ISOL PrePub (*vs* 10070 of 20447, 49.25 %; Fisher's exact test: $p < 0.001$) and ISOL Pub (*vs* 7252 of 14727, 49.24 %; Fisher's exact test: $p < 0.001$) groups. This result suggests that the cell preparation from INT Pub females comprised less mature OSNs. The proportion of VNO cells activated by KCl was nearly 10-fold lower (around 4 %) when compared to MOE cells. VSN responses to high KCl were significantly higher in the ISOL Pub (770 of 15895, 4.84 %) than in ISOL PrePub females (*vs* 682 of 16187, 4.21 %; Fisher's exact test: $p < 0.01$) and INT Pub females (*vs* 353 of 9369, 3.77 %; Fisher's exact test: $p < 0.01$).

3.1.2 Molecular characterization of dissociated MOE cells after Ca^{2+} imaging acquisition

We performed OMP immunocytochemistry to determine whether the Ca^{2+} transients observed in response to active male stimuli originated from mature (OMP-expressing) OSNs of the MOE (**Fig. 4A**).

First, we quantified the proportions of responses from OMP-positive (OMP+) MOE cells from the total cell responses per stimulus in **Fig. 4B**. The proportions of OMP+ cells activated by urine from active bucks ranged from 46 % to 63 %, but no significant differences were found between groups (Fisher's exact test: $p > 0.05$). By contrast, we observed a significantly higher proportion of OMP+ cells responding to active male hair in INT Pub does (195 of 334, 58.38 %) than in ISOL PrePub females (*vs* 114 of 284, 40.14 %; Fisher's exact test: $p < 0.001$) and in ISOL Pub females (*vs* 131 of 307; 42.67 %; Fisher's exact test: $p < 0.001$), but not between ISOL PrePub and ISOL Pub does (Fisher's exact test: $p > 0.05$). In order to confirm whether this result was due to a potential greater specificity of mature OMP+ neurons, we decided to relate these data to the total proportion of MOE OMP+ cells. (**Fig. 4C**). The proportion of OMP+ MOE cells was significantly higher in our INT Pub group (3452 of 5616, 61.47 %) compared with the ISOL PrePub (1638 of 4503, 36.38 %; Fisher's exact test: $p < 0.001$) and ISOL Pub (2795 of 5049, 55.36 %; Fisher's exact test: $p < 0.001$) groups. Furthermore, the proportion of OMP+ cells was higher in ISOL Pub females than in ISOL PrePub females (Fisher's exact test: $p < 0.001$). We computed the Spearman correlation coefficient between OMP+ responding cells and total OMP+ cells. We found a strong positive correlation between these two parameters (Spearman correlation: $r = 0.9212$, $p < 0.001$). To consider the group as a covariate, we built a linear model with permutations (lmPerm). While the group was not a significant predictor of OMP+ responding cells (lmPerm: estimate = 0.4006, $p > 0.05$), total OMP+ cells on each coverslip explained a large and significant amount of the variance in OMP+ responding cells (lmPerm: adjusted *R*-squared = 0.8696, estimate = 0.8989, $p < 0.001$). This analysis therefore indicates that the greater proportion of OMP+ cells responding to this stimulus in INT Pub females is rather due to the increase in the proportion of total OMP+ cells.

Next, we calculated the proportion of MOE OMP+ cells responding to a stimulus among the total population of MOE OMP+ cells (**Fig. 4D**). For active male urine, we observed a significantly higher proportion of responding OMP+ cells among total OMP+ cells in ISOL PrePub does (107 of 1638, 6.53 %) than in ISOL Pub females (*vs* 114 of 2795, 4.08 %; Fisher's exact test: $p < 0.01$) and INT Pub females (vs 157 of 3452, 4.55 %; Fisher's exact test: $p < 0.01$), but not between ISOL Pub and INT Pub does (Fisher's exact test: $p > 0.05$). For active male hair, the proportion of responding OMP+ cells in the OMP+ cell population was significantly higher in ISOL PrePub females (114 of 1638, 6.96 %) than in ISOL Pub females (*vs* 131 of 2795, 4.69 %; Fisher's exact test: $p < 0.01$). There was no significant

difference between ISOL PrePub and ISOL Pub females compared with INT Pub females (vs 195 of 3452, 5.65 %; Fisher's exact test: $p > 0.05$).

We also calculated the proportion of OMP+ cells on additional VNO coverslips (**Fig. S1**). The proportion of OMP+ VNO cells was significantly higher in our INT Pub females (555 of 1659, 33.45 %) than in our ISOL PrePub females (262 of 1664, 15.75 %; Fisher's exact test: $p < 0.001$) and ISOL Pub females (411 of 2483, 14.20 %; Fisher's exact test: $p < 0.001$). No significant differences were found between the ISOL PrePub group and the ISOL Pub group (Fisher's exact test: $p > 0.05$). Overall, these results do not fully recapitulate the observed response rates to high KCl (**Fig. 3C**), particularly in VNO cells, indicating that expression of OMP in mature sensory neurons does not necessarily correlate with high KCl activation.

3.2 Experiment 2: Can olfactory stimuli from sexually active bucks trigger precocious puberty in does?

3.2.1 Male sexual activity

Plasma testosterone concentrations increased during the experiment, exceeding the 10 ng ml^{-1} threshold by the end of August (**Fig. 5A**). The odor score and testicular weight of intact males also increased during the experimentation (**Fig. 5B-C**).

We also assessed the sexual behavior of intact bucks during bi-weekly introduction into the INT female pen (**Fig. 5D**). We observed no significant differences between months for each behavior (paired Wilcoxon rank sum tests: $p > 0.05$). There is a trend between August and October for occurrences of ano-genital sniffing (paired Wilcoxon rank sum test: $p = 0.073$). Interestingly, intact bucks showed lateral approach and mounting behavior from early September onwards. Together, these results indicate that males were sexually active during the testing period.

3.2.2 Onset of ovulatory activity in young does

There was no significant difference in weight between groups during the experiment (**Fig. S2**). Plasma progesterone measures showed that all females continued cyclic activity after their first ovulation (**Fig. 6A**). INT females exhibited three to four ovarian cycles starting between mid-September and early October, whereas in the other two groups, most does displayed two cycles (ISOL: $n = 8$; ODOR: $n = 9$), some only one (ISOL: $n = 1$; ODOR: $n = 3$) and others none (ISOL: $n = 2$; ODOR: $n = 1$). Only one female in the ODOR group showed three ovarian cycles. Moreover, most females in the ISOL and ODOR groups began cycling

between mid-October and early November. We calculated the area under the curve (AUC) to assess cumulative progesterone secretion (**Fig. 6B**) and found that AUC was significantly higher in the INT group than in the ISOL and ODOR groups (one-way ANOVA test: $F_{(2, 35)} = 29.63$, $p < 0.001$; Posthoc Tukey test: $p < 0.001$).

The age at puberty differed significantly between groups of does (**Fig. 6C**). Females exposed to intact bucks (INT) had a first ovulation earlier than females exposed to sexually active buck hair (ODOR) or isolated females (ISOL) (Log-rank test: $\chi 2 = 42.72$, $df = 2$, $p < 0.001$). Importantly, no significant difference was noted between ISOL and ODOR groups. Moreover, half of the does in the INT group were pubescent at 257 days of age (D257), while this ratio was only achieved at D282 and D285, respectively in the ISOL and ODOR groups. The delay compared with the INT group was therefore 25 days for the ISOL group and 28 days for the ODOR group. Together, these results indicate that goats exposed to male odors did not experience a male-induced early puberty onset, unlike those exposed to intact bucks.

4. Discussion

Data from our experiment 1 show that dissociated cells from the doe's two olfactory sensory organs (VNO and MOE) are able to respond to two sexually active buck stimuli (urine and aqueous hair extracts). Further, the endocrine status and sexual experience of the females modulate those responses. Active male hair, a stimulus reported to activate ARC neurons and LH secretion in adult females [22,23,25], activated more VNO and MOE cells in ISOL PrePub females than in the other two groups (MOE: + 1.86 to 2.45 %; VNO: + 1.1 to 2.68 %; **Fig. 3B**). We also found more MOE cell responses from sexually naïve females to active male urine (+ 0.78 to 2.12 %). These results challenge our initial hypothesis, since we assumed that females following a male-induced early puberty protocol (INT Pub) would show more responses due to their prior exposure. VNO and MOE cells from ISOL PrePub females show a higher proportion of responses, suggesting that the ability to detect and potentially transmit olfactory information provided by sexually active bucks may be enhanced during the prepubertal period in does. We therefore aimed to determine whether the observed responses originated from mature sensory neurons using anti-OMP immunocytochemistry.

We analyzed the proportion of responses from mature OSNs in the MOE. We hypothesized that exposure to males may have an impact on the specificity of cell responses to buck hair. Indeed, for INT Pub females, the majority of MOE cell responses (around 58%) originate

from mature OSNs, unlike ISOL PrePub (around 40%) and ISOL Pub (around 43%) females (**Fig. 4B**). However, these results are strongly related to the increase in the total proportion of mature OSNs observed in this group. Therefore, these results do not support the hypothesis that previous exposure to sexually active bucks could be responsible of a higher rate of mature OMP+ responding cells. Next, we investigated the proportion of mature OSNs responding among the overall population of OMP+ cells in the MOE. This proportion was higher in ISOL PrePub does than in ISOL Pub does, no matter the stimulus used (urine: + 2.45 %; hair: + 2.27 %; **Fig. 4D**). For active male urine, this proportion was also higher in ISOL PrePub females than in INT Pub females (+ 1.98 %). These data therefore suggest that puberty and exposure to male odors could modify cellular identity or response properties within the global population of mature OSNs.

Strikingly, many responses do not originate from OMP+ mature OSNs in any group. The identity of these OMP negative cells is unknown, but recent data in mice have shown the capacity and involvement of immature OSNs in the detection and transmission of odorants to the olfactory bulb [46]. Furthermore, immature olfactory neurons often express more than one OR gene [47], being thus possible that some of the responses observed in sexually naïve females (ISOL PrePub and ISOL Pub) originate from immature OSNs. Further research is needed to confirm this hypothesis, including molecular characterization using markers specific to immature neurons.

The total proportion of mature OSNs/VSNs in our primary cultures of dissociated MOE/VNO cells was first assessed. Interestingly, this proportion is always higher in both MOE and VNO of sexually experienced females (INT Pub) than in sexually naive females regardless they have reached puberty or not (ISOL PrePub: MOE, + 25.09 %, VNO, + 17.7 %; and ISOL Pub: MOE, + 6.12 %, VNO, + 19.25 %; **Fig. 4C** and **Fig. S1**). In mice, olfactory experience modulates the abundance of sensory neurons in the MOE and VNO, leading to differences in the expression of certain ORs and VRs [48]. Similar plasticity mechanisms are observed in rabbit VNO [49]. Thus, our results obtained in does could support the hypothesis of plasticity of both olfactory sensory epithelia modulated by exposure to male odors. Apart from the proportion of mature olfactory neurons, exploration of the expression of certain ORs [50] and V1Rs [51,52] in relation to sexual experience may also shed some light on the molecular mechanisms behind this olfactory plasticity. Besides sexual experience, our data show a higher proportion of mature OSNs in ISOL Pub females than in ISOL PrePub (+ 18.98 %) suggesting that the hormonal changes that drive the pubertal transition process could also

have a role in the potential plasticity of the MOE. Such regulatory mechanisms have been already described in mice, where female sex steroids can elicit a profound effect on the proliferation and activation/inhibition of VSNs [53,54]. However, in our study design, pubescent does were 3 to 4 months older than prepubescent ones. Age is thus a potential confounder that could be responsible for the observed difference in mature OSN proportion between ISOL Pub and ISOL PrePub and we cannot rule out this hypothesis.

The percentage of response to high KCl was higher in MOE cells (30-50%) than in VNO cells (3-5%) (**Fig. 3C**), similar to a previous study [30, in press]. However, OMP-positive cells were relatively abundant in VNO coverslips (up to 33.5 %), indicating that a majority of OMP+ VNO cells do not respond to high KCl. The reasons for these reduced responses are unclear, but our results indicate that high KCl activation cannot be considered as a reliable reporter of mature VSNs.

Our experiment 2 confirms that continuous exposure to intact sexually active bucks triggers first ovulation and thus precocious puberty in spring-born does. As shown in previous studies, our intact bucks became sexually active during September with an increase in plasma testosterone concentrations, odor score, testicular weight and the display of behaviors such as lateral approach and mounting [8,9]. On average, the group of exposed females (INT) reached puberty almost 1 month earlier than the ISOL group, which is consistent with previous studies [8,9].

Yet, this effect was not found in the ODOR group. We used active buck hair to stimulate females, since this olfactory stimulus is known to activate ARC neurons and LH secretion, sometimes leading to ovulation in adult females in anestrous [22–25]. We expected that ODOR females would have shown an earlier first ovulation than ISOL females to some extent [25]. However, olfactory stimulation failed to induce any significant change as females in both groups reached puberty within a similar timeframe (**Fig. 6C**). A possible explanation for this result is that prepubescent does are sexually naive and would require a process of "learning" the buck odor to get a full gonadotropic axis response leading to an ovulatory activity [55]. In sheep, a large proportion of sexually naïve young (1-year-old) and adult (2-5 years old) ewes show no LH response after exposure to ram fleece [56], whereas sexually experienced females show a similar LH response to ram odor to that obtained after exposure to male [56]. As our study did not measure LH secretion, we cannot exclude that sexually active buck hair triggers a partial response in exposed prepubescent females.

Another possible explanation could be related to the conditions of our exposure. In fact, we exposed females to free-access devices containing hair following the model of a previous study [25]. Our daily observations at different times of the day showed that the vast majority of females were interacting with the devices. However, coupling the devices with daily manual stimulation was perhaps insufficient to induce ovulatory activity in the exposed does. Other studies in adults have used face masks containing the stimulus of interest, with varying degrees of success in producing endocrine responses in females [24,25]. Unfortunately, this option would be difficult to implement on young, growing individuals, as it would require a significant habituation phase. Additional trials varying the experimental conditions are therefore necessary before concluding that olfaction alone is not sufficient to induce an early puberty onset. Lesional approaches in the MOE and VNO could provide evidence of a potential role of olfactory stimuli in this phenomenon. This still remains as a possible option because, in contrast to the male effect [57], somatosensory stimuli appear to be dispensable to induce early puberty in spring-born does [8]. The possibility that other sensory stimuli (visual and auditory) from the active male might be involved in this phenomenon cannot also be excluded.

5. Conclusion

In summary, our results show that sexually active buck stimuli are able to induce Ca^{2+} transients in dissociated MOE and VNO cells from does. Our data support the hypothesis that doe kids would be more responsive to active buck hair during the prepubertal period, perhaps through the activation of immature sensory neurons. Moreover, female sexual experience and the hormonal changes that drive the pubertal transition process could be involved in potential MOE and VNO plasticity mechanisms. This study also shows that under our conditions, sexually active buck hair alone is not sufficient to elicit an early ovulatory response in prepubescent females.

Authors contributions

Maxime A. Meunier: Conceptualization; formal analysis; investigation; writing – original draft; writing – review and editing. **Chantal Porte:** Investigation; writing – original draft; writing – review and editing. **Hélène Vacher:** Investigation; writing – original draft; writing –

review and editing. **Elliott Trives:** Investigation; software; writing – original draft; writing – review and editing. **Thiago S. Nakahara:** Investigation; writing – original draft; writing – review and editing. **Anne-Charlotte Trouillet:** Conceptualization; writing – original draft; writing – review and editing. **Philippe Chemineau:** Conceptualization; writing – original draft; writing – review and editing. **Pablo Chamero:** Conceptualization; writing – original draft; writing – review and editing. **Matthieu Keller:** Conceptualization; funding acquisition; supervision; writing – original draft; writing – review and editing.

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Disclosures

No competing interests declared.

Data Availability Statement

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

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Figure captions

Fig. 1. Structure of the doe nasal cavity. (**A**) Parasagittal view of the left side of the nasal cavity showing the nasal septum (S) and the vomeronasal organ (VNO, rectangle with dashed borders) after removal of mandible. (**B**) Midsagittal view of the right side of the nasal cavity showing the endoturbinates containing the main olfactory epithelium (MOE, rectangle with dashed borders). Br, brain.

Fig. 2. Experimental design. (**A**) From August 22nd, does were allocated into three groups: one isolated from males (ISOL, n = 11), one exposed to intact bucks (INT, n = 13), and one exposed to sexually active buck hair (ODOR, n = 14). Male stimulation of INT group was continuous with bucks separated from does by a barrier and introduced into the latter's pen twice a week for 1 h to observe sexual behavior. (**B**) Stimulation by the male odor represented by hair (neck, head, beard and shoulders) was also continuous. Hair was disposed in cloth bags placed in polyvinyl chloride devices (three per pen), suspended from a rope. Stimulation was amplified by placing a gauze containing hair over the nostrils of the females for 15 s and rubbing hair on the palate.

Fig. 3. Dissociated doe MOE and VNO cells respond to olfactory stimuli from sexually active bucks. (**A**) Examples of Ca^{2+} transients imaged on dissociated MOE (left) and VNO (right) cells from ISOL PrePub, ISOL Pub and INT Pub does stimulated with diluted urine (1:100) and aqueous extracts of active buck hair. (**B-C**) Analysis of ligand-evoked Ca^{2+} responses on dissociated MOE (left) and VNO (right) cells from ISOL PrePub (n = 4), ISOL Pub (n = 4) and INT Pub does (n = 5). Data are expressed as the percentage of responding cells to a given stimulus divided by the percentage of total cells [Fisher's exact test: *p < 0.05, **p < 0.01, ***p < 0.001, non-significant (ns)]. Number of responding cells over the number of cells analyzed is indicated in each bar. Cells were stimulated with (**B**) diluted urine (1:100) and aqueous extracts of active buck hair, but also with (**C**) high (150 mM) KCl solution as viability control.

Fig. 4. Molecular characterization of dissociated MOE cells after Ca^{2+} imaging acquisition. (**A**) Examples of Fura-2 ratio and post hoc immunocytochemistry images of prepubescent doe MOE cells indicating that cells activated by urine and the aqueous extracts of sexually active buck hair are OMP-positive (magenta). Nuclear labeling (Hoechst, cyan) was also performed (scale bars, 10 µm). Representative Ca^{2+} transients shown for imaged cells (white arrowheads) are indicated for each condition. (**B**) Percentage of responding

mature OSNs / total responding MOE cells for a given stimulus [Fisher's exact test: ***p < 0.001, non-significant (ns)]. MOE cells were obtained from ISOL PrePub (n = 4), ISOL Pub (n = 4) and INT Pub (n = 5) does. Number of responding mature OSNs over the number of total responding MOE cells is indicated in each bar. (**C**) Percentage of total mature OSNs (OMP-positive MOE cells) / total MOE cells in ISOL PrePub (n = 4), ISOL Pub (n = 4) and INT Pub (n = 5) does [Fisher's exact test: ***p < 0.001]. Number of total OMP-positive cells over the number of total cells is indicated in each bar. (**D**) Percentage of responding mature OSNs / total mature OSNs for a given stimulus [Fisher's exact test: **p < 0.01, non-significant (ns)]. MOE cells were obtained from ISOL PrePub (n = 4), ISOL Pub (n = 4) and INT Pub (n = 5) does. Number of responding mature OSNs over the number of total mature OSNs is indicated in each bar.

Fig. 5. Assessment of sexual activity in intact bucks (n = 4). Data are represented as mean ± SEM. (**A**) Plasma testosterone concentrations were measured weekly. The entry of bucks into the breeding season was indicated by values above the 10 ng ml^{-1} threshold. (**B**) Odor score was performed once a week by allocating 0 (neutral odor), 1 (weak male odor), 2 (moderate male odor) or 3 (strong male odor). (**C**) Testicular weight was estimated every two weeks. (**D**) Sexual behavior of bucks was evaluated for 1 h, twice a week during introduction into the female pen. The average occurrence of each behavior was compared between the months [paired Wilcoxon rank sum tests: #p = 0.073, *p < 0.05].

Fig. 6. Variations in plasma progesterone concentrations in young does. From August 22^{nd}, one group of does was isolated from males (ISOL, n = 11), one group was directly exposed to intact bucks (INT, n = 13) and one group was exposed to sexually active buck hair (ODOR, n = 14). Blood samples were collected twice a week. (A) Mean profiles of plasma progesterone secretion for each group. (**B**) Comparison of cumulative progesterone secretion per group represented by the area under the curve (AUC). Data are represented as mean ± SEM [one-way ANOVA test followed by Tukey post hoc analyzes: ***p < 0.001, non-significant (ns)]. (**C**) Proportion of ovulating does. Females were considered pubescent when two consecutive progesterone values were ≥ 1 ng ml^{-1} [Log-rank test: ***p < 0.001].

Supplemental figure captions

Fig. S1. Variations in the percentage of total OMP+ cells on VNO coverslips. Percentage of total mature VSNs (OMP-positive VNO cells) / total VNO cells in ISOL PrePub (n = 4),

ISOL Pub (n = 4) and INT Pub (n = 5) does [Fisher's exact test: ***$p < 0.001$]. Number of total OMP-positive cells over the number of total cells is indicated in each bar.

Fig. S2. Variations in body weight of young does. Does were allocated into three groups: one isolated from males (ISOL, $n = 11$), one exposed to intact bucks (INT, $n = 13$), and one exposed to sexually active buck hair (ODOR, $n = 14$). All individuals from the three groups were weighed monthly from 7 to 11 months of age. Data are shown as mean ± SEM per month and were analyzed using a mixed effects model ($p > 0.05$).

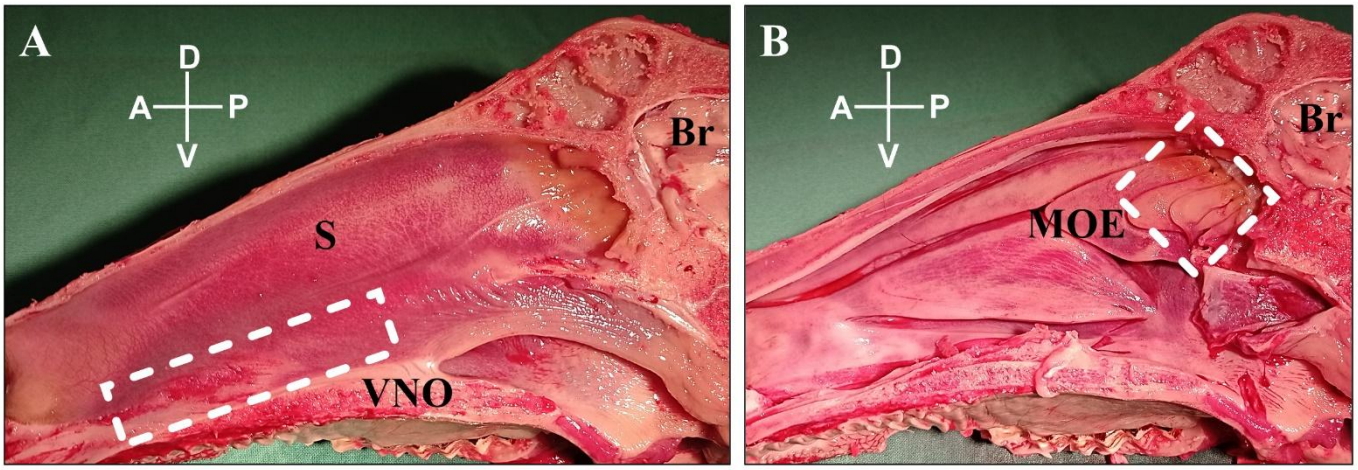
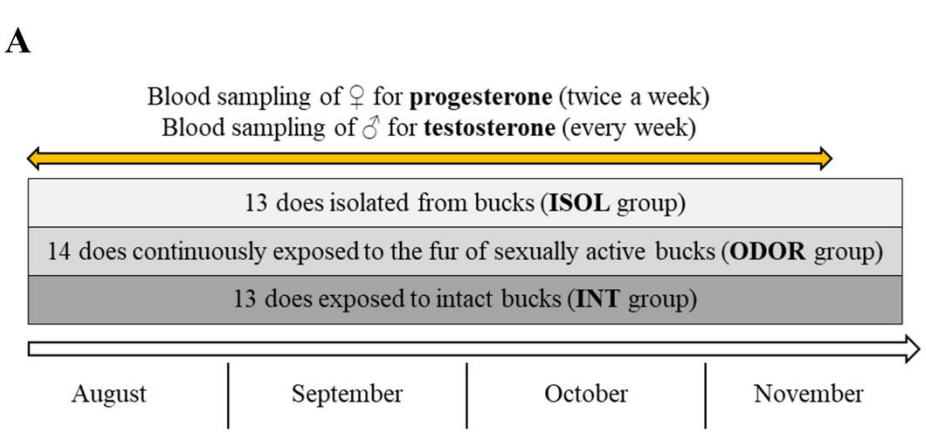


Fig. 1


A
Blood sampling of ♀ for **progesterone** (twice a week)
Blood sampling of ♂ for **testosterone** (every week)
13 does isolated from bucks (**ISOL** group)
14 does continuously exposed to the fur of sexually active bucks (**ODOR** group)
13 does exposed to intact bucks (**INT** group)
August
September
October
November

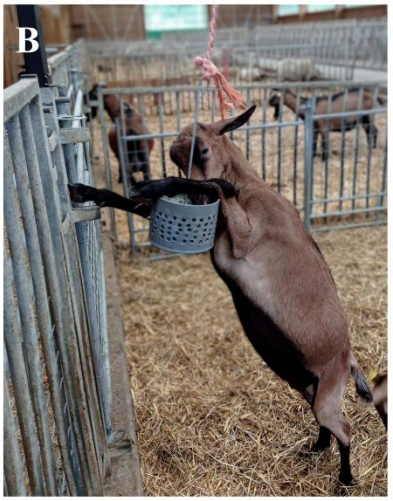
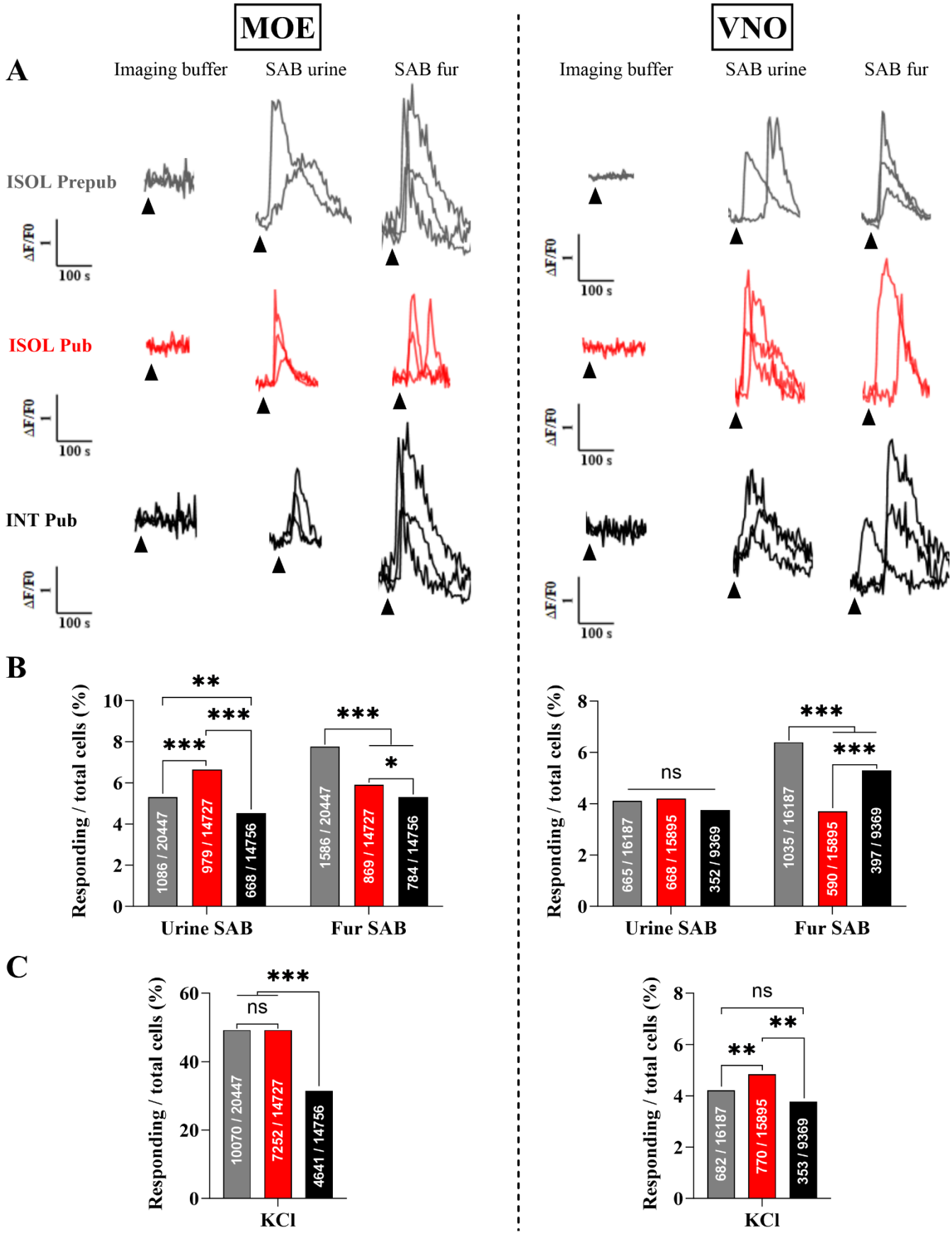

B

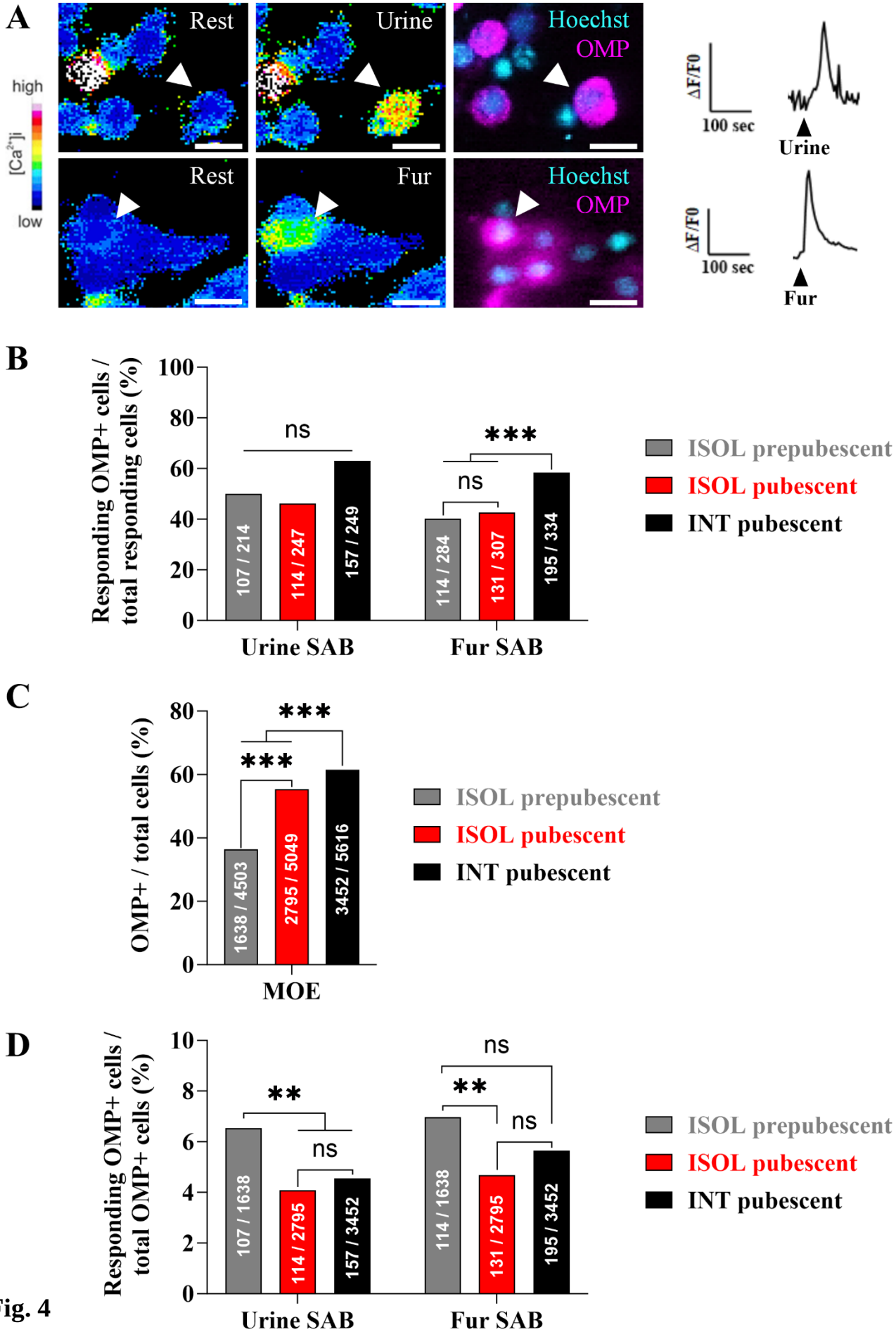
Fig. 2


MOE
VNO
A
Imaging buffer
SAB urine
SAB fur
ISOL Prepub
ISOL Pub
INT Pub
ΔF/F0
1
100 s
B
Responding / total cells (%)
1086 / 20447
979 / 14727
668 / 14756
1586 / 20447
869 / 14727
784 / 14756
665 / 16187
668 / 15895
352 / 9369
1035 / 16187
590 / 15895
397 / 9369
Urine SAB
Fur SAB
ns
C
10070 / 20447
7252 / 14727
4641 / 14756
682 / 16187
770 / 15895
353 / 9369
KCl

821

822 **Fig. 3**

823


A
Rest
Urine
Hoechst
OMP
high
[Ca2+]i
low
Rest
Fur
Hoechst
OMP
ΔF/F0
100 sec
Urine
ΔF/F0
100 sec
Fur
B
Responding OMP+ cells / total responding cells (%)
100
80
60
40
20
0
ns
***
ns
107 / 214
114 / 247
157 / 249
114 / 284
131 / 307
195 / 334
Urine SAB
Fur SAB
ISOL prepubescent
ISOL pubescent
INT pubescent
C
OMP+ / total cells (%)
80
60
40
20
0
***
***
1638 / 4503
2795 / 5049
3452 / 5616
MOE
ISOL prepubescent
ISOL pubescent
INT pubescent
D
Responding OMP+ cells / total OMP+ cells (%)
10
8
6
4
2
0
**
ns
ns
**
ns
107 / 1638
114 / 2795
157 / 3452
114 / 1638
131 / 2795
195 / 3452
Urine SAB
Fur SAB
ISOL prepubescent
ISOL pubescent
INT pubescent

824 **Fig. 4**

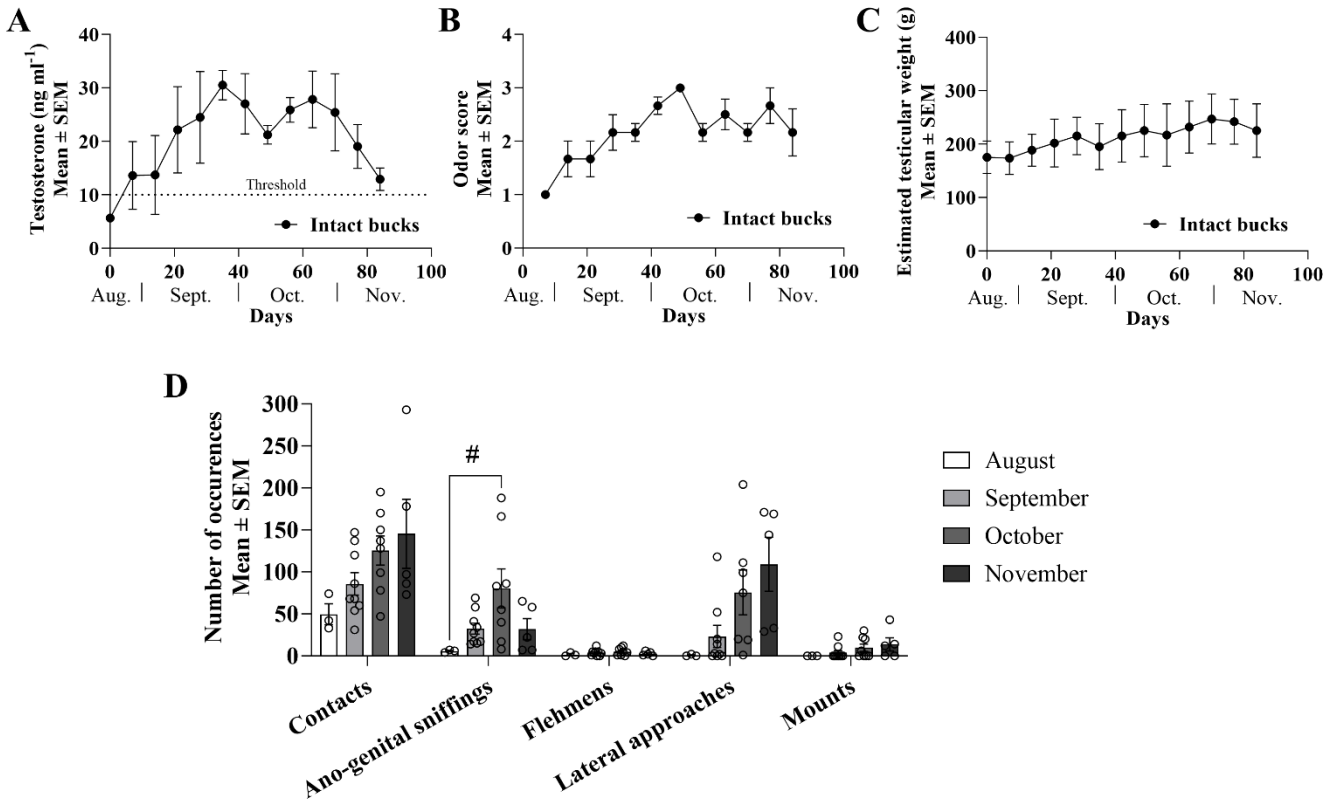

A
Testosterone (ng ml-1)
Mean ± SEM
Threshold
Intact bucks
Aug.
Sept.
Oct.
Nov.
Days
B
Odor score
Mean ± SEM
Intact bucks
C
Estimated testicular weight (g)
Mean ± SEM
Intact bucks
D
Number of occurences
Mean ± SEM
#
August
September
October
November
Contacts
Ano-genital sniffings
Flehmens
Lateral approaches
Mounts

Fig. 5

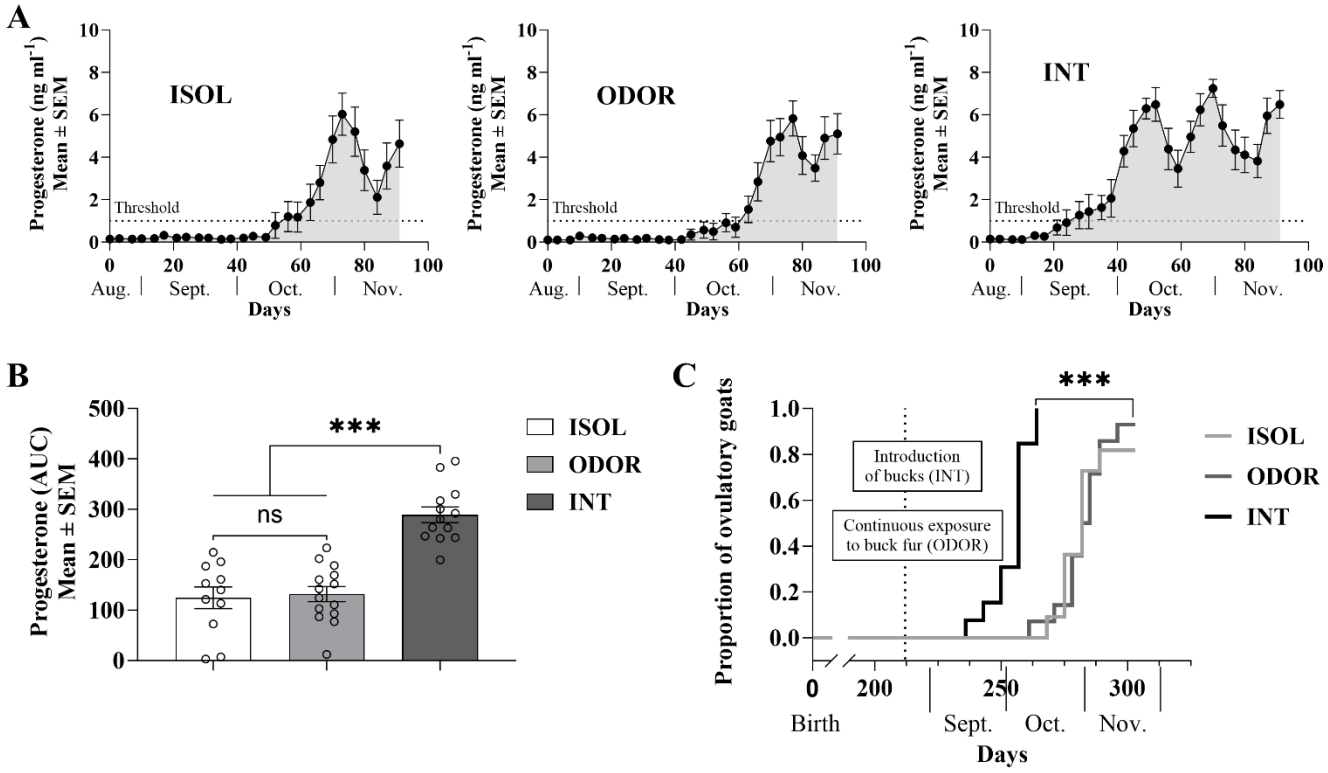
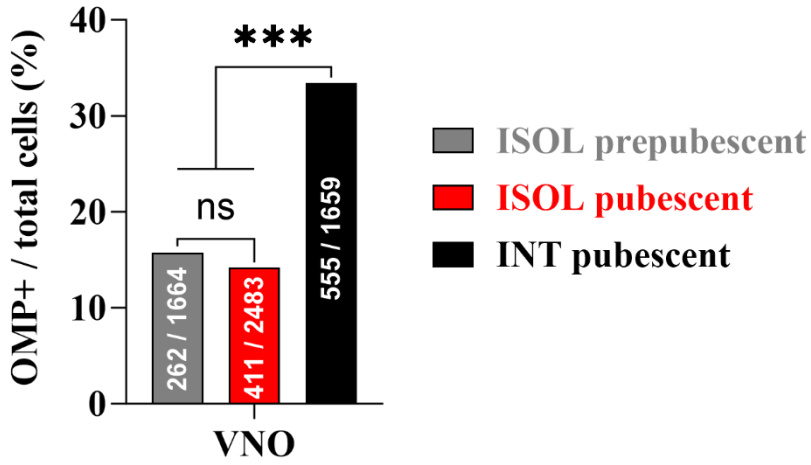

A
ISOL
ODOR
INT
Progesterone (ng ml-1) Mean ± SEM
Threshold
Aug.
Sept.
Oct.
Nov.
Days
B
Progesterone (AUC) Mean ± SEM
***
ns
ISOL
ODOR
INT
C
Proportion of ovulatory goats
Introduction of bucks (INT)
Continuous exposure to buck fur (ODOR)
Birth
Sept.
Oct.
Nov.
Days

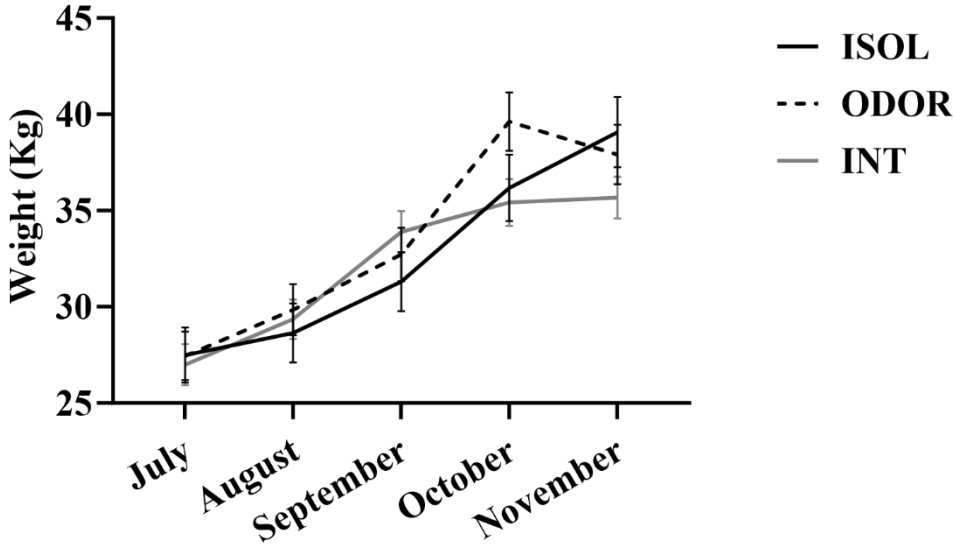
Fig. 6



832

833 **Supp. 1**

834



835

836 **Supp. 2**

837