# Olfactory Cue Mediated Neonatal Recognition in Sheep, Ovis aries

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## Abstract

The strong bond between ewe and lamb formed shortly after parturition is an important factor in lamb survival. Evidence exists that a ewe can distinguish her lamb by its unique smell, but the constituents of such a putative olfactory cue have not yet been identified. We have now identified 133 volatile organic compounds associated with the wool of Döhne Merino lambs that we presume may be involved in neonatal recognition. Quantitative analysis and comparison of odor profiles of the twins of 16 ewes (9.69% sample group) of a flock of 165 twin-bearing ewes revealed that the wool volatiles of twins are qualitatively and quantitatively similar, but differ from those of other twins or non-twin lambs in the flock. The 88 constituents present in at least 20% of the analyzed wool samples were considered as variables for multivariate analysis.

A P-value $< 0.001$ was calculated, indicating that the pairing of twins according to the qualitative and quantitative composition of the wool was significant. Bioassays carried out during the lambing seasons of 2009 and 2010 confirmed the previously established role of lamb odor in ewe-lamb recognition. However, when alien lambs were dressed in jackets sprayed with synthetic mixtures formulated to match the chemical composition of the effluvia of the ewes' own lambs, ewes rejected the aliens. This is possibly because the VOCs were not released in quantitative ratios sufficiently accurate to emulate the odor of the ewes' own lambs.

**Key Words**: Semiochemical communication, Kin recognition, Headspace analysis, Sorptive sample enrichment, Sample enrichment probe, SEP technique, GC-MS analysis .

## Introduction

Since Aristotle reportedly (Lynch et al., *1992*) described the most successful way of achieving fostering in sheep was to skin a dead lamb, place the skin on a foster lamb, and confine the ewe with the foster lamb, this approach has been practiced by many sheep farmers. Sheep are seasonal breeders with large numbers of ewes giving birth during a relatively short lambing season. It is, therefore, crucial that a mother recognizes her lamb in order to provide nourishment selectively to her offspring. Although auditory and visual cues also are used, ewes appear to rely on an olfactory cue, described in other animals as an odor signature (Yamazaki et al., *1976*) or signature mixture (Wyatt, *2010*), which provides final assurance before allowing lambs at the udder (Lindsay, *1988*).

When ewes are not in parturition and not lactating, olfactory cues play an inhibitory role in maternal responsiveness, resulting in ewes displaying indifferent or hostile behavior toward neonatal lambs (Dwyer, *2008*). However, for a short period, around the time of parturition, ewes become highly interested in neonatal lambs and may even steal lambs from other ewes. During parturition, odor produced by or associated with a lamb is a powerful stimulus, and neural structures such as the olfactory bulb undergo extensive changes when exposed to such odors (Kendrick et al., *1992*). The bond between ewe and lamb is established through contact within 4–6 h after birth. If this fails to happen, maternal interest wanes and the ewe will not accept the lamb. However, once the bond is formed, ewe and lamb can be separated for relatively long periods without disruption of bond integrity (Lindsay, *1988*). The common factor that characterizes most post-partum deaths of lambs is impediment or disturbance of bond formation between ewes and offspring (Lindsay, *1988*).

In order to curtail financial losses that result from rejection of newborn lambs, a well-known phenomenon in all sheep races, farmers and scientists have examined methods of facilitating adoption of rejected lambs by ewes with ample milk. In addition to the Aristotelian method, other methods of odor transfer are practiced, albeit with limited success, by sheep farmers and have been subjected to scientific scrutiny including, for example, the transfer of the odor of a foster mother's own lamb to a rejected or orphaned lamb (Price et al., *1984*, *2003*; Alexander et al., *1987*, *1989a*, *b*). Although sheep have been domesticated for about 11,000–12,000 years, and are regarded as one of the most studied animal species (Adams and McKinley, *1995*), the olfactory cue that mediates ewe-lamb bonding has not yet been characterized. There are several sources from where the volatile organic compounds (VOCs) constituting a lamb's unique olfactory cue could be released. One possibility is a maternal label originating from amniotic fluid, milk, saliva, or some other secretion/excretion of the ewe.

Rietdorf (*2002*) investigated three possible sources of sheep maternal odor: colostrum, amniotic fluid, and inguinal gland secretion. Because ewes are strongly attracted to amniotic fluid for a few hours after parturition (Lévy et al., *1983*; Vince et al., *1985*), farmers often smear a newborn lamb with a ewe's amniotic fluid so as to strengthen the bond (Lévy et al., *2004*). However, the amniotic fluid has no effect on maternal acceptance, indicating that the fluid contains cues for general attractiveness rather than individual recognition (Lévy et al., *2004*). Alternatively, a lamb could be born with its own individual odor, with amniotic fluid having a supporting role in recognition by inducing a ewe to lick and groom a lamb, thus enabling the ewe to become acquainted with the lamb's olfactory profile. The interest that ewes display in the hindquarters, head, and neck areas of their lambs is well documented (Alexander et al., *1983*; Price et al., *1984*; Lynch et al., *1992*; Houpt, *2005*).

However, from our observations, it is apparent that, in general, a ewe has no preference for a specific area on a lamb's body during the initial identification process; she simply inspects the closest part of the lamb. The lamb is finally accepted at the udder with its head turned away from the head of the ewe, at which stage the ewe, perhaps for practical reasons, shows increased interest in the lamb's hindquarters. These observations are in accord with other findings that indicate that the discrimination by ewes between their own and alien lambs is mediated primarily by olfactory cues from the lamb's wool and skin, and not from a specific area on the lamb's body or amniotic fluid (Alexander and Stevens, *1981*; Lynch et al., *1992*; Brennan and Kendrick, *2006*).

Furthermore, Kendrick et al. (*1992*) found that lamb odors had no influence on the activity of olfactory bulb neurons of ewes during the period before birth, but when tested after birth these odors were potent; the smell of a lamb's wool being almost as effective a stimulus as the smell of the whole lamb. Subsequently, Lévy et al. (*2004*) showed that ewes could recognize lambs solely through VOCs, without direct contact. Cues responsible for the individual olfactory signature of a lamb are not effective at distances greater than 250 mm (Alexander and Shillito, *1977*; Alexander, *1978*; Poindron et al., *2003*), suggesting that the cues are either not very volatile or are present at very low concentrations.

Poindron et al. (*2006*) and Romeyer et al. (*1993*) showed that ewes use phenotype matching to recognize lambs. A ewe implanted with monozygotic twin embryos, and allowed to learn the odor of one of the twins after birth, recognized the second monozygotic lamb without being previously exposed to it. However, when this experiment was repeated with dizygotics, the ewe did not identify the twin after first being exposed to the other dizygotic (non-identical) twin. This experiment also shows that at least some features of the lamb's odor are genetically based (Wyatt, *2003*).

Here, we report the results of qualitative and quantitative analyses of VOCs present in the cranial wool of Döhne Merino lambs, as well as results of preliminary field tests with synthetic mixtures of the identified VOCs.

# Material and Methods

## *General*

All (borosilicate) glassware used for the collection of samples was washed thoroughly with distilled water, heated (400°C) for at least 30 min. to remove all traces of adsorbed organics, and then cooled immediately prior to use. Samples (1 ml) of solvents dichloromethane (DCM) (Romil, Super Purity, Cambridge, UK or Pestanal Grade, Riedel de-Haën, Seelze, Germany) and tert-butyl methyl ether (TBME) (Sigma-Aldrich, anhydrous, 99.8%, St Louis, MO, USA) were concentrated to 10 µl in a nitrogen atmosphere (Reiter et al., *2003*) and analyzed for impurities. These solvents were sufficiently pure for the extraction of semiochemicals. Syringes were cleaned by flushing barrels and rinsing plungers with DCM.

## *Sample Collection and Preparation*

A ewe flock of Döhne Merinos at Mariendahl, an experimental farm of Stellenbosch University located 14 km outside Stellenbosch, was available for study. The Döhne Merino sheep race produces a high percentage of twins. Singleton- and twin-bearing ewes were housed in separate paddocks. After birth, the singleton-bearing ewes plus lambs were moved to another paddock, while the twin-bearing ewes with lambs were moved to lambing pens for 2–3 d, after which they were housed in their own paddock. Sixteen (9.69%) of a flock of 165 twin-bearing ewes were used as a sample group.

Using a pair of scissors, wool samples (ca 200–400 mg) were collected from the foreheads of 32 lambs on the morning after birth. Cranial wool was used because the head of a lamb should be less contaminated with foreign matter than the rest of the body. The samples were stored in glass vials (25 ml) with Teflon-faced septa at −20°C until analyzed. The wool harvested from one lamb was sufficient for several analyses (Supplementary material, Fig. S1).
The procedures that were followed in our research were approved by the Stellenbosch University Ethics Committee: Animal Care and Use (Ethics number 11NC_BU01).

## *Analytical Methods*

Gas chromatographic (GC) analyses were carried out with a Carlo Erba HRGC gas chromatograph (Milan, Italy) with a Grob split/splitless sample inlet and flame ionization detector operated at 220°C and 280°C, respectively. Gas chromatographic data were acquired with a DELTA Chromatography Data System, Version 5.0 (Digital Solutions, Brisbane, Australia). The capillary columns were manufactured by the Laboratory for Ecological Chemistry (LECUS, Stellenbosch University) and were provided with integrated retention gaps of 1–2 m. The following columns were used: column A [glass, 40 m × 0.25 mm i.d., coated with 0.25 µm of apolar PS-089-OH (DB-5 equivalent)], column B [glass, 40 m × 0.25 mm i.d., coated with 0.25 µm of polar AT-1000 (FFAP equivalent)], enantioselective column C [glass, 30 m × 0.3 mm i.d., coated with 0.25 µm of OV-1701-OH containing 10% heptakis(2,3-di-O-methyl-6-O-tert-butyldimethylsilyl)-β-cyclodextrin], and enantioselective column D [glass, 30 m × 0.3 mm i.d., coated with 0.25 µm of OV-1701-OH containing 10% heptakis(2,3-di-O-acetyl-6-O-tert-butyldimethylsilyl)-β-cyclodextrin)].
Hydrogen was the carrier gas at a linear flow velocity of 50 $cm.sec^{-1}$ (column temperature 40°C). Samples were injected in the split mode, at a column temperature below 30°C. The column temperature was increased ballistically to 40°C, after which columns A and B were temperature programmed at 2°$C.min^{-1}$ to 280°C and 250°C, respectively. The final temperature was held for 20 min. The enantioselective columns were programmed at 1°$C.min^{-1}$ from 40 to 220°C. Gas chromatographic-low resolution electron impact mass spectrometric analysis (GC-LRMS) of material from wool samples was carried out on a Carlo Erba QMD 1000 instrument (Milan, Italy), using the columns and GC parameters specified above. Electron impact (EI) mass spectral data were acquired at 70 eV from m/z 25–350. Helium was the carrier gas at a linear flow velocity of 28.6 $cm.sec^{-1}$ (column temperature 40°C). Inlet and interface temperatures were 220°C and 250°C, respectively. The ion source temperature was set at 180°C, and the pressure in the source housing was ca. $2 \times 10^{-5}$ mm Hg at a column temperature of 40°C, decreasing to ca. $1 \times 10^{-5}$ mm Hg towards the end of the temperature program.

A scan rate of 0.9 $sec.scan^{-1}$ and an interscan delay of 0.1 sec were used. This instrumentation also was used for retention time comparisons of the volatiles in the wool samples (wool volatiles) with authentic synthetic reference compounds. Gas chromatography-mass spectrometry (GC-MS) was used to monitor the syntheses of reference compounds, and to confirm the identification of the wool volatiles by retention time comparison with commercially available synthetic compounds (Sigma-Aldrich, Cape Town) available from previous research projects, or compounds synthesized during the present study (Supplementary material). High resolution mass spectrometric data were acquired on a gas chromatograph coupled to a time-of-flight mass spectrometer (GC-TOF-HRMS), employing a Waters GCT Premier GC-MS instrument (Waters, Milford, MA, USA) fitted with capillary column E (30 m × 0.25 mm i.d., 0.25 µm film, DB-5 ms, Agilent JW Scientific, Folsom, CA, USA). Helium was the carrier gas at 1 $ml.min^{-1}$.

An inlet temperature of 260°C was used, and samples were introduced in the split mode (split ratio 1:5) at a column temperature below 30°C. The column temperature was then increased ballistically to 40°C, after which the oven was programmed at 2°$C.min^{-1}$ from 40 to 280°C (isothermal 5 min.), followed by a 2°$C.min^{-1}$ ramp to 300°C (isothermal for 20 min). EI-MS data were acquired at 70 eV, scanning the range m/z 35–600 at a rate of 0.2 sec/scan, with an inter-scan delay of 0.05 sec. The source temperature was 180°C and perfluoro-tri-n-butylamine was used as mass reference. The

identification of the compounds associated with the cranial wool of the experimental lambs also was substantiated by the determination of retention indices (Kovats, *1958*).

$^{1}H$ and $^{13}C$ NMR spectra of synthesized reference compounds were recorded in $CDCl_3$ on Varian VNMRS 300, Unity INOVA 400 and UNITY INOVA 600 NMR instruments (Varian, Palo Alto, CA, USA).

## Sampling Methods

### *Conventional Sample Extraction*

Dichloromethane (DCM) and tert-butyl methyl ether (TBME) were tested for the extraction of wool volatiles. TBME (1 ml) was found to be more effective for extracting wool samples (300–500 mg). Methyl hexanoate (14.8 µg/ml) was added as an internal standard. Synthetic analogs of all the compounds identified also were used as external standards. Extracts were concentrated in a nitrogen atmosphere by placing a vial into a 100-ml beaker, covering the beaker with aluminum foil, and purging the solvent vapor from the beaker without directing the nitrogen flow into the vial (Reiter et al., *2003*). During evaporation, the extracts were transferred to smaller Reacti-Vials. The concentration process took about 15 h to yield a final volume of ca 10 µl. Aliquots (1 µl) of extracts were subjected to GC-MS analysis by using split and septum purge flows of 10 and 1 $ml.min^{-1}$, respectively.
Headspace Sampling by Solid Phase Microextraction (SPME)

Headspace volatiles of a sample of wool (70 mg) in a bottle (8 ml) were sampled by SPME for 15 h at 50°C in a conventional laboratory oven with air circulation.

Headspace Sampling by Sample Enrichment Probe (SEP)

A second generation SEP50 sample enrichment probe (MasChrom Analisetegniek, Stellenbosch, South Africa), consisting of a fused silica stalk, or shaft, carrying a 50 mm sleeve (47 mg) of polydimethylsiloxane (PDMS) tubing (Burger et al., *2006*, *2011*), was used to trap VOCs from wool samples for GC and GC-MS analyses. The Teflon face of a septum was pierced centrally with the sharpened back end of the SEP, and the septum positioned on the stalk so as to enable headspace sampling from an 8-ml vial. The wool sample (70 mg) was placed in the vial, the SEP50 installed with its lower tip clearing the sample, and, after tightening the vial cap, the SEP was exposed to the headspace at 70°C for 1 h. The volatile compounds retained in the PDMS were desorbed at 220°C in the GC-MS. The SEP was left in the injector until completion of an analysis (Burger et al., *2006*).

## Dimethyl Disulfide Derivatization

The double bond positions of unsaturated VOCs of wool were determined by dimethyl disulfide (DMDS) derivatization and GC-MS analysis of the resulting adducts, similar to the method of Vincenti et al. (*1987*). An aliquot of a TBME extract of wool volatiles was concentrated to 5 µl in a Reacti-Vial, using a slow stream of purified nitrogen. The concentrate was re-dissolved in carbon disulfide (50 µl), and treated with an excess of iodine solution (5 µl of 60 mg of $I_2$ in 1 ml of TBME) and DMDS (50 µl).

The Reacti-Vial was sealed, and the reaction mixture was heated at 60°C for 40 h. The reaction was quenched with 5% aqueous sodium thiosulfate, and the aqueous layer separated from the organic layer by centrifuging the mixture at 3,000 rpm. The organic layer was transferred to another Reacti-Vial and washed with water (2 × 20 µl), utilizing centrifugation to facilitate phase separation. The extract was concentrated and used for GC-MS analysis.

## Determination of Absolute Configuration of Chiral Compounds

Enantioselective columns C and D were used to separate enantiomers of the chiral constituents present in wool samples.

## Statistical Analysis of VOCs

Of the 133 constituents identified, only those present in 20% or more of the wool samples were considered as variables for statistical analysis, reducing the number of constituents included in the analysis to 88. The chromatographic data used for multivariate analyses still contained a large number of variables (peak areas for these identified constituents, $N \leq 88$) relative to the number of sample units (wool samples, $N \leq 32$). Peak areas were normalized across all samples to produce comparable variables with zero means and unit standard deviation (Kowalski and Bender, *1972*). Permutation tests were carried out to determine whether the wool of twin lambs could be grouped according to the

qualitative and quantitative composition of VOCs present in their wool. The following null-hypothesis was tested: twin lambs cannot be grouped according to the qualitative and quantitative composition of their wool volatiles. Thus, the calculated P-value provided an indication of whether the similarities found between twin lambs could be attributed to the fact that they are twins or to random variation in the data.

Using this principle, the similarity in the odor profile of the wool of a pair of twin lambs was assessed by executing 10,000 permutation tests. The 88 constituents and their quantities in the wool of day-old lambs born during the lambing season of 2007 were used to construct a principal component analysis (PCA) biplot. Biplots can be considered as multivariate scatterplots that simultaneously give a graphical presentation of samples (lambs) and variables (identified constituents). Lambs are displayed as points on the graph while the identified constituents are displayed as linear axes.

The significance of an axis of a biplot is similar to that of an ordinary scatterplot: if a line is drawn from any point in a biplot perpendicular to a biplot axis, the value of the variable at that point can be read on the axis. In addition, the angle between any two axes approximates the correlation between two relevant variables. Constituents displayed as axes that are 90 degrees in relation to one another have no correlation with each other, and those constituents lying on axes close to one another have a high level of correlation with one another. An axis is labeled at the positive value of its calibration.

PCA biplots, permutation tests, and investigations into predictivity, were performed using R (Vienna, Austria), as described in Aldrich et al. (*2004*) and Gardner-Lubbe et al. (*2008*).

## Bioassays

Bioassays were performed with singleton- and twin-bearing Döhne Merino ewes during the lambing season at Mariendahl from March to April 2009 and 2010. Singleton-bearing ewes, with their lambs, were placed in a 0.2-ha paddock within 12 h after parturition, while twin-bearing ewes, with lambs, were isolated in lambing pens (1.2 × 1.6 m) inside a naturally illuminated and ventilated shed. All bioassays were conducted in these pens, outside in an enclosed concrete-surface arena (12 × 8 m), or in paddocks (ca. 10,000 $m^2$) with natural vegetation.

Exploratory bioassays were carried out during 2009 and 2010 by dressing lambs in treated cotton fleece jackets (which served as an odor-disseminating medium), and presenting alien lambs to ewes. In order to prevent, or at least curtail, odor diffusion through the jackets during the 5 min of the bioassay, alien lambs were fitted first with disposable diapers (Huggies, New Born, Kimberly-Clark SA, South Africa). In addition, the bodies and legs of experimental lambs were wrapped in several layers of low-density polyethylene-based film (Clorox, Rockdale, Australia). Jackets were made from new, white or pale grey, fleece cloth that had been repeatedly washed in hot water and sodium dodecyl hydrogen sulfate in a domestic washing machine and air-dried on a washing line. Pieces of the cloth were subjected to SEP-headspace analysis to ensure that the material was free from contaminants that could otherwise interfere with the bioassays.

Openings were made in the jackets for the legs, ears, eyes, and snouts of lambs, before placing the jackets on lambs. The jackets were fastened ventrally and under the chins of lambs by safety pins. The tail ends of some of these jackets were folded together so as to form artificial tails, and these were kept in shape with rubber bands (Fig. S2). Hoodless jackets were made with openings for the legs. These covered the body of a lamb from the buttocks to the shoulders. Some of the bioassays carried out during April 2010 were done with experimental ewes that had been conditioned to their own lambs wearing jackets. For these and other experiments requiring lambs to wear jackets for periods of up to 72 h, excess cloth was cut away from the hind legs of male lambs to prevent them from wetting jackets.

In control experiments, one of the lambs of a twin-bearing ewe was removed from the lambing pen for the duration of the experiment. The other lamb was presented to a ewe from a non-adjacent pen (so as to preclude the possibility that she could have been in occasional semiochemical contact with the experimental lamb). The lamb was then diapered, wrapped and presented to its own mother and then to the other ewe, after which a new cotton jacket was added, and the lamb presented again to its mother and the other ewe. Finally, it was undressed and presented to its own mother.

Drastic measures were not used to facilitate acceptance of an alien lamb. An alien lamb was considered accepted if it was allowed at the udder and if the ewe directed no butts (Supplementary material, videos available at http://scholar.sun.ac.za/handle/10019.1/3980) or butt attempts toward it over a period of 5 min after first contact. If a

ewe rejected an alien lamb and displayed aggressive behavior toward it, the experiment was terminated immediately and the lamb removed from the pen or experimental arena. In order to allow experimental lambs to drink, the snouts and area around the eyes were not completely covered, and thus lambs were dressed mostly in hoodless jackets. In these experiments, the lamb (N = 5) was presented sideways to the ewe so that the ewe could first sniff at the jacket. After completion of the experiments, the animals were kept under observation until it was clear each lamb was not permanently rejected by its mother.

Wool extracts also were tested. Approximately 8 g of wool were sheared from the bodies of day-old lambs and Soxhlet-extracted with either pentane (N = 15), diethyl ether (N = 4), DCM (N = 4) or ethyl acetate (N = 1) for 2 h. The extracts were sprayed uniformly on new, washed fleece cloth jackets. The respective solvents were allowed to evaporate before the bioassays were carried out, always within 24 h after the wool had been harvested. In a further experiment, approximately 16 g of wool, collected from both twins of a ewe, were extracted in pentane (N = 2) or DCM (N = 4). To restrict co-evaporation of VOCs from the jackets, extracts were added to a freshly cleaned, new jacket in a flange flask. The flask with its contents was cooled to −30°C, after which most of the solvent was removed under reduced pressure at the lowest temperature possible (between −30°C and −50°C), in order to restrict co-evaporation of VOCs from the treated jackets. The residual solvent traces were allowed to evaporate before the alien lambs were dressed in the jackets.

Finally, in exploratory experiments, the responses of twin-bearing ewes to alien lambs dressed in jackets sprayed with synthetic mixtures of identified wool VOCs were investigated. The synthetic mixtures were dissolved in pentane to match the composition of the wool VOCs of an experimental ewe's own lambs (analyzed as above). The bioassays were carried out within 24 h after the wool had been harvested. With the exception of a few unidentified constituents, authentic synthetic samples of all the VOCs identified in the cranial wool of the experimental lambs were available.

The interpretation of the results of the behavioral experiments was based on the consensus opinion of two or three observers.

## Results and Discussion

As a working hypothesis, we presumed that the odor profiles of twin lambs were identical, or at least similar, and quantitatively different from those of other lambs. Based on the assumption that the odor of a lamb is directly related to the VOC composition in the headspace of a wool sample (i.e., to the relative percentages in which all of the compounds are present in the sample's headspace, and thus in the effluvium of the lamb), it should be possible to identify and quantify the chemicals constituting the ewe–lamb recognition cue by intra- and inter-twin comparisons of the effluvia of a representative sample of lambs. The results of GC-MS analyses, as exemplified in Fig. 1, showed that the odors in twins were indeed remarkably similar, in contrast to the differences found between the odors of lambs born to different ewes (e.g., Fig. 2).

Exploratory experiments showed that the wool of day-old lambs contained only trace quantities of VOCs, and about 5–10% of high-boiling point organics (lanolin). The minute quantities of VOCs precluded the use of solvent extraction; Soxhlet extraction and the removal of solvent from extracts was expected to result in unacceptable quantitative reproducibility and, also, the injection of large quantities of organic compounds with high boiling points could have a deleterious effect on the performance of capillary GC columns. Thus, headspace analysis was used. SPME was found to be too insensitive (results not shown). An adapted version of closed-loop stripping (Grob, *1973*) also was evaluated.

Although reasonable results were obtained (data not shown), this technique was rejected because the air-circulating pump became contaminated by semi-volatile compounds that could not be removed by circulating water, methanol, acetone, methanol and DCM (in that order) through the pump and purging solvent vapors from the pump by overnight pumping of purified air. Because of its high sensitivity and simplicity, the SEP technique (Burger et al., *2006*, *2011*) eventually was used for sample enrichment of the wool VOCs. The recently introduced term "enrichment" has gained wide acceptance, and is now preferred for sampling techniques in which the constituents of a gas sample, or the headspace of solids or liquids, are concentrated in the sample preparation step prior to introduction of the sample into the injector of the analytical instrument. A typical total ion trace (TIC) of the cranial wool volatiles of a day-old lamb is depicted in Fig. 3.

Nevertheless, several wool samples also were extracted with DCM for determination of the total composition of the collected wool and for physical data required for the calculation of the composition of the concentration of the volatiles and semi-volatiles in the wool from the headspace data. The wool of day-old lambs typically contained 5–10% moisture, 1–4% inorganic particles, and 4–8% extractable organic material (commonly known as lanolin). The lanolin contained traces of volatile and semi-volatile organic compounds that eluted from an apolar capillary column at temperatures up to 280°C.

A total of 133 constituents (Table 1), comprising saturated and unsaturated alkanes, branched and unbranched primary alcohols, branched and unbranched aldehydes, unsaturated aldehydes, aromatic aldehydes, branched and unbranched methyl ketones, ethyl ketones, branched and unbranched carboxylic acids, unsaturated carboxylic acids, benzoic acid, esters, γ-lactones, terpenoids, 3-octanol, 2-pentylfuran, dimethyl sulfone, 3-ethyl-4-methyl-1H-pyrrole-2,5-dione, 3-ethyl-3-methyl-pyrrolidine-2,5-dione, 3-methyl-4-vinyl-1H-pyrrole-2,5-dione, and 2-ethyl-3-methylpyrrolidine-2,5-dione, were unambiguously identified by GC-LRMS and GC-HRMS analyses, Kovats retention index (RI) determination, and GC-MS comparison with authentic synthetic standards (Table 1). These compounds had masses from 60.05 to 410.39, and boiling points from 119°C to 360°C. The enantiomeric compositions of the chiral constituents [3-methyl-2-undecanone, a series of γ-substituted γ-lactones (alkan-4-olides), 3-methylpentanal and 2-ethyl-3-methylpyrrolidine-2,5-dione; Table 2] of wool were determined by GC-MS comparison with the corresponding synthetic compounds, using enantioselective columns C and D, and data published by Maas et al. (*1994*) and Burger et al. (*2008*).

The terpenoids eluted as single peaks and were presumably present in the wool as pure isomers. However, since the individual enantiomers or racemates were not available for retention time comparison, it could not be established whether this assumption was correct or not. A large number of the carboxylic acids, hydrocarbons, and alcohols identified in this study have been identified previously in lanolin (Motiuk, *1979a*,*b*, *1980*; Schlossman and McCarthy, *1979*). In the present investigation, these compounds were not considered as potential components of the recognition cue because of their high boiling points. The constituents of such a cue must be reasonably volatile because bond formation does not require direct physical contact between the ewe and lamb (Lévy et al., *2004*).

The 133 compounds identified in this study were all present in at least 10% of the samples collected during 2007. The number and varying concentrations of these compounds are consistent with the constraints on the design of chemical communication systems used in kin recognition which, according to Alberts (*1992*), requires a wide variety of compounds present in different concentrations. Approximately 50% of the total average peak area of the VOCs present in the headspace of the wool of the day-old Döhne Merino lambs consisted of only five compounds: ethyl tetradecanoate (20%), nonanal (12%), 6,10,14-trimethyl-2-pentadecanone (9%), tetradecanal (4%), and pentadecanal (4%). The use of statistical analysis to elucidate active compounds in complex semiochemical mixtures is limited by the possibility that constituents identified as principal components in multivariate analyses might not necessarily be key components of an olfactory cue. In order to determine whether any basis existed for using twins in this study, an attempt was made to quantify intra-twin similarities and inter-twin differences in the odors of neonatal lambs by multivariate analysis of the data obtained from a randomly selected 9.69% sample of the ewe flock.

The permutation tests of the analytical data calculated a P-value $< 0.001$, indicating that the pairing of twin lambs, according to the qualitative and quantitative composition of the wool, did not occur by chance, and was significant. Assuming that the odor of a lamb is related directly to the composition of the VOCs in the headspace of a wool sample, our GC-MS analyses (Figs. 2 and 3) showed that the odors of twins are remarkably similar, but there are differences between odors of lambs born to different ewes. Twin lambs thus possess odor profiles that are unique, with odor profiles more similar to each other than to those of other randomly selected non-twin lambs (See also Supplementary material, Fig. S3). The PCA biplot depicted in Fig. 4a provides the optimal two-dimensional presentation of the data matrix. Not all 32 samples and 88 variables are equally well represented in the biplot and axis predictivity (Gardner-Lubbe et al., *2008*). The quality of a biplot is an overall measure of the accuracy of the two-dimensional approximation of the data matrix and hence also of the reliability of the analytical data. Furthermore, sample predictivities and axis predictivities provide detailed information about how accurately each datum point is represented in the biplot and the degree of accuracy in the predictions made from the biplot axes.

Predictivity values range from 0 to 1, with a value of 1 representing the best predictivity. In Fig. 4a, b, only the 21 axes with predictivities >0.800 are displayed. The quality of display for the PCA biplots in Fig. 4a, b is 54%, which reflects the proportion of variation in the data accounted for in the first two dimensions of the two-dimensional display (Gower and Hand, *1996*). The other 46% is explained in the remaining 30 dimensions. In Fig. 4b, the axes are not displayed in order

so as to draw attention to the positions of the twin lambs. Each square represents a sample of wool collected from a day-old lamb, and the last three digits of the lamb's number are indicated in the biplot. Data pertaining to the lambs of each of the 16 twins are connected with black lines in Fig. 5a, b. The group of lambs concentrated in the encircled area indicates that these lambs possess odor profiles that are generally similar. The fact that non-twins (e.g., lambs 12 and 434) are closer in Fig. 4a, b than some twins can perhaps be explained in terms of possible variables that were not chosen in this experiment.

The 21 constituents with predictivities > 0.800 in order of decreasing predictivity are 2,6,10,14-tetramethylhexadecane (C91), 2-pentadecanone (C79), octadecane (C89), 1-pentadecanol (C87), (5E)-6,10-dimethyl-5,9-undecadien-2-one (C60), 15-methylhexadecanal (C97), unidentified constituent C84, (E)-2-tetradecenal (C78), 2-dodecanone (C54), tridecanal (C67), decanoic acid (C53), decanal (C35), 6,10,14-trimethyl-2-pentadecanol (C95), 13-methyltetradecanal (C77), hexadecane (C72), heptadecane (C80), nonadecane (C100), 2-heptadecanone (C99), unidentified constituent C83, 6,10,14-trimethyl-2-pentadecanone (C93) and dodecanal (C57). Interestingly, all the aldehydes have maxima on the left-hand side of the plot. All the methyl ketones, except 2-dodecanone, and all the hydrocarbons and decanoic acid are highly correlated with each other. In 2009, eight, day-old twins from five of the ewes that belonged to the sample group in 2007, were available for further investigation. These twins and those used in 2007 were sired by different rams. As for the wool volatiles collected in 2007, in 2009 only the volatiles present in 20% or more of the samples collected were considered as variables for statistical analysis, which reduced the number of variables to 84 constituents.

The similarity in the odor profiles of the wool of a pair of twin lambs was again assessed by executing 10,000 permutation tests. A P-value of 0.006 was calculated, indicating that the null hypothesis was rejected, and that the twin lambs born in 2009 possessed odor profiles that were unique and more similar than those of randomly selected non-twins. A higher P-value was obtained for lambs investigated in 2009 than for those investigated in 2007, but this could be attributed to the smaller number of wool samples available in 2009. The flock of ewes used in this project is being used for a long-term sheep breeding study at the Faculty of AgriSciences, and thus we could not carry out an experiment examining whether wool volatiles of offspring produced by the same parents are quantitatively and qualitatively similar from one lambing season to the next. A comparison of wool volatiles from lambs born in 2007 and 2009 from the same ewes, but with different fathers, showed that odor profiles of the 2 years were not different, with nearly all the constituents identified in wool in 2007 also present in 2009.

However, the PCA biplot (Fig. 5) shows a definite separation between wool volatiles from lambs born in 2007 and those born in 2009, indicating substantial quantitative differences. This provides evidence that the odor profiles of lambs born of the same ewe, but sired by different rams, are not identical. We are studying this aspect in more detail. The quality of display of the PCA biplot (only the 20 constituents with predictivities >0.800 are displayed) in Fig. 5 is 56%. The constituents, in order of decreasing predictivity, are tridecane (C46), 13-methyltetradecanal (C77), 3-octanone (C16), 1-heptanol (C15), hexanal (C4), unidentified constituent C64, tridecanal (C67), (E)-2-tetradecenal (C78), octanal (C19), (E)-2-nonenal (C30), 2-pentadecanone (C79), tetradecanal (C73), dodecanal (C57), hexadecane (C72), 6-methyl-2-heptanone (C14), (E)-2-octenal (C23), heptanal (C8), N-methyl-2-piperidinone (C29), hexadecanal (C90) and 2,5-dimethylpyrimidine (C11). It is clear from Fig. 5 that lambs born in 2007 have larger quantities of all these compounds. The figure suggests that the two nitrogen containing constituents, N-methyl-2-piperidinone (C29) and 2,5-dimethylpyrimidine (C11), are highly correlated.

All the hydrocarbons, ketones and aldehydes, except (E)-2-octenal (C23), also correlate reasonably well with each other. Price et al. (*1984*), placed nylon stockinettes worn by lambs for 72 h on alien lambs, which were then presented to ewes. Initially, 38% of alien lambs were accepted immediately and another 50% accepted over a further 36 h. More drastic measures have been used in other studies to force ewes to accept alien lambs treated with various odors or synthetic materials. For example, ewes were tethered to prevent them from displaying their typical udder denial behavior in experiments that continued over periods of up to 72 h (Alexander et al., *1987*, *1989b*). We carried out similar experiments. However, the experimental lambs were diapered and wrapped in polyethylene film to impede the diffusion of odor of the lambs into the treated jackets. In our control experiments, carried out to test the effect of diapers and polyethylene film, the majority of ewes rejected the wrapped and dressed alien lambs (Table 3). A smaller majority of ewes rejected their own wrapped and dressed lambs. Although the lambs did not bleat much, it is possible that these ewes could have recognized their own lambs by auditory or visual cues. It was apparent, nevertheless, that the diapers plus polyethylene film were sufficiently impermeable to restrict diffusion of wool volatiles into the cotton jackets during the short bioassays (ca 5 min). The results in Table 3 draw attention to the problems involved in the interpretation of various behavioral responses of ewes when approached by an alien lamb.

The mothering instinct of ewes varies: some tolerate the presence of an alien lamb as long as it keeps its distance, while others react aggressively, butting the lamb. The behavior of ewes also varies depending on the number of lambs they have mothered. Young ewes are normally more suspicious and more aggressive than more experienced ewes, perhaps because older ewes have become accustomed to the presence of observers. In bioassays, typical behavioral patterns of ewes were observed, ranging from total rejection to enthusiastic acceptance, including, immediate and repeated aggressive butting of the lamb, one or more less aggressive buts, moving in circles to prevent the lamb from getting at the udder, stamping of a hind foot on the side of the approaching lamb, stepping gently aside, allowing the lamb to approach, allowing the lamb to drink for a few moments before moving away or butting it, allowing the lamb to drink for longer periods before moving away or, finally, accepting the lamb and uttering low grunts of acceptance and/or encouragement.

The reaction of the ewe also depended on how thirsty the lamb was, or on or how persistent it was at getting to the udder. It was clear that some lambs smelled or in some other way realized that an experimental ewe was not its mother. In some cases, the ewe and alien lamb simply avoided each other, a situation that made it difficult to decide whether the ewe rejected the lamb or vice versa. A small minority of ewes did not accept any lamb. In another set of control experiments, we observed a ewe aggressively butting a jacketed alien lamb without having sniffed at it and afterwards even rejecting her own twin lambs for more than an hour. In further experiments carried out in 10,000-$m^2$ paddocks, lambs of singleton-bearing ewes wore hoodless cotton fleece jackets for 72 h. Alien lambs, dressed in these jackets and presented to the respective ewes, were all accepted by ewes, without any of the typical signs of rejection (acceptance rate 100%; N = 6). The experimental lambs then were undressed and returned to their mothers and were all immediately accepted. One of these jackets was stored in a glass jar and the VOCs in the headspace analyzed by GC-MS.

Not all the previously identified VOCs were detected (Table 1), probably because of partial evaporation of the more volatile constituents during the bioassay. On the other hand, no compounds other than those listed in Table 1 were detected. Because our qualitative chemical analyses were carried out on lambs born to twin-bearing ewes, we conducted similar bioassays with the twin-bearing ewes. An acceptance rate of lambs of only 62% (N = 13) was obtained. The difference in acceptance rates between singleton- and twin-bearing ewes could possibly be ascribed to increased stress on the twin-bearing ewes due to the experiments being carried out in a concrete arena or in small lambing pens in a shed housing about 40 other ewes with twins (as opposed to the paddocks for the singleton-ewe trial). Alternatively, the absence of the second twin during the experiment may also have contributed to the stress of these ewes. Similar experiments, spraying extracts of 8 g of lamb wool on new washed fleece cloth jackets were carried out. In five (20%; pentane, N = 4; dichloromethane, N = 1) of the experiments, the alien lambs were accepted by ewes.

Although the small sample size precluded meaningful statistical analysis, we suspect that selective co-evaporation of some of the more volatile compounds with the solvent could have contributed to the low acceptance rate. Headspace analyses of jackets treated with these wool extracts, or with mixtures of synthetic VOCs, revealed that VOCs are not released from the treated jackets in the same ratios in which they are present in the wool or in the synthetic mixtures. These results showed that the volatile, apolar compounds, in particular, are prone to co-evaporation with the solvent. It is also possible that the dissemination of VOCs from cotton cloth differed from that of lamb's wool, or that some constituents were converted to other products during Soxhlet extraction. A more concentrated extract of 16 g (collected from both twins of the ewes) of wool was applied to the jackets, with solvent evaporated at low temperature so as to minimize the co-evaporation of VOCs. Ewes (N = 6) rejected all the treated alien lambs, although they did sniff at the jackets for longer than in previous bioassays before rejecting the aliens.

Finally, twin-bearing ewes rejected all (N = 4) the alien lambs dressed in jackets sprayed with mixtures of synthetic VOCs. With the exception of a few unidentified constituents, authentic synthetic samples of all the VOCs identified in the cranial wool of lambs were available. Therefore, for these experiments we did not have to select only the 21 constituents with predictivities >0.800, as in the statistical analyses of our analytical results. Our observations in these experiments suggested that cues other than lamb odor might be used by ewes when confronted with a decision of whether to accept or reject a lamb. In the experiments we could not preclude alien lambs from bleating, nor could we cover the heads and snouts of the lambs in such a way that they could still see and drink while preventing ewes from finding unprotected parts of the lambs to sniff at. The jackets appeared to hamper the normal persistence of lambs to get at the udder, and the awkward gait of lambs dressed in this manner could have triggered their rejection.

Future work will focus on finding a more reliable bioassay, and on devising methods for dissemination of the VOCs in natural concentrations and ratios.

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## Table 1

Volatile organic compounds of the cranial wool of Döhne Merino lambs collected during the lambing seasons of 2007 and 2009. Constituents are grouped according to compound class

| No. | Compound | Column | | Remarks |
|---|---|---|---|---|
| | | Apolar | Polar | |
| C10 | Nonane | ✓ | | b,c,d,k,m |
| C20 | Decane | ✓ | | b,c,d,k,m,o |
| C28 | Undecane | ✓ | | b,c,d,o |
| C36 | Dodecane | ✓ | ✓ | b,c,d,o |
| C46 | Tridecane | ✓ | | b,c,d,j,o |
| C58 | Tetradecane | ✓ | ✓ | b,c,d,j,n,o |
| C72 | Hexadecane | ✓ | | b,c,d,j,o |
| C80 | Heptadecane | ✓ | | b,c,d,j,o |
| C89 | Octadecane | ✓ | | b,c,d,j,o |
| C100 | Nonadecane | ✓ | | b,c,d,j,o |
| C44 | 1-Tridecene | ✓ | | b,c,d,f,o |
| C55 | 1-Tetradecene | ✓ | | b,c,d,f,m,o |
| C66 | 1-Pentadecene | ✓ | | b,c,d,f,o |
| C118 | 1-Pentacosene | ✓ | | b,c,e |
| C120 | 1-Hexacosene | ✓ | | b,c,e |
| C2 | 1-Pentanol | ✓ | | b,c,d,k |
| C15 | 1-Heptanol | ✓ | ✓ | b,c,d,o |
| C25 | 1-Octanol | ✓ | ✓ | b,c,d,j,o |
| C33 | 1-Nonanol | ✓ | ✓ | b,c,d,o |
| P10 | 1-Tridecanol | | ✓ | b,c,d |
| C75 | 1-Tetradecanol | ✓ | ✓ | b,c,d,j,l |
| C87 | 1-Pentadecanol | ✓ | | b,c,d,j,l,o |
| C98 | 1-Hexadecanol | ✓ | ✓ | b,c,d,j,l,m |
| C108 | 1-Octadecanol | ✓ | | b,c,d,j,l |
| C114 | 1-Eicosanol | ✓ | | b,c,d,j |
| C116 | 1-Heneicosanol | ✓ | | b,c,e,j |
| P1 | 3-Methyl-1-butanol | | ✓ | b,c,d |
| C6 | 4-Methyl-1-pentanol | ✓ | ✓ | b,c,d |
| P2 | 3-Octanol | | ✓ | b,c,d |
| C4 | Hexanal | ✓ | | b,c,d,k |
| C8 | Heptanal | ✓ | ✓ | b,c,d,k,o |
| C19 | Octanal | ✓ | ✓ | b,c,d,o |
| C27 | Nonanal | ✓ | ✓ | b,c,d,o |
| C35 | Decanal | ✓ | ✓ | b,c,d,o |
| C45 | Undecanal | ✓ | | b,c,d,o |
| C57 | Dodecanal | ✓ | ✓ | b,c,d,o |

| No. | Compound | Column | | Remarks |
|---|---|---|---|---|
| | | Apolar | Polar | |
| C67 | Tridecanal | ✓ | ✓ | b,c,d,o |
| C73 | Tetradecanal | ✓ | ✓ | b,c,d,o |
| C81 | Pentadecanal | ✓ | ✓ | b,c,d,o |
| C90 | Hexadecanal | ✓ | | b,c,d,o |
| C101 | Heptadecanal | ✓ | | b,c,d,o |
| C105 | Octadecanal | ✓ | | b,c,d |
| C1 | 3-Methylpentanal | ✓ | | b,c,d,h |
| C24 | 7-Methyloctanal | ✓ | | b,c,d,e,m,o |
| C31 | 8-Methylnonanal | ✓ | | b,c,d,e,o |
| C41 | 9-Methyldecanal | ✓ | | b,c,d,e,o |
| C51 | 10-Methylundecanal | ✓ | ✓ | b,c,d,e,o |
| C68 | 12-Methyltridecanal | ✓ | | b,c,d,e |
| C77 | 13-Methyltetradecanal | ✓ | | b,c,d,e,o |
| C86 | 14-Methylpentadecanal | ✓ | | b,c,d,e,m,o |
| C97 | 15-Methylhexadecanal | ✓ | | b,c,d,e,m |
| C23 | (E)-2-Octenal | ✓ | ✓ | b,c,d,e,o |
| C30 | (E)-2-Nonenal | ✓ | ✓ | b,c,d,e,o |
| C40 | (E)-2-Decenal | ✓ | ✓ | b,c,d,e,o |
| C50 | (E)-2-Undecenal | ✓ | ✓ | b,c,d,e,f,o |
| C62 | (E)-2-Dodecenal | ✓ | | b,c,d,e,m,o |
| C78 | (E)-2-Tetradecenal | ✓ | | b,c,d,e |
| C13 | Benzaldehyde | ✓ | | b,c,d,n,o |
| C22 | Phenylacetaldehyde | ✓ | | b,c,d,n,o |
| C17 | 2-Octanone | ✓ | ✓ | b,c,d,k,o |
| C26 | 2-Nonanone | ✓ | | b,c,d,o |
| C34 | 2-Decanone | ✓ | ✓ | b,c,d,o |
| C43 | 2-Undecanone | ✓ | | b,c,d,o |
| C54 | 2-Dodecanone | ✓ | ✓ | b,c,d,o |
| C65 | 2-Tridecanone | ✓ | ✓ | b,c,d,o |
| C71 | 2-Tetradecanone | ✓ | ✓ | b,c,d |
| C79 | 2-Pentadecanone | ✓ | ✓ | b,c,d,o |
| C99 | 2-Heptadecanone | ✓ | ✓ | b,c,d,o |
| C14 | 6-Methyl-2-heptanone | ✓ | ✓ | b,c,d,k,o |
| C47 | 3-Methyl-2-undecanone | ✓ | | b,c,d,e,g |
| C16 | 3-Octanone | ✓ | | b,c,d,o |
| P4 | 3-Decanone | | ✓ | b,c,d |
| P3 | Acetic acid | | ✓ | b,c,d |
| P5 | Propanoic acid | | ✓ | b,c,d |

| No. | Compound | Column | | Remarks |
|---|---|---|---|---|
| | | Apolar | Polar | |
| P6 | Butanoic acid | | ✓ | b,c,d |
| P7 | Hexanoic acid | | ✓ | b,c,d |
| P8 | Heptanoic acid | | ✓ | b,c,d |
| P9 | Octanoic acid | | ✓ | b,c,d,j |
| C42 | Nonanoic acid | ✓ | ✓ | b,c,d,j |
| C53 | Decanoic acid | ✓ | ✓ | b,c,d,j |
| C69 | Dodecanoic acid | ✓ | ✓ | b,c,d,j,m |
| C85 | Tetradecanoic acid | ✓ | ✓ | b,c,d,j,o |
| C94 | Pentadecanoic acid | ✓ | ✓ | b,c,d,j,n,o |
| C104 | Hexadecanoic acid | ✓ | ✓ | b,c,d,j,m,o |
| C107 | Heptadecanoic acid | ✓ | | b,c,d,j |
| C112 | Octadecanoic acid | ✓ | ✓ | b,c,d,j |
| C115 | Eicosanoic acid | ✓ | ✓ | b,c,d,j |
| C117 | Heneicosanoic acid | ✓ | | b,c,d,j |
| C119 | Docosanoic acid | ✓ | | b,c,d,j |
| C82 | 12-Methyltridecanoic acid | ✓ | | b,c,d,e,j |
| C102 | 14-Methylpentadecanoic acid | ✓ | ✓ | b,c,d,e,j |
| C106 | 15-Methylhexadecanoic acid | ✓ | | b,c,d,e,j |
| C113 | 17-Methyloctadecanoic acid | ✓ | | b,c,d,e,j |
| C103 | (Z)-9-Hexadecenoic acid | ✓ | ✓ | b,c,d,f |
| C110 | (Z,Z)-9,12-Octadecadienoic acid | ✓ | ✓ | b,c,d |
| C111 | (Z)-9-Octadecenoic acid | ✓ | ✓ | b,c,d,f |
| C32 | Benzoic acid | ✓ | ✓ | b,c,d |
| C88 | Ethyl tetradecanoate | ✓ | ✓ | b,c,d,o |
| C92 | Isopropyl tetradecanoate | ✓ | | b,c,d,e,o |
| C48 | Nonan-4-olide | ✓ | ✓ | b,c,d,g,o |
| C61 | Decan-4-olide | ✓ | | b,c,d,g |
| C76 | Dodecan-4-olide | ✓ | | b,c,d,g |
| C109 | Hexadecan-4-olide | ✓ | | b,c,d,g |
| C56 | 6,10-Dimethyl-2-undecanone | ✓ | | b,c,d,h |
| C60 | (5E)-6,10-Dimethyl-5,9-undecadien-2-one | ✓ | | b,c,d,o |
| C91 | 2,6,10,14-Tetramethyl-hexadecane | ✓ | | b,c,d,h |
| C93 | 6,10,14-Trimethyl-2-pentadecanone | ✓ | ✓ | b,c,d,h,o |
| C95 | 6,10,14-Trimethyl-2-pentadecanol | ✓ | | b,c,d,h |
| C122 | Squalene | ✓ | ✓ | b,c,d |
| C5 | 2-Methylpyrimidine | ✓ | ✓ | b,c,d |
| C11 | 2,5-Dimethylpyrimidine | ✓ | ✓ | b,c,d |
| C29 | N-Methyl-2-piperidinone | ✓ | ✓ | b,c,d |

| No. | Compound | Column | | Remarks |
|---|---|---|---|---|
| | | Apolar | Polar | |
| C37 | 3-Ethyl-4-methyl-1H-pyrrole-2,5-dione | ✓ | ✓ | b,c,i,n,o |
| C38 | 3-Ethyl-4-methylpyrrolidine-2,5-dione | ✓ | ✓ | b,i,g,n |
| C39 | 3-Methyl-4-vinyl-1H-pyrrole-2,5-dione | ✓ | ✓ | b,c,i,n |
| C9 | Dimethyl sulfone | ✓ | ✓ | b,c,d,n,o |
| C18 | 2-Pentylfuran | ✓ | | b,c,d,o |
| C124 | Cholest-5-en-3β-ol | ✓ | | b,c,d,j |
| C3 | Unidentified | ✓ | | |
| C7 | Unidentified | ✓ | | |
| C12 | Unidentified | ✓ | ✓ | n |
| C21 | Unidentified | ✓ | | |
| C49 | Unidentified | ✓ | ✓ | n,o |
| C52 | Unidentified | ✓ | | o |
| C59 | Unidentified | ✓ | | |
| C63 | Unidentified | ✓ | ✓ | |
| C64 | Unidentified | ✓ | | o |
| C70 | Unidentified | ✓ | | o |
| C74 | Unidentified | ✓ | | m |
| C83 | Unidentified | ✓ | | m |
| C84 | Unidentified | ✓ | | b,m |
| C96 | Unidentified | ✓ | | b,c,o |
| C121 | Unidentified | ✓ | | |
| C123 | Unidentified | ✓ | | |

**a**: Constituents identified using apolar column A are numbered from C1 to C124 and those identified using polar column B numbered from P1 to P10
**b**: Low-resolution electon ionization mass spectrum
**c**: Library spectrum (NBS and/or NIST)
**d**: Retention time comparison with synthetic compound
**e**: Retention index
**f**: Double bond localization by dimethyl disulfide derivatization and gas chromatography-mass spectrometry
**g**: Absolute configuration given in Table 2
**h**: Absolute configuration not determined
**i**: Tentative identification
**j**: Compounds previously identified in lanolin (Schlossman and McCarthy, *1979*; Motiuk, *1979a*, *1979b*, *1980*)
**k**: Compounds previously identified in wool (Lisovac and Shooter, *2003*)
**l**: Compounds previously identified in inguinal gland of ewes (Rietdorf, *2002*)
**m**: Compounds identified in the wool during the lambing season of 2007 that were not identified in the lambing season of 2009
**n**: Compounds identified in the wool during the lambing season of 2009 that were not identified in the lambing season of 2007
**o**: Compounds identified in cotton fleece jacket left on lamb for 3 days

## Table 2

Enantiomeric composition of chiral constituents of wool determined by gas chromatography using columns C and D[a]

| Compounds | | Enantiomeric ratio (R:S) |
|---|---|---|
| C38 | 3-Ethyl-4-methylpyrrolidine-2,5-dione[b] | 54:46 (N = 1, C[c]) |
| C47 | 3-Methyl-2-undecanone | 91:9 to 100:0 (N = 4, C) |
| C48 | Nonan-4-olide | 69:31 to 78:22 (N = 5, C) |
| C61 | Decan-4-olide | 58:42 to 74:26 (N = 3, C) |
| C76 | Undecan-4-olide | 66:34 (N = 1, C) |
| C109 | Hexadecan-4-olide | 54:46 (N = 1, D[d]) |

[a]The ratios were determined by integration of single ion plots of the base peaks of the individual enantiomers. The quantitative data are given in the order of elution of the enantiomers from column C or D. In some samples, one or both of the enantiomers co-eluted with other constituents and could thus not be quantified, which accounts for the difference in the number of listed samples (N)

[b]Order of elution unknown

[c]Enantioselective column C

[d]Enantioselective column D

## Table 3

Efficacy of diapering and wrapping experimental lambs in polyethylene foil in order to curtail the release of the recognition cue during bioassays[a]

| | Alien lamb | Own lamb | Alien lamb | Own lamb | Alien lamb | Own lamb |
|---|---|---|---|---|---|---|
| | Naked | Diapered and wrapped | | Diapered, wrapped and jacketed | | Naked afterwards |
| Ewe 79 Lamb 298 | Rejected[b] | Rejected | Rejected without being sniffed at | Rejected | Rejected | No sign of rejection; lamb did not attempt to drink[c] |
| Ewe 298 Lamb 79 | Avoided by ewe | Rejected; did not attempt to drink | Rejected | Rejected | Rejected | Accepted immediately[d] |
| Ewe 204 Lamb 96 | Avoided by ewe | Accepted at the udder, then rejected | Did not attempt to drink; ewe showed no aggression | Ewe avoided lamb; no aggression | Did not attempt to drink | No sign of rejection; lamb did not attempt to drink |
| Ewe 188 Lamb 78 | Rejected | Accepted at the udder | Rejected | Accepted at the udder | Rejected | Accepted immediately |
| Ewe 78 Lamb 188 | Avoided by ewe | Accepted at the udder | Avoided ewe; did not attempt to drink | Avoided by ewe; did not attempt to drink | Ewe avioded lamb | Accepted immediately |
| Totals[e] | Rejected 100% | Rejected 60% | Rejected 80% | Rejected 80% | Rejected 80% | Accepted 100% |

[a]The experiments were carried out in the absence of the other twin of the twin-bearing ewes. The wrapping and dressing procedure is described in the text

[b]Based on butting of the lamb and/or various ways of avoiding contact with the lamb

[c]Probably due to the lamb being awkwardly dressed

[d]Immediately accepted at the udder; in some cases accompanied by ewe's typical low grunts encouraging the lamb to drink

[e]Based on consensus of opinion of two observers

## Figures

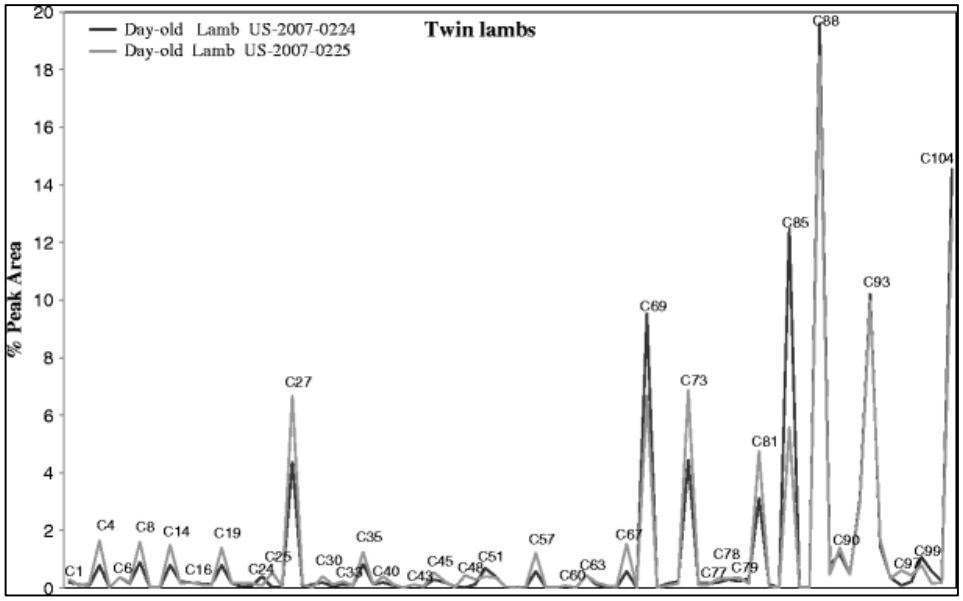


Fig. 1 Comparison of the percentage peak areas derived from mass chromatograms of the sample-enrichment-probe-enriched wool volatiles of day-old twin lambs US-2007-0224 (black) and US-2007-0225 (grey) analyzed on apolar column A. The peaks are numbered according to the information in Table 1. The minor VOCs P1–P10 were not visible in these chromatograms. Note that the concentrations of compounds 87 to 96 are so similar that the black chromatogram of lamb US-2007-0224 is only visible at the apexes of the peaks

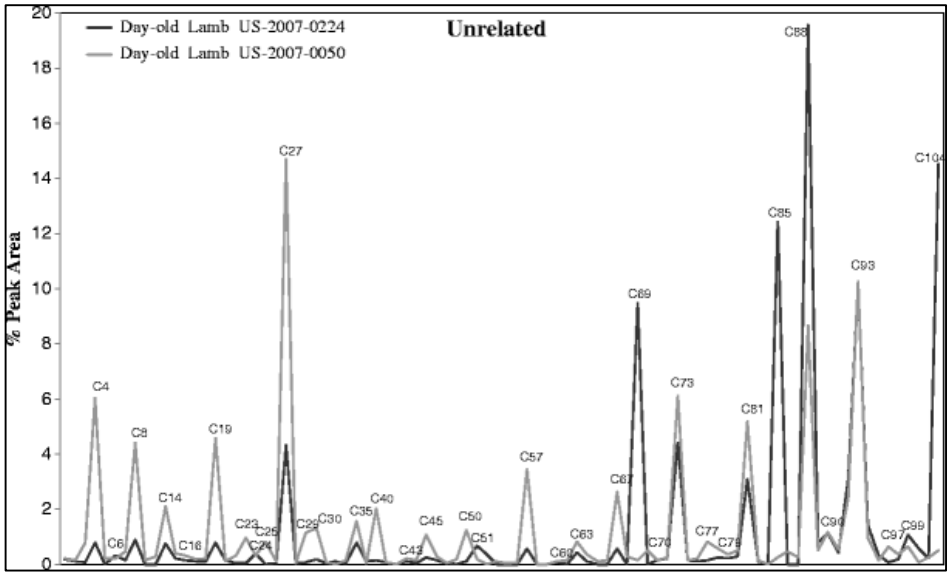


Fig. 2 Comparison of the percentage peak areas derived from mass chromatograms of the sample-enrichment-probe-enriched wool volatiles of the unrelated lambs US-2007-0224 (black) and US-2007-0050 (grey). The peaks are numbered according to Table 1

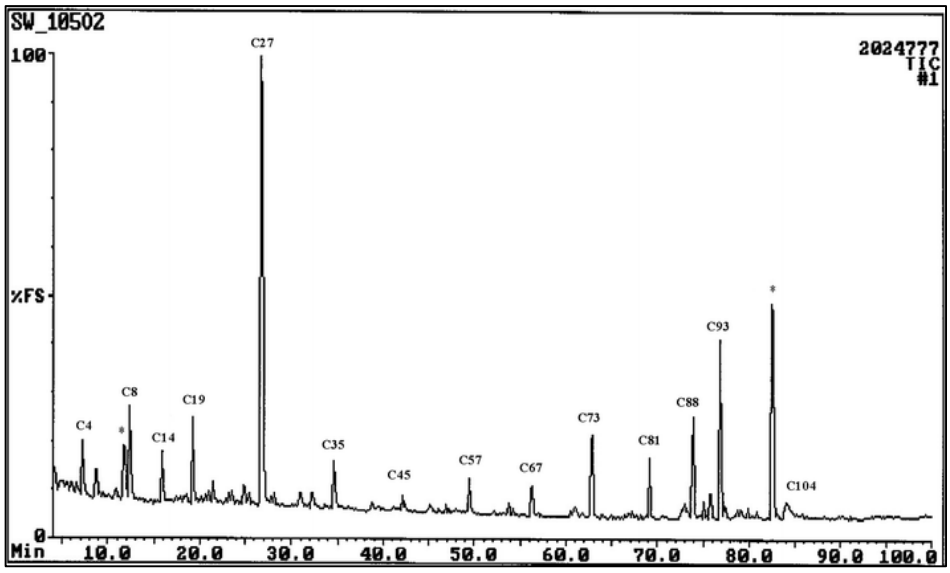


Fig. 3 Total ion chromatogram (TIC) of the headspace of the wool of Döhne Merino lamb US-2007-0105 analyzed on apolar column A. The peaks are numbered according to Table 1. Two impurities, present in all samples, are indicated by an asterisk

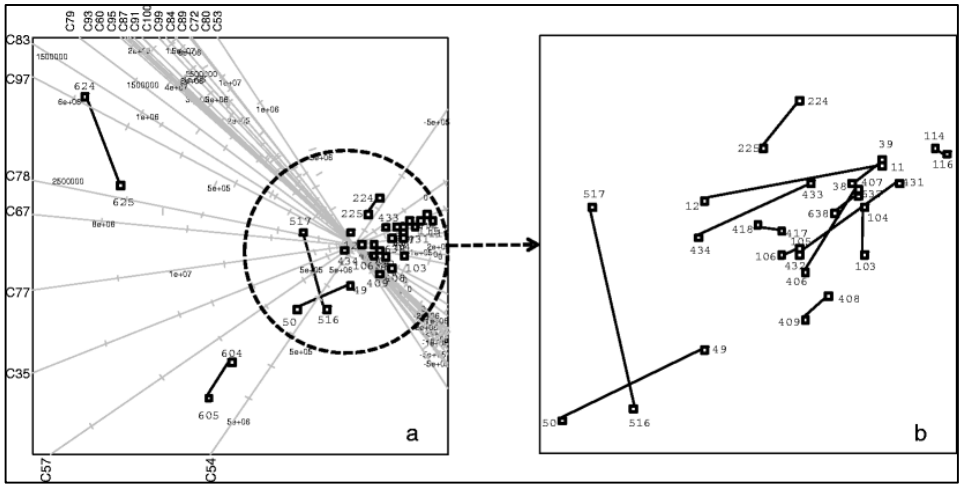


Fig. 4 a Principal component analysis biplot for the wool volatiles collected from day-old lambs during the lambing season of 2007; b PCA biplot displaying a magnified view of the crowded area in Fig. 4a. Lambs possessing similar odor profiles are circled. For identities of chemical codes refer to Table 1

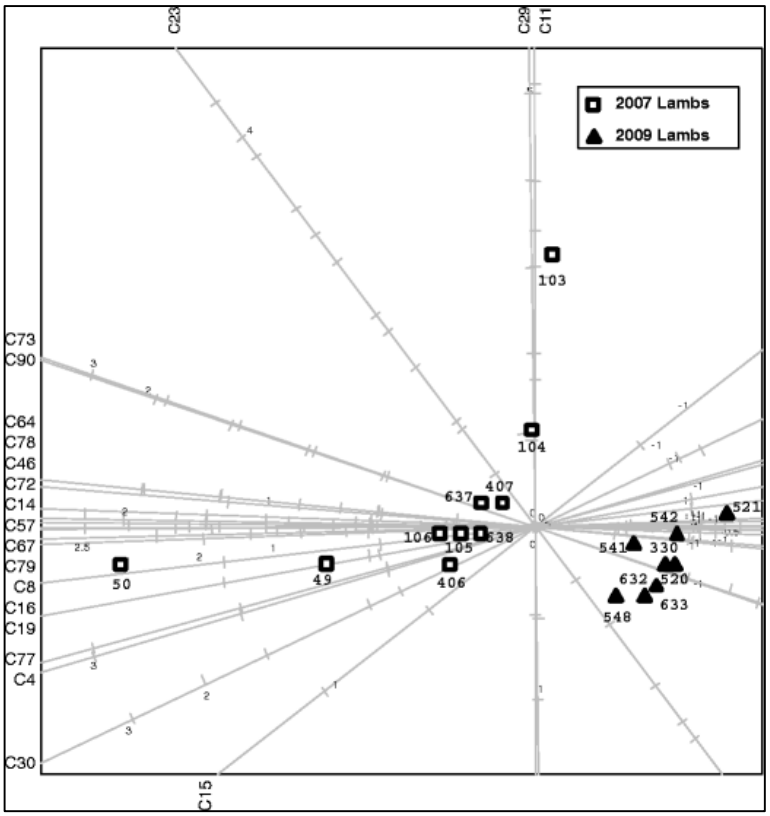


Fig. 5 Principal component analysis biplot for the wool volatiles collected from day-old lambs during the lambing seasons of 2007 and 2009. For identities of chemical codes refer to Table 1

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## Supplementary Information

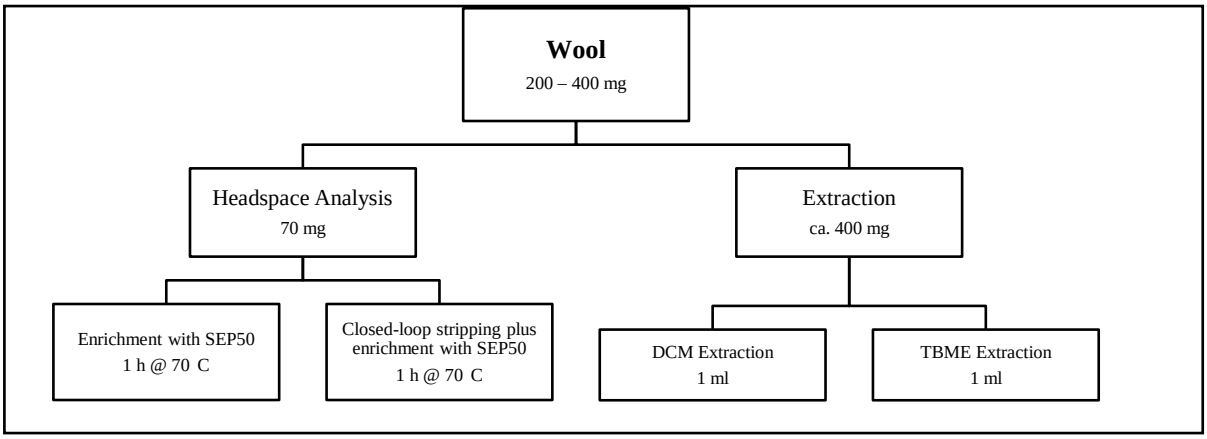


Fig. S1. Wool sample allocation for gas chromatography-mass spectrometry analysis

Fig.S2. Diapered and wrapped alien lamb dressed in a cotton fleece jacket worn for 24 hr by the ewe's own lamb.

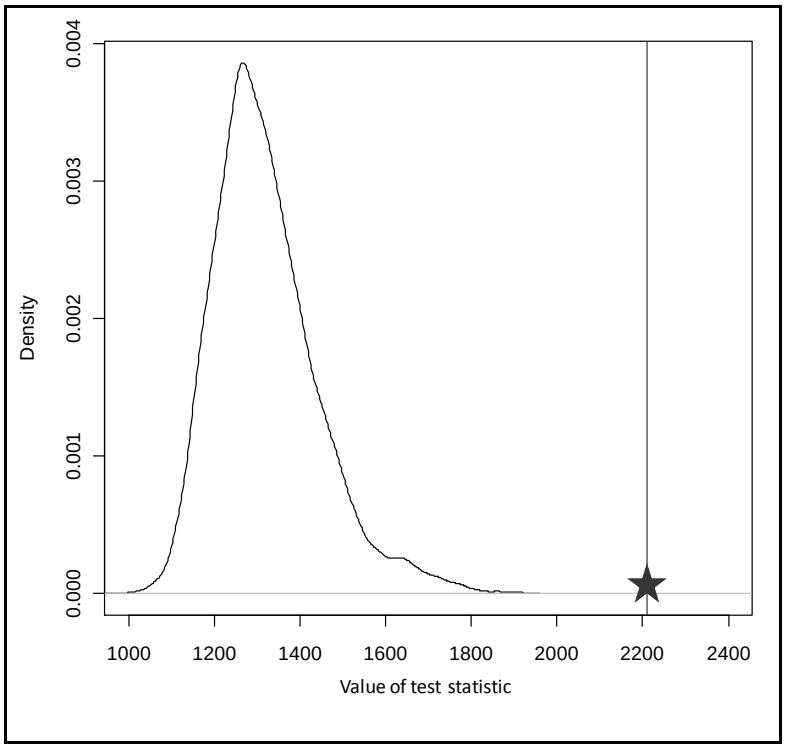


Fig. S3. Permutation distribution of the test statistic showing the differences in the composition of the wool volatiles collected from day-old twin lambs compared to the rest of the group of lambs. The calculated *P*-value < 0.001 is indicated by the vertical line on the right hand side of the figure. Twin lambs thus possess odor profiles that are unique and their odor profiles are more similar than those of other randomly selected non-twin lambs.

**Synthesis of Reference Compounds**.

Some of the compounds that were required as standards for the confirmation of the structures of constituents identified in this study were available from previous studies in our laboratory, or from the South African distributors of the products of Sigma-Aldrich, Merck, Saarchem, NT Laboratories and B.D.H.; others were synthesized from commercially available starting materials as described below. The structures of all of the compounds that were synthesised in the present investigation were verified by GC-MS, $^{1}H$ and $^{13}C$ NMR spectrometry. Some of the synthesized compounds belong to homologous series of the respective long-chain compounds. The NMR spectra of compounds belonging to these homologous series are practically identical

and the $^{13}C$ NMR data of only one example of each compound class are included in the information below.

*Preparation of 2,6,10,14-tetramethylhexadecane*. A solution of (6*E,*10*E*)-7,11,15-trimethyl-3-methylenehexadeca-1,6,10,14-tetraene (Burger *et al.*, 1978) (0.25 g, 0.92 mmol) in glacial acetic acid (50 ml) was hydrogenated using platinum on activated charcoal (10% Pt/C) as catalyst. The hydrogenation was carried out at approximately atmospheric pressure and the consumption of hydrogen was monitored volumetrically. The reaction was allowed to run to completion, the reaction mixture diluted with an

equal volume of distilled water, and then extracted with $CHCl_3$ (3 x 10 ml). The combined extracts were washed with water to neutral pH and dried on anhydrous $MgSO_4$, after which the drying agent was filtered off and the solvent removed on a rotary evaporator to yield 2,6,10,14-tetramethylhexadecane in quantitative yield with a purity of 91% (GC-MS). MS (70 eV): *m/z* (%) 282($M^+$, 0.07), 267(0.13), 253(0.17), 197(3), 183(4), 155(3), 141(5), 127(7), 113(8), 99(14), 85(44), 71(84), 57(100), 43(52). $^{13}C$ NMR (CDCl3, 101 MHz): δ = 11.45 (q, C-16), 19.26 (q, C-20)3, 19.32 (q, C-19*)4, 19.81 (q, C-18*), 22.68 (q, C-17*), 22.77 (q, C-1*), 24.54 (t, C-8*), 24.55 (t, C-12*), 24.86 (t, C-4*), 28.04 (d, C-2), 29.63 (t, C-15), 32.83 (d, C-6*), 32.87 (d, C-10*), 34.49 (d, C-14), 37.01 (t), 37.35 (t), 37.45 (t), 37.46 (t), 37.52 (t), 39.44 (t, C-3).

Note. * exchangeable assignments

*Preparation of branched primary alcohols*. Primary isoalcohols and ante-isoalcohols were synthesized from the corresponding branched carboxylic acids according to a scaled-down version of the protocol of Tietze and Eicher (1981: 416), as exemplified by the synthesis of 15-methyl-1-hexadecanol. The resulting alcohols were used as starting materials for the

preparation of the branched aldehydes described below. Note. Methyl substituents are numbered in order of their attachment to carbon atoms with ascending numbers in the parent-chain.

15-Methyl-1-hexadecanol. 15-Methylhexadecanoic acid (8.00 mg; 0.03 mmol) in TBME (200 µl) was placed in a 2 ml Reacti-Vial, with a small-vaned magnetic follower, and treated with an excess of a clear standardized solution of $LiAlH_4$ in dry diethyl ether. The Reacti-Vial was sealed and the reaction mixture stirred at 60°C for 3 hr. The excess $LiAlH_4$ was decomposed by the dropwise addition of water (500 µl) with stirring. The white precipitate was dissolved by the addition of a few drops of conc. $H_2SO_4$ (350 µl). The organic phase was separated from the aqueous phase and the aqueous phase extracted with TBME (100 µl). The combined extracts were washed to neutral pH with water and dried on anhydrous $MgSO_4$. The drying agent was filtered off and the solvent removed on a rotary evaporator to yield 15-methyl-1-hexadecanol (6.00 mg, 79%), with a purity of 95% (GC-MS). MS (70 eV): *m/z* (%) 238(1.3), 210(4), 182(4), 111(25), 97(42), 83(60), 69(78), 57(87), 55(86), 43(100), 41(74).

8-Methyl-1-nonanol MS (70 eV): *m/z* (%) 140($M^+$, 4), 126(14), 112(11), 111(17), 97(14), 85(50), 83(30), 71(51), 70(52), 69(79), 57(74), 56(58), 55(65), 43(100), 41(84).

11-Methyl-1-tridecanol. MS (70 eV): *m/z* (%) 196(0.04), 168(1.5), 166(2), 139(4), 125(25), 97(50), 83(72), 71(80), 69(100), 57(85), 55(94), 43(50), 41(62).

12-Methyl-1-tridecanol. MS (70 eV): *m/z* (%) 196(0.5), 168(4), 140(6), 125(4), 111(7), 97(33), 83(56), 69(84), 57(84), 56(100), 55(88), 43(93), 41(81).

13-Methyl-1-tetradecanol. MS (70 eV): *m/z* (%) 210(0.02), 140(0.2), 125(3), 111(4), 97(18), 83(32), 69(100), 57(33), 56(92), 55(95), 43(72), 41(62).

14-Methyl-1-pentadecanol. MS (70 eV): *m/z* (%) 224(0.8), 196(4), 168(5), 140(4), 125(6), 111(21), 97(39), 83(59), 69(81), 57(86), 56(86), 55(84), 43(100), 41(78).

14-Methyl-1-hexadecanol. MS (70 eV): *m/z* (%) 238(0.5), 210(3), 125(13), 111(30), 97(65), 83(79), 70(98), 57(92), 55(100), 43(72), 41(88).

*Preparation 2-pentadecanol*. Following the protocol of Brown and Geoghegan (1967), 1-pentadecene (4.80 g, 0.022 mol) was added to a solution of mercuric acetate (7.01 g, 0.022 mol), in water (22 ml) and purified tetrahydrofuran (22 ml), in a 150-ml round-bottom flask containing a magnetic follower (stir bar). The reaction mixture was stirred for 1.5 hr at room temperature to complete the oxymercuration stage. Sodium hydroxide solution (20 ml, 3 M) was then added, followed by a solution of sodium borohydride (20 ml, 0.5 M) in sodium hydroxide (3.0 M). The mercury was allowed to settle and the reaction mixture diluted with purified diethyl ether (20 ml). The organic phase was separated from the aqueous phase and dried on anhydrous $MgSO_4$. The drying agent was filtered off, and the solvent removed on a rotary evaporator to yield 2-pentadecanol (2.88 g, 57%), with a purity of 82% (GC-MS). MS (70 eV): *m/z* (%) 210(2), 125(6), 111(8), 97(15), 83(12), 69(12), 57(20), 55(23), 45(100), 43(41).

This product was used for the preparation of 2-pentadecanone as described below.

*Preparation of 6,10,14-trimethyl-2-pentadecanol*. 6,10,14-Trimethyl-2-pentadecanone (hexahydrofarnesyl acetone) (50 µl, 0.15 mmol) in TBME (100 µl) was placed in a 2 ml Reacti-Vial with a magnetic follower and reduced with $LiAlH_4$, as described above for the primary alcohols, to yield 6,10,14-trimethyl-2-pentadecanol (31.87 mg, 91%), with a purity of 93% (GC-MS). MS (70 eV): *m/z* (%) 210(0.3), 196(1), 182(2), 167(1), 140(3), 125(17), 111(24), 97(44), 83(25),

71(63), 69(60), 57(100), 55(70), 45(69), 43(61), 41(38). $^{13}$C NMR ($CDCl_3$, 101 MHz): δ = 19.70 (q, C-16), 19.77 (q, C-17), 22.64 (q, C-18*), 22.74 (q, C-15*), 23.44 (t, C-8), 23.53 (q, C-1), 24.68 (t, C-4*), 24.83 (t, C-12*), 28.00 (d, C-14), 32.79 (d, C-10*), 32.81 (d, C-6*), 37.05 (t, C-5*), 37.15 (t, C-7*), 37.30 (t, C-9*), 37.41 (t, C-11*), 39.39 (t, C-13*), 39.75 (t, C-3*), 68.19 (d, C-2).

*Preparation of aldehydes*. Branched and unbranched aldehydes were synthesized by oxidation of the corresponding primary alcohols with pyridinium chlorochromate (PCC), according to Harwood et al. (2003), in yields ranging from 53 to 84%, as exemplified by a microscale preparation of 15-methylhexadecanal from 15-methyl-1-hexadecanol.

15-Methylhexadecanal. 15-Methyl-1-hexadecanol (6.00 mg, 0.023 mmol), dissolved in DCM (1 ml) was added dropwise to a magnetically stirred suspension of PCC (7.58 mg, 0.352 mmol) in DCM (200 µl) at 0ºC. The reaction mixture was stirred at room temperature for 2 h, diluted with dry diethyl ether (50 µl) and the supernatant solution decanted from the black residue. The residue was washed with diethyl ether (3 x 30 µl), employing centrifugal separation of the phases, and the combined ether and DCM phases filtered through a short column of silica gel. The solution was dried on anhydrous $MgSO_4$, then the drying agent filtered off and the solvent removed on a rotary evaporator to yield 15-methylhexadecanal (5.00 mg, 84%), with a purity of 98% (GC-MS). MS (70 eV): m/z (%) 254($M^+$, 0.1), 236(0.3), 210(1), 208(2), 180(2), 154(2), 152(3), 137(4), 123(8), 109(14), 96(23), 95(31), 82(40), 81(43), 69(61), 67(47), 57(100), 55(71), 43(91), 41(62). 13C NMR (CDCl3, 150.88 MHz): δ = 22.70 (q, C-16, C-17), 28.01 (d, C-15), 29.10 (t), 29.21 (t), 29.27 (t), 29.39 (t), 29.47 (t), 29.63 (t), 29.67 (t), 29.70 (t), 29.75 (t), 29.98 (t), 33.82 (t, C-3), 39.10 (t, C-14), 43.96 (t, C-2), 203.03 (d, C-1).

3-Methylpentanal. MS (70 eV): *m/z* (%) 100($M^+$, 6), 82(2), 71(19), 58(39), 57(50), 56(100), 44(55), 43(79), 41(86). $^{13}C$ NMR (CDCl3, 75.38 MHz): δ = 11.23 (q, C-5), 19.45 (q, C-6), 29.69 (t, C-4), 31.68 (d, C-3), 41.15 (t, C-2), 203.21 (d, C-1).

8-Methylnonanal. MS (70 eV): *m/z* (%) 138(0.4), 128(4), 114(6), 110(7), 95(34), 82(45), 81(31), 71(31), 69(45), 67(27), 57(93), 55(56), (100), 41(95).

11-Methyltridecanal. MS (70 eV): *m/z* (%) 212(M+, 0.1), 194(0.4), 184(0.7), 183(1), 166(2), 165(3), 139(3), 138(2), 137(5), 123(6), 109(26), 96(28), 95(46), 83(42), 82(38), 81(40), 70(59), 69(40), 67(36), 57(95), 55(78), 43(67), 41(100).

12-Methyltridecanal. MS (70 eV): *m/z* (%) 212($M^+$, 0.1), 194(0.4), 184(1), 168(2), 166(2), 153(2), 151(2), 140(3), 138(3), 137(4), 123(7), 110(8), 109(16), 96(20), 95(37), 83(40), 81(47), 71(35), 69(61), 67(45), 57(100), 55(74), 43(92), 41(67).

13-Methyltetradecanal. MS (70 eV): *m/z* (%) 208(2), 182(3), 180(2), 154(2), 152(2), 137(3), 124(6), 123(6), 109(12), 96(26), 95(28), 82(46), 71(25), 69(43), 68(31), 67(31), 57(79), 55(60), 43(100), 41(83).

14-Methylpentadecanal. MS (70 eV): *m/z* (%) 222(1), 212(1), 196(1), 194(2), 166(2), 164(2), 152(2), 138(3), 137(4), 124(5), 123(6), 109(14), 96(28), 95(30), 83(31), 82(51), 81(30), 69(43), 57(79), 55(70), 43(100), 41(81).

14-Methylhexadecanal. MS (70 eV): *m/z* (%) 236(1), 225(1), 208(1), 181(1), 151(2), 137(5), 123(9), 109(21), 96(30), 95(41), 82(45), 81(45), 70(64), 69(49), 67(35), 57(100), 55(79), 43(77), 41(92). $^{13}C$ NMR ($CDCl_3$, 150.88 MHz): δ = 11.44 (q, C-16), 19.26 (q, C-17), 24.73 (t, C-12), 29.10 (t, C-11), 29.27 (t), 29.53 (t, 2 x C), 29.62 (t), 29.67 (t), 29.70 (t), 29.75 (t), 30.06 (t), 31.96 (t, C-3), 34.44 (d, C-14), 36.68 (t, C-13), 43.95 (t, C-2), 203.00 (d, C-1).

Tetradecanal. MS (70 eV): *m/z* (%) 212($M^+$, 0.13), 194(1), 184(1), 168(5), 166(3), 138(7), 124(9), 110(38), 96(32), 82(38), 69(27), 68(28), 67(28), 57(82), 55(77), 43(100), 41(95). $^{13}C$ NMR ($CDCl_3$,

101 MHz): δ = 14.11 (q, C-14), 22.11 (t, C-13), 22.71 (t, C-12), 29.09 (t), 29.19 (t), 29.27 (t), 29.38 (t), 29.45 (t), 29.61 (t), 29.67 (t), 29.69 (t), 31.94 (t, C-3), 43.92 (t, C-2), 203.00 (d, C-1).

Pentadecanal. MS (70 eV): *m/z* (%) 226($M^+$, 0.2), 208(2), 182(4), 180(3), 152(5), 138(6), 124(8), 110(14), 96(40), 82(44), 69(22), 68(23), 67(22), 57(65), 55(61), 43(93), 41(100).

Hexadecanal. MS (70 eV): *m/z* (%) 240($M^+$, 0.2), 222(2), 194(3), 166(3), 152(3), 138(6), 124(10), 110(16), 109(15), 96(45), 82(53), 69(25), 68(26), 67(25), 57(61), 55(58), 43(100), 41(94).

Heptadecanal. MS (70 eV): *m/z* (%) 254($M^+$, 0.2), 236(2), 210(3), 208(3), 180(2), 152(3), 138(6), 137(5), 124(10), 123(8), 110(15), 109(14), 96(43), 82(47), 69(23), 68(23), 67(22), 57(60), 55(57), 43(100), 41(85).

*Preparation of methyl ketones*. Methylketones were prepared by PCC oxidation, according to Harwood et al. (2003), from the corresponding secondary alcohols, as described in the previous section.

2-Tetradecanone. MS (70 eV): *m/z* (%) 212($M^+$, 4), 197(2), 154(4), 153(3), 127(2), 111(2), 110(2), 96(6), 85(11), 71(42), 59(48), 58(100), 55(18), 43(96), 41(33). $^{13}$C NMR (CDCl3, 75.38 MHz): δ = 14.10 (q, C-14), 22.68 (t, C-13), 23.88 (t, C-4), 29.18 (t), 29.35 (t), 29.40 (t), 29.47 (t), 29.53 (t), 29.61 (t), 29.65 (q, C-1), 29.81 (t), 31.92 (t, C-12), 43.81 (t, C-3), 209.30 (d, C-2).

2-Pentadecanone. MS (70 eV): *m/z* (%) 226($M^+$, 7), 211(3), 168(6), 138(3), 127(5), 110(5), 96(11), 85(17), 71(39), 59(68), 58(92), 43(100), 41(55).

*Preparation of 6,10-dimethyl-2-undecanone.* 6,10-Dimethyl-5,9-undecadien-2-one (geranylacetone) (0.25 g, 1.29 mmol) was hydrogenated in glacial acetic acid (50 ml) using platinum on activated charcoal (10% Pt/C) as catalyst at atmospheric pressure. The consumption of hydrogen was monitored and was terminated after the consumption of a volume of hydrogen equivalent to the reduction of two double bonds. The reaction mixture was then diluted with an equal volume of distilled water and extracted with $CHCl_3$ (3 x 10 ml). The combined extracts

were washed with water to neutral pH and dried on anhydrous $MgSO_4$, after which the drying agent was filtered off and the solvent removed on a rotary evaporator to yield 6,10-dimethyl-2-undecanone (0.24 g, 95%), with a purity of 88% (GC-MS). MS (70 eV): *m/z* (%) 198($M^+$, 0.4), 180(4), 140(3), 123(3), 109(11), 95(11), 85(16), 71(28), 58(77), 43(100), 41(23). $^{13}C$ NMR ($CDCl_3$, 100.58 MHz): δ = 19.55 (q, C-12), 21.44 (t, C-4), 22.69 (q, 2 x C), 24.73 (t, C-8), 27.96 (d, C-10), 29.83 (q, C-1), 32.65 (d, C-6), 36.52 (t, C-7), 37.10 (t, C-5), 39.32 (t, C-9), 44.14 (t, C-3), 209.35 (d, C-2).

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