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## Description of an experimental setup to investigate the impact of olfactory stimuli on behavioural responses in sheep (*Ovis aries*), with a particular focus on minimizing experimental bias through a habituation phase

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### Abstract:

In sheep, the emotional valence of olfactory stimuli needs to be studied as olfaction is crucial for social and dietary preferences. To this end, it is necessary to evaluate the behavioural responses induced by olfactory stimuli (OS). These latter have often been explored with animals tested alone, in odorized versus non-odorized food choice tests or with constraining procedures. All these conditions are likely to induce emotional behaviours, which questions the specificity and reproducibility of behavioural responses induced by the OS. This study aimed to create an experimental setup for investigating the impact of OS on behavioural responses while addressing social and food resources and avoiding other confounding factors. Particularly, we focused on minimizing experimental bias through a habituation phase. To test our setup, we examined the consistency of behavioural responses measured in the control situation over time in adult ewes and lambs, by using principal component analysis. We found that the behavioural responses of individuals are similar over time and that the animals were not stressed by the experimental setup. Then, we concluded that it is adapted to explore the repulsive or attractive properties of the OS. Moreover, based on specific behaviours like vocalization, agitation, exploration, social contact, it could be possible to explore the emotional valence of OS.

**Category of the Short Note:** original study

**Experimental approach:** *in vivo*

**Specie:** *Ovis aries*

**Keywords:** ecological context, 3Rs, social and feeding behaviours



## Introduction

Olfaction plays a crucial role in the development and expression of social (1-3) and dietary (4,5) preferences in sheep. Sheep also use olfaction to detect and avoid sources of danger, such as predators (6,7) or parasitic diseases (8).

Different experimental strategies have been used to study the role of olfaction on behaviour. For example, congener recognition has been studied in animals that have undergone pharmacological treatment to render them anosmic, *i.e.* unable to perceive odours (3). To test the repellent effect of certain predator olfactory stimuli (OS), animals were fasted, alone and exposed to a novel environment (9). In this case, it is common to use faeces placed in or near the food, to subject the animal to a choice test and to compare the quantities of food ingested in the presence or absence of the odour (9,10). Although olfaction is a sensory modality that has been mentioned for its potential role in improving animal welfare (11,12) and in recognising the emotions of others (13,14), studies on the effects of OS on emotions in sheep remain rare.

According to appraisal theory, emotion, which is short-lived, is induced by a specific event and modulated by internal and external factors (15). It is therefore essential to control and standardise these factors in order to demonstrate that an emotion is specifically induced by a stimulus, particularly an OS, and not simply by these other factors. However, in the studies mentioned above, which were not designed to measure emotions, several confounding factors co-exist (novel environment, starvation, social isolation etc), which even on their own are likely to induce emotion. In this context, some studies have attempted to reduce stress bias by including a habituation phase or by testing animals without complete separation from their companions (6,7). Furthermore, to control the OS diffusion, some authors choose to use odorized masks (6). But this is detrimental to the measurement of emotion by impeding the emission of vocalisations, a key emotional indicator (16,17). These studies highlight the difficulty of ensuring the repeatability and reproducibility of measuring the emotional valence of odours in accordance with the 3Rs.

In this short-note, we present an experimental setup designed to minimize potential bias by emphasizing a rigorous habituation phase before olfactory testing, that could be used to explore the emotional valence of OS. In this aim we measured behavioural responses in a non-stressful environment, with social and food resources, and without severe experimental procedures to the animal, and evaluate their consistency over time. The protocol firstly excludes any procedures that invasively alter the individual's olfactory abilities, and secondly avoids confounding factors inherent in other experimental setups such as social isolation, starvation, novelty and constraint. We have opted to publish the detailed habituation protocol and its associated data here, separate from our full behavioural analysis, to ensure transparency and reproducibility, and to highlight the critical role of habituation in reducing experimental bias. We propose that this experimental setup will reduce potential experimental bias by focusing on the habituation phase and improve reproducibility of data. To this end, the experimental setup was tested on three groups of Ile de France sheep of different ages: adult ewes aged 4-6 years, male and female lambs aged 3 and 4 months (see Table 1 in the Material and Methods section).

## Results

### Strategy for limiting stress bias

The aim was to avoid stress that may be caused by a novel environment, the test room, the experimental setup (including a box used for delivering the OS, Figure 1) and all events associated with the procedure. Firstly, in order to limit the movements and manipulations of the animals by the experimenter, we recommend that the tests be carried out close to where the animals live, that a waiting area be provided in a room adjacent to the test room, and that the different rooms be connected by corridors (Figure 1C). Finally, we recommend habituating and training the animals before exposing them to the experimental OS, the aimed being to mimic the protocol of OS presentation.

The habituation and training were performed on morning progressively with different steps: familiarization with experimenters in the housing-park, reducing the distance between animals and experimenters, introduction of round green plastic trough, movement in the different rooms in groups of 3 to 5 animals and eating pellets from the green plastic trough placed in the polymethyl methacrylate (PMMA) box (Figure 1). Based on previous studies (16,17), we recommend to perform habituation, training included, for 5 to 10 days. We suggest determining the order of passage based on the animals' voluntary presentation and never leaving the individual alone. Success of habituation and training was assessed by the absence of vocalisations on entering the test corridor, the absence of agitation in the test corridor, the entry of the head into the PMMA box and the ingestion of the pellets.

For the adult ewes, the habituation and training lasted five days (Figure 2A). During the first two days, in the housing-park, they were in presence of the experimenters and then of the round green plastic trough. At this stage, some animals were already eating in the trough. On the third day, the ewes were introduced to the test corridor in groups of three to four. They began the training phase, which aimed to encourage them to put their heads in the PMMA box and eat the pellets in in the green plastic trough inside it (Figure 1D). If this failed, it was possible to separate the training of eating in the round green plastic trough from the training of putting the head in the PMMA box. The two last days, three to four experimental ewes went to the waiting area (Figure 1C). One by one, they entered in the test room, being in visual contact with the social resource when they entered inside the test corridor (Figure 1E), the other experimental animals being in the waiting area (Figure 1C). At this stage, the individual was exposed to a cotton pad impregnated with 1 ml of demineralised water placed in the box, under the grid (Figure 1B,E). After the habituation and training period, the majority of ewes (27/30) approached the PMMA box and put their heads inside to ingest the pellet ration, yielding an overall habituation success rate of 90%.

For the lambs, the habituation and training began two weeks before the experiment. For the first three days, in the housing-park, they were in presence of the experimenters and then of the round green plastic trough. On the fourth day, they were introduced to the test corridor in groups of 5 for 10 minutes and in groups of 3 for 5 minutes the following day, and began the training phase. At the end of the first five days, most lambs did not enter their heads in the PMMA box, confirming the need of a longer training period (Figure 2B). Subsequently, for the last five days of training, each lamb was individually placed in the test corridor with access to social and food resources and exposed to a cotton pad impregnated with demineralised water. After the habituation and training period, except a 4-month old female which did not eat, they do not express agitation or vocalization.

The experimental setup has been used to evaluate behavioural responses to different OS in adult ewes (Figure 2A) and lambs (Figure 2B) over one month. During this period, the adult ewes were submitted to three periods of OS exposures (3 different OS each time) with two distinct periods of habituation and training (Figure 2A). In total, the adult ewes have been exposed to demineralised water four times after the habituation and training period (days 8, 11, 22 and 29). For the lambs, three and four-month old, two OS exposures occurred (3 and 4 different OS) with only on long period of habituation and training. In total, the lambs have been exposed to demineralised water three times after the habituation and training period (days 15, 19, 22). During the five minutes of exposure to demineralised water and OS, a total of 29 behavioural variables were measured.

Only the behavioural responses collected in presence of demineralised water were used to test their consistency over time and validate the experimental setup. The data collected during OS exposures are available for adult ewes (<https://doi.org/10.57745/XSND95>) and lambs (<https://doi.org/10.57745/AIYZUV>).

#### *Evaluation of the consistency of behaviours*

Because of the large numbers of behavioural variables, we opted to perform a Principal Component Analysis (PCA), which enables us to visualise and reduce a high-dimensional dataset to a low-dimensional one. The

PCA results highlight the dimensions explained by the original behavioural variables, as well as the distribution of individuals according to the day of demineralised water exposure. If behaviours are consistent over time, we do not expect individuals to be discriminated according to the days. In other words, we expect the variables to be all the same, whatever the day of exposure.

Whatever the age of experimental animals (adult ewes, three- or four-months-old lambs), the PCAs revealed a consistency in behavioural responses across demineralised water exposures (Figure 2). Regardless of age, Dimension 1 was primarily explained by variables related to feeding behaviour - such as the time spent in the zone near the food resource - accounting for 36.4% of the variance in ewes, 30.9% in 3-month-old lambs, and 39% in 4-month-old lambs. Dimension 2, however, differed between groups of age. For adult ewes and 4-month-old lambs, it was mainly associated with locomotion-related behaviours and the frequency of entries into the entrance zone, explaining 17.4% and 17.3% of the variance, respectively (Figure 2). In contrast, for 3-month-old lambs, Dimension 2 was also influenced by feeding-related behaviours and accounted for 18.1% of the variance (Figure 2B). Additionally, for both adult ewes and 4-month-old lambs, a distinct cluster of points appeared to the left of the main group in the PCA plots (Figure 2). These isolated clusters corresponded to individuals that either did not eat during the experiment or spent very little time feeding.

## **Conclusion**

The experimental setup we designed allows to investigate the impact of an olfactory stimulus on behavioural responses while minimising stress and individual variability. We successfully designed an experimental setup by providing social and food resources and avoiding pharmacological interventions, social isolation, starvation, novelty, and physical constraint. It is therefore suited for studying emotional responses specifically induced by an olfactory stimulus, in a non-stressful environment. In addition, the presence of social and food resources could lead to a motivational conflict in the experimental setup, pitting one against the other. This allows us to identify different behavioural profiles based on animals' motivation, whether social or food-related. Rather than being a limitation, this variability is a key feature of our study, as it helps distinguish individuals with different motivational tendencies. We can assume that animals position themselves along a gradient, from those that are more food-oriented to those that are more socially-oriented (18).

## **Perspectives**

The experimental setup presented in this short note can be used to assess the emotional valence of OS by analysing behavioural responses. An attractive OS is expected to elicit increased proximity to the odour source, and low locomotion and vocalisation, while a repulsive OS should lead to agitation, vocalisations, avoidance of the odour source, and increased social-seeking behaviour. Additionally, our setup will facilitate the exploration of phenomena such as social buffering (19) and emotional contagion (20). Social buffering is a phenomenon characterized by the presence of conspecifics reducing, or even eliminating, the impact of events with negative emotional valence (19). For testing social buffering with our experimental setup, we reduce the level of social resource by reducing the number of congeners in the presence of a negative valence OS. While social skills and affinities also play a role, assessing them would require prior social network characterisation. The hypothesis here is to observe an increased level of negative emotion in animals exposed to the negative valence OS, at the same time as the social resource is reduced. Emotional contagion is a phenomenon whereby an animal feels and expresses the emotions of other animals without being exposed to the trigger of the emotion (20). To study emotional contagion with this experimental setup, it is necessary to focus on animals in the social resource park and measure their emotion-related behaviours when exposed to an animal feeling an emotion induced by an OS.

By employing this approach, we anticipate contributing to a better understanding of the role of olfaction in sheep, which could have implications for animal welfare and husbandry practices, in which essential oils are reported to help control parasites and stress.

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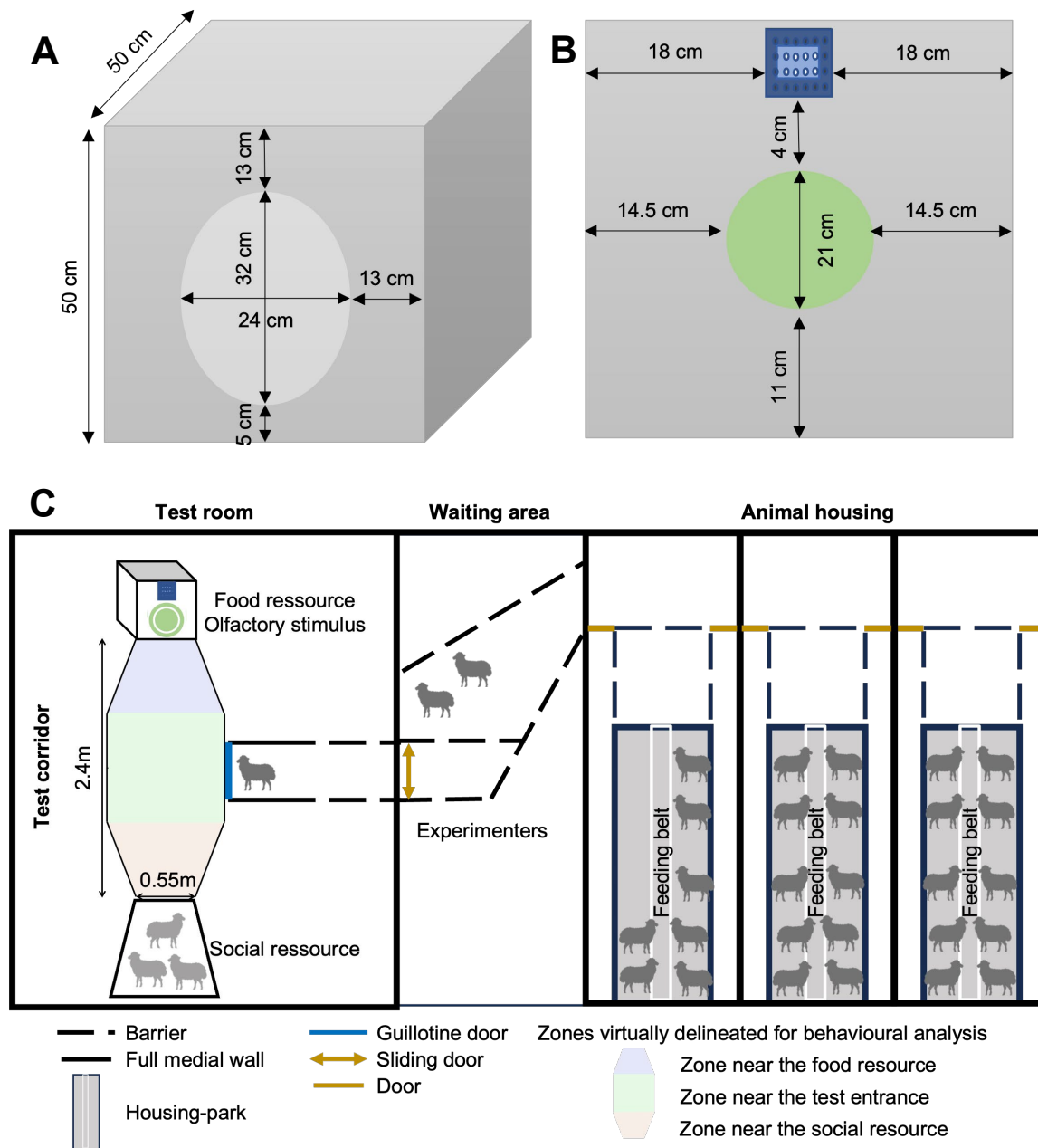
### **Ethics approval / References**

This protocol was approved by the local ethics committee for animal experimentation (CEEA VdL, Tours, France authorization 2022-1511-1). Because the experimental protocol does not present any suffering procedures for the individuals, it is classified out of scope in accordance with the European directive 2010/63/UE for animal protection and welfare used for scientific purposes.

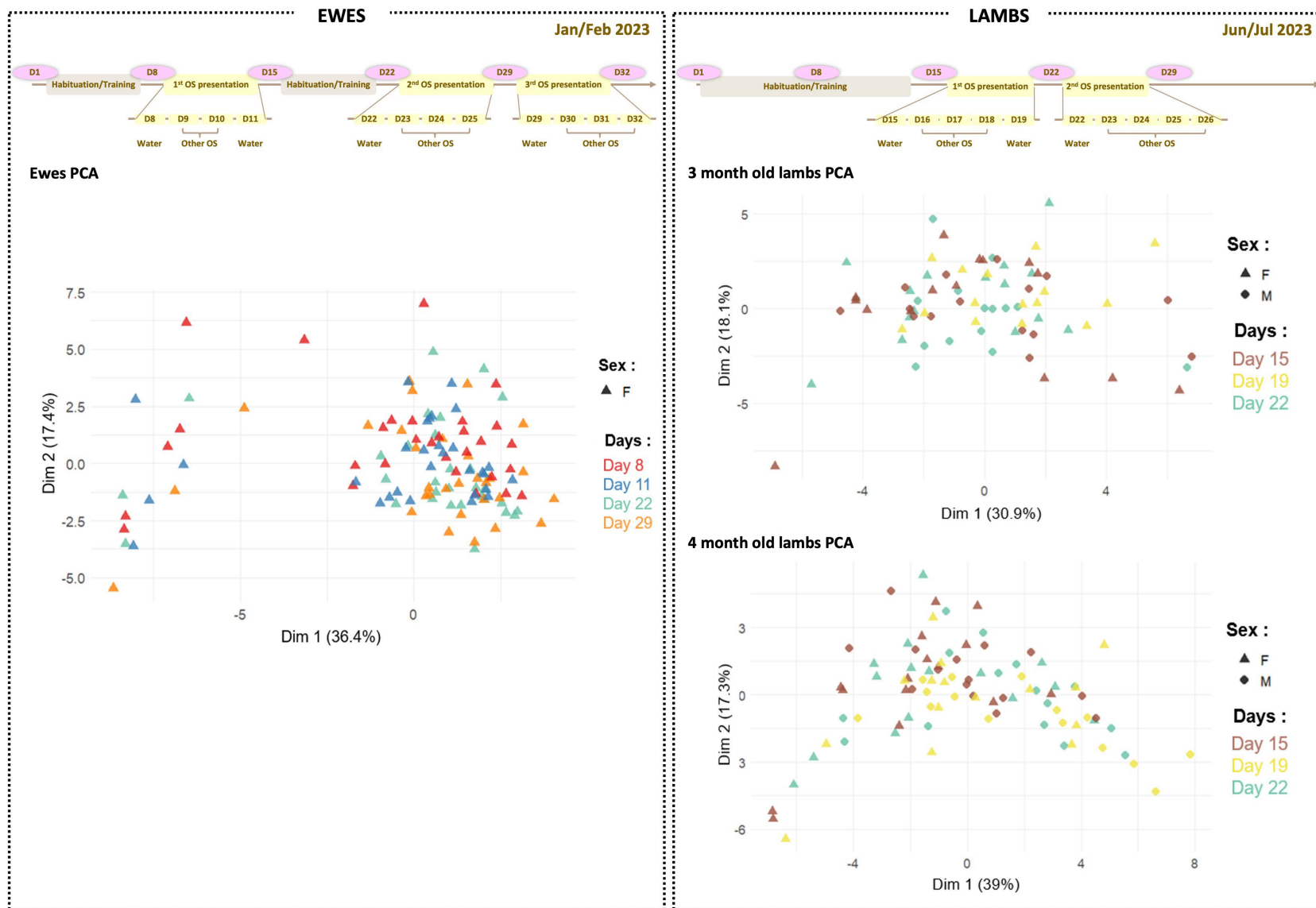
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**Figure 1.** **A.** Three-quarter view with external dimensions of the PMMA box and the single opening (grey oval) that allows the animals to place their head inside. **B.** Top view with internal dimensions of the PMMA box with the location of the round green plastic trough (green circle) and the location of the OS impregnated cotton placed under a grid (blue rectangle). **C.** Schema of the experimental building, waiting and testing rooms. The corridor measures 2.4 meters in length, with each zone measuring 80 cm. **D.** Training phase: Animals are trained to eat in the round green plastic trough (green circle) in presence of 2 to 3 other animals. **E.** Animal during the test with the head in the PPMA box.



**Figure 2. A.** Organisation of OS exposures for ewes and PCA performed with all behaviours measured in control situation ( $n=30$  ewes). Ewes were subjected four times to demineralised water. **B.** Organisation of OS exposures for 3-month-old and 4-month-old lambs and PCA performed with all behaviours measured in control situation ( $n=30$  3-month-old and  $n=30$  4-month-old lambs). Lambs were subjected three times to water. Each demineralised water exposure is symbolized by different colours and each sex by different symbol. The PCA highlighted two main dimensions. Dimension 1 was primarily explained by variables related to feeding behaviours, including food intake, total time spent eating, latency to eat, time spent with the head inside the box, and latency to insert the head into the box. For both ewes and 4-month-old lambs, dimension 2 was mostly driven by variables reflecting locomotion-related behaviour, such as the number of crossed zones. For 3-month-old lambs, Dimension 2 was also influenced by feeding behaviour variables, including the occurrence of presence in the food zone.

## MATERIALS AND METHODS

### Animals

To ensure reproducibility and repeatability of the results, we invite experimenters to apply the STRANGE framework proposed by Webster and Rutz (21). The aim is to describe the animals according to different characteristics such as breed or genetic, age, life history, sociality etc., each being a source of individual variability. The experimental setup was tested with 30 adult and 60 young Ile-de-France sheep from the UEPAO (INRAE Centre Val de Loire, Nouzilly, France doi:10.15454/1.5573896321728955E12) (Table 1). All were mothered and weaned at around 3 months of age. The experiment with the 30-adult sheep was conducted between January and February 2023 with ewes having experienced one or two parturitions and mothering. The experiment with the 3-month-old lambs was conducted in June, one week after weaning, and the experiment with the 4-month-old lambs was conducted in July, one month after weaning. During the experimental period, the animals were housed in the experimental building on straw bedding. The animals were provided with *ad libitum* hay and a supply of pellets to cover their maintenance and growth requirements (300 g per adult ewe and *ad libitum* for lambs).

Table 1. Distribution of date of birth and body weight of the groups of animals involved in the experiment

	Date of birth		Body weight at the beginning of the experiment	
	Median [IQ1-IQ3]	Max Min	Median [IQ1-IQ3]	Max Min
<b>Adult ewes N=30</b>	30/05/2018 [9/03/18-6/09/18]	8/03/2017 29/11/2019	76.5 kg [72.5-81.7] kg	55.1 87.9 kg
<b>3-month lambs N=15 females and 15 males</b>	<i>Female</i> 10/03/2023 [06/03/23-13/03/23]	<i>Female</i> 02/03/2023 19/03/2023	<i>Female</i> 28.4 kg [27.3-29.5] kg	<i>Female</i> 25.3 30.2 kg
	<i>Male</i> 11/03/2023 [07/03/23-13.5/03/23]	<i>Male</i> 13/03/2023 15/03/2023	<i>Male</i> 29.7 kg [28.8-30.3] kg	<i>Male</i> 28.2 30.8 kg
<b>4-month lambs N=15 females and 15 males</b>	<i>Female</i> 09/03/2023 [07/03-11/03]	<i>Female</i> 04/03/2023 19/03/2023	<i>Female</i> 34.3 kg 30.0-35.3	<i>Female</i> 26.5 36.7 kg
	<i>Male</i> 11/03/2023 [07/03-14.5/03]	<i>Male</i> 13/03/2023 23/03/2023	<i>Male</i> 30.3 kg [29.1-31.6]	<i>Male</i> 26.5 32.6 kg

### Setup for presenting an olfactory stimulus

Each OS was presented for 5 minutes in a hermetically sealed PMMA box with a single opening to allow the animals to put their head inside (Figure 1A). The PMMA sides were screwed and taped, rather than glued, to limit the potentially disturbing smell of the glue. A round, green plastic trough was placed at 11 cm from the opening and the OS was placed just behind the trough, at the bottom of the PMMA box, against the back side (Figure 1B). The choice of 11 cm as the distance between the opening and the trough was made based on the size of the animals' heads. The decision to place the OS behind the food was motivated by two reasons. Firstly, we did not want to put the OS in or near the food to be sure that the behaviours were due to the OS. Secondly, the OS was placed at the back of the box, as far from the opening as possible, to minimise diffusion of the odour outside of the box. The OS was applied to a cotton pad (8 cm x 10 cm), fixed under a perforated metal grid (14 cm x 14 cm) (Figure 1B). To ensure reliable comparisons between different OS, a separate PMMA box was used for each OS. This procedure aimed to minimize odour accumulation while maintaining a neutral testing environment. Before and during the OS-exposure sessions, the animals were exposed to a cotton pad impregnated with 1 ml of demineralised water. This exposure was considered the control.

### Experimental setup

The experimental setup includes a corridor with the food and social resources at each end. The animal enters the corridor through a guillotine door equidistant from each end (Figure 1C). The social resource

consisted of a group of companions selected to match the experimental animals in terms of breed, age, sex and physiological stage. These companion animals were familiar to the experimental subjects, as they had been housed together prior to the experiment, although not in the same housing location during the testing phase. Adult ewes were tested in presence of four companions and the lambs in presence of three companions. The test corridor and the social resource pen are separated by a barrier to maintain visual, auditory and physical interaction between the experimental and companion animals. The food resource was provided by distributing a pellet ration adapted to the animal's needs placed in the round green plastic trough (adult: 300 g, lamb: 200 g), inside the PMMA box (Figures 2D-E).

### Measurement of behaviours

During the test, animals were continuously recorded by two cameras: a video surveillance camera (AXIS M3025) fixed to the ceiling and a digital camera (SONY HDR-CX240E camera) placed facing the PMMA box in front of the animal's face. The videos were analyzed with Boris V.8.27.1 software (23) and a total of 29 behavioural variables has been extracted from BORIS or calculated (Table 2).

Table 2. List of behavioural variables

Type of behaviours	Behavioural variables
Feeding	Number of occurrence and time spent eating in the trough or inside the box, latency to eat, and time spent during the first occurrence to feeding. The animals are considered to be feeding when they are observed taking pellets and masticating. Total time spent eating and total number of occurrences to eat were calculated by summing the time spent eating and the number of occurrences to eat, in the box and the trough, respectively. The time spent in the box without eating was calculated by subtracting the eating time from the total time spent in the box.
Location and Locomotion	Latency to enter different zones (social, food or entrance), number of occurrences, and time spent in each zone, as well as the time spent during the first occurrence in the social or food zone. The animals are considered to be in a zone when both front legs are inside it. When they are positioned between two zones, their location is determined by the orientation of the muzzle. For evaluating locomotion, we calculated the number of crossed zones. Time spent in the food resource zone without eating was determined by subtracting the eating time from the total time spent in that zone.
Interaction with the PMMA box (food resource and OS location)	Latency to insert the head into the box, time spent with the head inside the box, time spent with the head inside the box without eating, number of occurrences of muzzle in contact with the internal side of the box, and time spent with the muzzle in contact with the internal side of the box.
Vocalisation	Latency and number of occurrences of vocalization
Excretions	Presence of urine and faeces

### Data analyses

To assess the consistency of behavioural responses over time, we conducted a PCA on the full set of observed behavioural variables measured in the control condition in presence of a cotton pad impregnated with demineralised water (four exposures for adult and three exposures for lambs). All data analyses were conducted in R (V.4.3.2). We included data from habituation days 1 to 5 in the PCA, justified by the observed stabilization of exploratory behaviour from day 3 onwards. The PCA was performed using the function on the covariance matrix (Pearson correlation) in R. These measures were done during protocols in which the animals have been exposed to different OS.

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