Temporal Changes in the Neonatal Recognition Cue of Dohne Merino Lambs (*Ovis aries*)

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Abstract

The strong bond that is formed between ewe and lamb after parturition is important for lamb survival. Evidence exists that the ewe mainly employs olfactory recognition cues to distinguish her lamb from other lambs in the flock. We have found that volatile organic compounds in the wool of Merino lambs that presumably constitute the neonatal recognition cue in sheep undergo temporal changes, at least during the first 100 days of their lives. To compensate for changes in the composition of the recognition cue, ewes are compelled to sample the changing effluvia of lambs to refresh their memories of their lambs' odor in order to preserve the exclusive olfactory attachment to their lambs. These changes could be the reason for the well-known regular sniffing of ewes at their lambs. Parallel changes in the effluvia of twin lambs ensure the retention of the previously observed intra-twin similarity of the recognition cues of twin lambs. The coherent manner in which these changes take place probably contributes to the cohesion in a flock of sheep.

Key Words: Semiochemical communication, Kin recognition, Headspace analysis, Sample enrichment probe; SEP technique, Gas Chromatography-Mass Spectrometry (GC-MS)

Introduction

Sheep are seasonal breeders, and large proportions of ewes give birth during a relatively short lambing season. Recognition of a lamb is, therefore, crucial for the mother to provide nourishment selectively to her offspring. The attraction to the amniotic fluid results in the mother licking and grooming the lamb, low-pitched bleating and the acceptance of the new-born lamb at the udder. These behavioral patterns are directed towards the formation of an exclusive olfactory attachment between the ewe and lamb. This relies on the ewe's ability to memorize the individual olfactory cues from her offspring (Lévy et al. 2004).

The bond between ewe and lamb is established through contact with the new-born lamb within 4–6 h after birth. If this bond is not formed, maternal interest wanes and the ewe will not accept the lamb. Once the bond has been formed, however, ewe and lamb can be separated for relatively long periods without disrupting the integrity of the bond (Lindsay 1988). The selective ewe–lamb bond did not develop in ewes that were rendered anosmic (Lévy et al. 2004), indicating that maternal selectivity and ewe–lamb bond depends strongly on the individual odor of the offspring. Although ewes are capable of recognizing their lambs by auditory and visual cues (Morgan et al. 1975; Shillito and Alexander 1975; Poindron and Carrick 1976; Alexander and Shillito 1977; Terrazas et al 1999), Alexander and Stevens (1981) and Lynch et al. (1992) have presented evidence showing that the discrimination of ewes between their own and alien lambs is mediated primarily by an olfactory cue from the lamb's wool and skin — not from a specific area on the lamb's body, nor from the amniotic fluid. Ewes primarily rely on olfactory cues as final assurance before allowing lambs at the udder (Lindsay, 1988).

Exploring the possibility that the neonatal recognition pheromone originates from the wool of the lamb, Burger et al. (2011b) identified 133 volatile organic compounds (VOCs) associated with the wool of Dohne Merino (*Ovis aries*) lambs that are presumably constituents of a putative neonatal recognition cue in this species. Quantitative analysis, using the sample enrichment (SEP) technique (Burger et al. 2011a), and subsequent comparison of the odor profiles of twin lambs revealed that the wool volatiles of twins are

both qualitatively and quantitatively practically identical, but differ from those of other twins or non-twin lambs in the flock ($P < 0.001$). The analytical results also highlighted the accuracy with which analyses of the VOCs of the cranial wool samples were carried out using the SEP technique. However, attempts at fostering lambs using synthetic mixtures were unsuccessful (Burger et al. 2011b). The fostering experiments were carried out by dressing alien lambs in cotton fleece jackets sprayed with solutions of synthetic mixtures emulating the composition of the wool VOCs of the lambs of the experimental ewes. The failure of these experiments could be ascribed to various imponderables, such as the unpredictable difference in the rate of release of different compound classes from cotton wool jackets instead of lamb's wool, and the co-evaporation of the solvent and some highly volatile constituents of the synthetic mixture (Burger et al. 2011b). The possibility also has to be considered that the lamb's unique recognition cue could wear off or change in composition as the lamb grows older, in which case the formulation of synthetic mixtures for bioassays and fostering experiments would require accurate information on the extent of the change in the recognition cue and the rate at which it takes place.

The present study was carried out to gain information on the properties of the recognition cue, such as the consistency of its composition and its persistence (longevity), information that could in future studies contribute to ensuring the success of bioassays with mixtures of synthetic analogues of the wool VOCs.

Materials and methods

Sample collection

A flock of Dohne Merino ewes and their lambs was available at Stellenbosch University's experimental farm Mariendahl for the collection of wool samples. At Mariendahl, single-bearing ewes and their lambs are released into large camps soon after parturition, whereas twin-bearing ewes are kept in small pens until the lambs are stronger. Although not required for the envisaged research, collection of wool samples from twin lambs in their small pens was preferred as more convenient and less stressful for the lambs than collecting samples in large camps. Cranial wool was chosen for analysis because the heads of the lambs were expected to be less contaminated with foreign matter than the rest of their bodies. We have also observed that ewes almost invariably sniff at lambs on first contact. To follow possible temporal changes in the odor of lambs, cranial wool samples were collected from the lambs (n = 10) the morning after birth (Day 1) and on Day 7 during the lambing season (March to

April) in 2007. After about 7 days twin-bearing ewes and their offspring are normally also released into the large camps housing the single-bearing ewes. Here they are not readily available for sample collection until Day100 when the lambs are all brought in for performance evaluation and treatment. In 2010, samples were therefore collected from the lambs (n = 6) on Days 1, 7 and 100. All samples were stored in glass vials (25 ml) with Teflon-faced septa at −20 °C until analyzed. No microbial activity was detected in wool samples stored at this temperature.

The procedures followed in our research were approved by the Stellenbosch University Ethics Committee: Animal Care and Use (Ethics number 11NC_BU01).

Analytical procedures

Sample enrichment of the headspace VOCs of the collected cranial wool samples using the SEP technique (Burger et al. 2011a), gas chromatographic (GC), low- and high-resolution gas chromatographic-mass spectrometric analysis (GC-MS analysis), enantioselective GC analysis, Kovats retention index (RI) determination and GC-MS comparison of the tentatively identified VOCs with authentic synthetic analogues were carried out as described by Burger et al. (2011b).

Statistical analysis

The vast amount of data generated during this investigation into the composition of VOCs present in the wool of the lambs made multivariable methods of analysis indispensable in attempts at reaching meaningful interpretation of the data. The changes in the relative concentrations of the wool VOCs over a period of 1 week and 100 days in 2007 and 2010, respectively, were assessed to determine whether the odor of the lambs changes in a predictable and uniform manner. The VOCs from cranial wool samples collected from 10-week-old lambs (5 twins) born in 2007, and from 6 lambs (3 twins) born in 2010 were analyzed and the resulting data used for statistical analysis. In 2007 and 2010, 87 and 81 constituents, respectively, were used as variables in statistical analyses.

Biplots can be considered as multivariate scatterplots that simultaneously give a graphical presentation of samples (lambs as points on the graph) and variables (identified constituents as linear axes on the graph). 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 off from the axis. In addition to

this usage of biplot axes, the angle between any 2 axes is an approximation of the correlation between the 2 relevant variables. Constituents lying on axes close to one another have a high level of correlation with one another and constituents displayed as axes that are 90 degrees in relation to one another have no correlation with each other. An axis is labeled at the positive value of its calibration. 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.

Of the 133 wool VOCs identified in the cranial wool of neonatal lambs (104 in the headspace and 29 in extracts of the wool), 87 headspace VOCs identified in the lambing season of 2007 and 81 headspace VOCs identified in 2010 were used as variables for statistical analysis. Peak areas were normalized across all samples to produce comparable variables with zero means and unit standard deviation (Kowalski and Bender, 1972). The qualitative and quantitative data of the 87 constituents (2007) and 81 constituents (2010) were used to construct principal component analysis (PCA) biplots. The biplots were constructed, and permutation tests and investigations into axis predictivity were carried out using R (Vienna, Austria), as described by Aldrich et al. (2004), Garden-Lubbe et al. (2008) and Gower et al. (2011).

Results

Consistence of composition of the recognition cue

Assuming that the recognition cue is produced by the lamb, a series of experiments was carried out to gain insight into the consistency of the composition of the neonatal recognition cue. Samples of the cranial wool of Dohne Merino lambs (n = 10) were collected the morning after birth (Day 1) and on Day 7 during the lambing season in 2007. Comprehensive qualitative and quantitative headspace analyses of the VOCs of these wool samples revealed temporal changes, mainly with respect to the quantitative composition of the VOCs. In view of these results, the experiment was repeated and extended in 2010 to include the collection of cranial wool samples from the experimental lambs (n = 6) on Day 100. The results of the previous experiment were confirmed. A summary of the relevant data is given in Table 1.

Table 1 Compounds from the cranial wool of Döhne Merino lambs collected during the lambing seasons of 2007 and 2010 arranged according to compound class.

No.	Compounds	2007[a]		2010			Remarks [b]
		Day 1	Day 7	Day 1	Day 7	Day 100	
C10	Nonane	✓	✓				a,b,c,j,l
C20	Decane	✓	✓				a,b,c,j,l
C28	Undecane	✓	✓			✓	a,b,c
C36	Dodecane	✓	✓	✓	✓	✓	a,b,c
C46	Tridecane	✓	✓	✓	✓	✓	a,b,c,i
C58	Tetradecane	✓	✓	✓	✓	✓	a,b,c,i
C72	Hexadecane	✓	✓	✓	✓	✓	a,b,c,i
C80	Heptadecane	✓	✓	✓	✓	✓	a,b,c,i
C89	Octadecane	✓	✓	✓	✓	✓	a,b,c,i
C100	Nonadecane	✓	✓		✓	✓	a,b,c,i
C91	2,6,10,14-Tetramethylhexadecane	✓	✓	✓	✓	✓	a,b,c,g
C44	1-Tridecene	✓	✓		✓	✓	a,b,c,e
C55	1-Tetradecene	✓	✓				a,b,c,e,l
C66	1-Pentadecene	✓	✓	✓	✓	✓	a,b,c,e
C118	1-Pentacosene			✓	✓	✓	a,b,d,m
C2	1-Pentanol	✓	✓				a,b,c,j,l
C15	1-Heptanol	✓	✓	✓	✓	✓	a,b,c
C25	1-Octanol	✓	✓	✓	✓	✓	a,b,c,i
C33	1-Nonanol	✓	✓	✓	✓	✓	a,b,c
C75	1-Tetradecanol	✓	✓	✓	✓	✓	a,b,c,i,k
C87	1-Pentadecanol	✓	✓	✓	✓	✓	a,b,c,i,k
C98	1-Hexadecanol	✓	✓	✓	✓	✓	a,b,c,i,k
C108	1-Octadecanol			✓	✓	✓	a,b,c,i,k,m
C114	1-Eicosanol					✓	a,b,c,i,m
C6	4-Methyl-1-pentanol	✓	✓	✓	✓	✓	a,b,c
C95	6,10,14-Trimethyl-2-pentadecanol	✓	✓			✓	a,b,c,g
C4	Hexanal	✓	✓	✓	✓	✓	a,b,c,j
C8	Heptanal	✓	✓	✓	✓	✓	a,b,c,j
C19	Octanal	✓	✓	✓	✓	✓	a,b,c
C27	Nonanal	✓	✓	✓	✓	✓	a,b,c
C35	Decanal	✓	✓	✓	✓	✓	a,b,c
C45	Undecanal	✓	✓	✓	✓	✓	a,b,c
C57	Dodecanal	✓	✓	✓	✓	✓	a,b,c
C67	Tridecanal	✓	✓	✓	✓	✓	a,b,c
C73	Tetradecanal	✓	✓	✓	✓	✓	a,b,c
C81	Pentadecanal	✓	✓	✓	✓	✓	a,b,c
C90	Hexadecanal	✓	✓	✓	✓	✓	a,b,c
C101	Heptadecanal	✓	✓	✓	✓	✓	a,b,c
C1	3-Methylpentanal	✓	✓				a,b,c,g,l
C24	7-Methyloctanal	✓	✓				a,b,c,d,l
C31	8-Methylnonanal	✓	✓			✓	a,b,c,d
C41	9-Methyldecanal	✓	✓				a,b,c,d,l
C51	10-Methylundecanal	✓	✓				a,b,c,d,l
C68	12-Methyldodecanal	✓	✓				a,b,c,d,l
C77	13-Methyltetradecanal	✓	✓				a,b,c,d,l
C86	14-Methylpentadecanal	✓	✓		✓	✓	a,b,c,d
C97	15-Methylhexadecanal	✓	✓			✓	a,b,c,d
C23	(E)-2-Octenal	✓	✓	✓	✓	✓	a,b,c,d
C30	(E)-2-Nonenal	✓	✓	✓	✓	✓	a,b,c,d
C40	(E)-2-Decenal	✓	✓	✓	✓	✓	a,b,c,d
C50	(E)-2-Undecenal	✓	✓	✓	✓	✓	a,b,c,d,e
C62	(E)-2-Dodecenal	✓	✓		✓	✓	a,b,c,d

C78	(E)-2-Tetradecenal	✓	✓	✓	✓	✓	a,b,c,d
C5	2-Methylpyrimidine	✓	✓				a,b,c,l
C9	Dimethyl sulfone			✓	✓	✓	a,b,c,m
C11	2,5-Dimethylpyrimidine	✓	✓				a,b,c,l
C17	2-Octanone	✓	✓				a,b,c,j,l
C26	2-Nonanone	✓	✓	✓	✓	✓	a,b,c
C34	2-Decanone	✓	✓	✓	✓	✓	a,b,c
C43	2-Undecanone	✓	✓	✓	✓		a,b,c
C54	2-Dodecanone			✓	✓	✓	a,b,c,m
C65	2-Tridecanone	✓	✓	✓	✓		a,b,c
C71	2-Tetradecanone	✓	✓	✓	✓	✓	a,b,c
C79	2-Pentadecanone	✓	✓	✓	✓	✓	a,b,c
C99	2-Heptadecanone	✓	✓	✓	✓	✓	a,b,c
C16	3-Octanone	✓	✓				a,b,c,l
C14	6-Methyl-2-heptanone	✓	✓	✓	✓	✓	a,b,c,j
C47	3-Methyl-2-undecanone	✓					a,b,c,d,f,l
C56	6,10-Dimethyl-2-undecanone	✓					a,b,c,g,l
C60	(5E)-6,10-Dimethyl-5,9-undecadien-2-one	✓	✓	✓	✓	✓	a,b,c
C93	6,10,14-Trimethyl-2-pentadecanone	✓	✓	✓	✓	✓	a,b,c,g
C48	Nonan-4-olide	✓	✓	✓	✓	✓	a,b,c,f
C61	Decan-4-olide				✓	✓	a,b,c,f,m
C76	Dodecan-4-olide			✓	✓	✓	a,b,c,f,m
C109	Hexadecan-4-olide				✓	✓	a,b,c,f,m
C42	Nonanoic acid	✓	✓	✓	✓		a,b,c,i
C53	Decanoic acid	✓	✓	✓	✓	✓	a,b,c,i
C69	Dodecanoic acid	✓	✓	✓	✓		a,b,c,i
C85	Tetradecanoic acid	✓	✓	✓	✓	✓	a,b,c,i
C94	Pentadecanoic acid				✓	✓	a,b,c,i,m
C104	Hexadecanoic acid	✓	✓	✓	✓	✓	a,b,c,i
C112	Octadecanoic acid			✓	✓		a,b,c,i,m
C115	Eicosanoic acid					✓	a,b,c,i,m
C103	(Z)-9-Hexadecenoic acid			✓	✓	✓	a,b,c,e,m
C110	(Z,Z)-9,12-Octadecadienoic acid					✓	a,b,c,m
C111	(Z)-9-Octadecenoic acid			✓		✓	a,b,c,e,m
C88	Ethyl tetradecanoate	✓	✓	✓	✓		a,b,c
C92	Isopropyl tetradecanoate	✓	✓	✓	✓		a,b,c,d
C18	2-Pentylfuran	✓	✓				a,b,c,l
C22	Phenylacetaldehyde			✓	✓	✓	a,b,c,m
C29	*N*-Methyl-2-piperidinone	✓	✓		✓		a,b,c
C37	3-Ethyl-4-methyl-1H-pyrrole-2,5-dione			✓	✓	✓	a,b,h,m
C38	2-Ethyl-3-methyl-pyrrolidine-2,5-dione			✓			a,h,f,m
C39	3-Methyl-4-vinyl-1H-pyrrole-2,5-dione			✓	✓		a,b,h,m
C124	Cholest-5-en-3β-ol			✓	✓	✓	a,b,c,i,m
C21	Unidentified	✓	✓			✓	
C59	Unidentified	✓					l
C63	Unidentified	✓	✓				l
C64	Unidentified	✓	✓				l
C70	Unidentified	✓	✓				l
C74	Unidentified	✓	✓				l
C83	Unidentified	✓	✓			✓	
C96	Unidentified	✓					a,b,l

C3	Unidentified		✓	✓	✓	✓	✓

[a]As published in Burger *et al*., 2011b. [b]Reliability of identification is indicated by the following: a, low-resolution EI mass spectrum; b, library spectrum (NBS and/or NIST); c, retention time comparison with synthetic compound; d, kovats retention index; e, double bond localization by DMDS derivatization and GC-MS analysis; f, absolute configuration given in Burger et al. (2011b); g, absolute configuration not determined; h, tentative identification; i, compounds previously identified in lanolin (Schlossman and McCarthy, 1979; Motiuk, 1979a, 1979b, 1980); j, compounds previously identified in wool (Lisovac and Shooter, 2003); k, compounds previously identified in inguinal gland of ewes (Rietdorf, 2002); l, compounds only identified during the lambing season of 2007; m, compounds only identified during the lambing season of 2010.

Of the 133 wool VOCs identified in the cranial wool of neonatal lambs during the 2007 and 2010 lambing seasons, 104 were identified as wool headspace VOCs and 29 were present in extracts of the wool. The headspace VOCs comprised the following: saturated and unsaturated hydrocarbons, branched and unbranched primary alcohols, branched and unbranched aldehydes, unsaturated aldehydes, branched and unbranched ketones, saturated and unsaturated carboxylic acids, carboxylic acid esters, butanolides (γ-lactones), 2-pentylfuran, dimethyl sulfone, 2-methylpyrimidine, 2,5-dimethylpyrimidine, 3-ethyl-4-methyl-1H-pyrrole-2,5-dione, 3-methyl-4-vinyl-1H-pyrrole-2,5-dione and 2-ethyl-3-methylpyrrolidine-2,5-dione, all of which were unambiguously identified by the analytical techniques nentioned above. The additional 29 VOCs identified in wool extracts in our previous study (Burger et al. 2011b) were not considered relevant in the present study and hence only the 104 VOCs present in headspace samples identified in the cranial wool of twin Dohne Merino lambs are included in Table 1.

Considerable quantitative differences were found between the VOCs enriched from the headspace gas of cranial wool samples collected from the same lambs on Days 1 and 7 after they were born during the lambing season of 2007, as well as on Days 1, 7 and 100 during the lambing season of 2010. Typical differences are illustrated in Figure 1 in which examples of the total ion chromatograms (TICs) of the VOCs enriched on the three sampling occasions in 2010 are depicted.

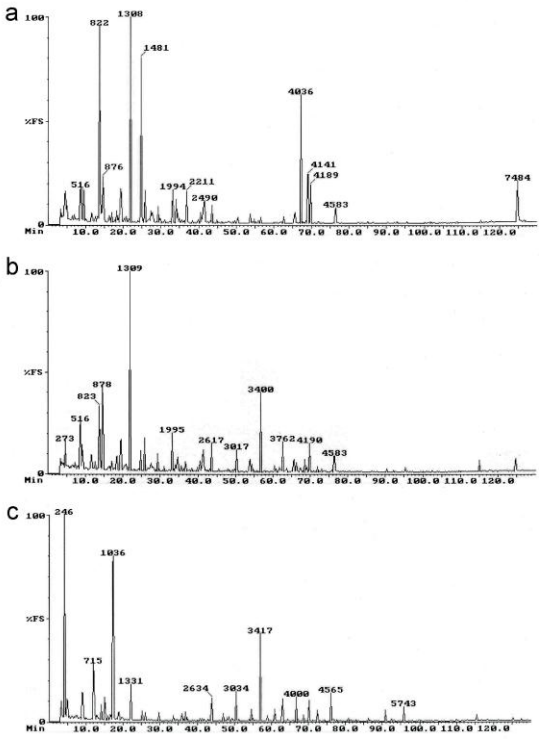

Figure 1 TICs of the headspace VOCs from cranial wool collected on (a) Day 1, (b) Day 7 and (c) Day 100 from Dohne merino lamb US-2010-0205 during the lambing season of 2010.

These differences can also be visualized by superimposing reconstructed TICs of wool VOCs collected from the same lamb on the respective sampling days. The sharp contrast between the difference in quantitative composition of the cranial wool VOCs of lambs on the 3 sampling occasions, and the remarkable similarity of the VOCs from day-old dizygotic twin lambs (Burger et al. 2011b) is illustrated in Figures 2a and 2b, respectively.

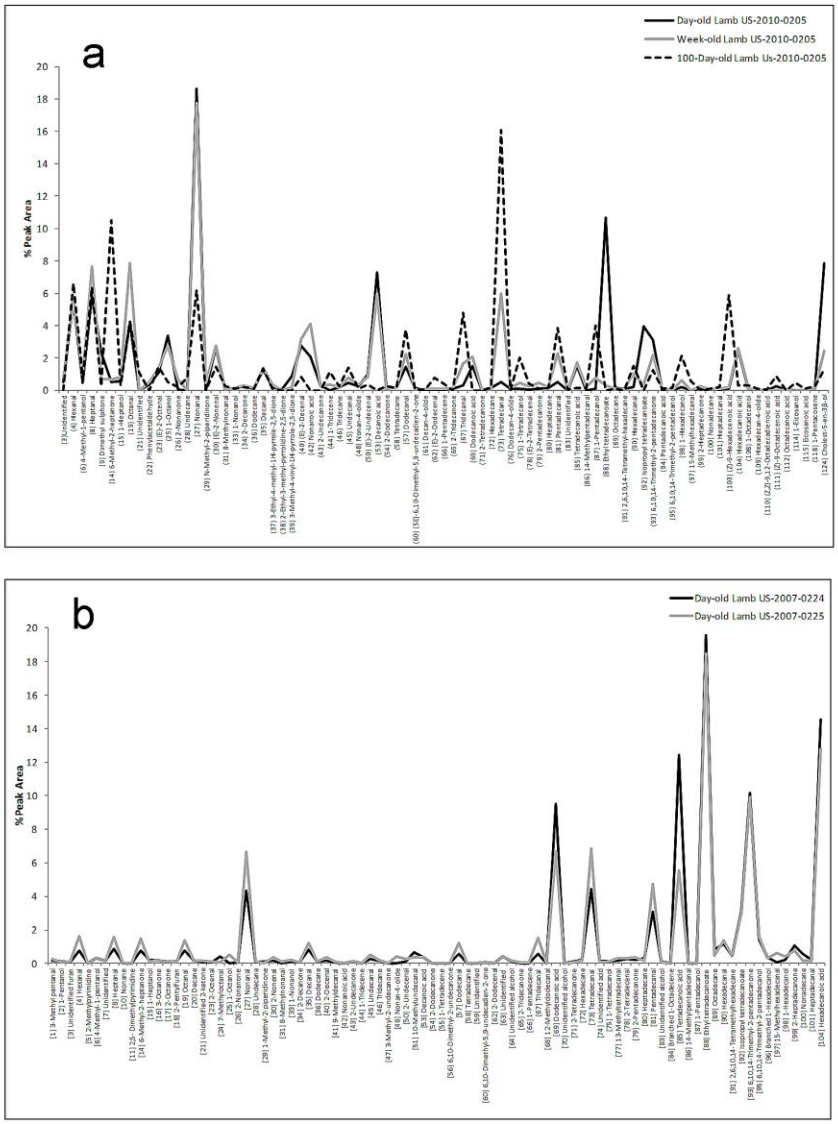

Figure 2 Quantitative comparison (percentage peak areas) of the headspace VOCs of cranial wool collected, (a) during the lambing season of 2010 on Day 1 (black), Day 7 (grey) and Day 100 (dashed line) from lamb US-2010-0205; and (b) during the lambing season of 2007 from day-old twin lambs US-2007-0224 (black) and US-2007-0225 (grey) by superimposing the reconstructed gas chromatograms. Note that the concentrations of compounds 88 and 93 are so similar that the black chromatogram of lamb US-2007-0224 is only visible at the apexes of the peaks (Burger et al. 2011b).

The differences in the average percentage peak areas (>1%) of the wool VOCs present in wool samples collected from lambs on different days during the lambing seasons in 2007 and 2010 are shown in Figure 3.

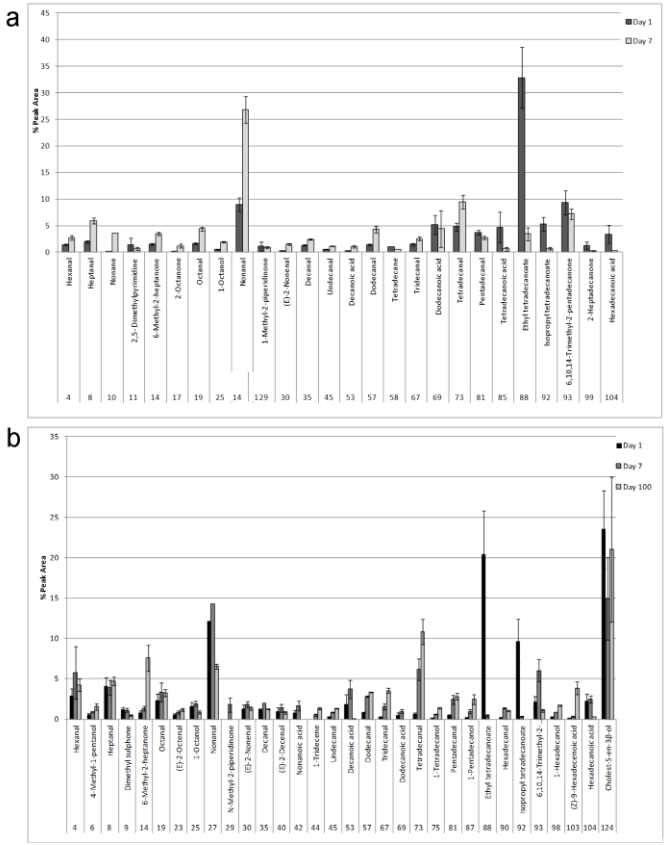

Figure 3 Comparison of the average percentage peak areas (> 1%) of the headspace VOCs of cranial wool collected during the lambing season of 2007 (a) on Day 1 (black), Day 7 (grey), and during the lambing season of 2010 (b) on Day 1 (black), Day 7 (dark grey) and Day 100 (light grey).

Approximately 78% of the total average peak area of the total ion chromatograms (TICs) of the VOCs present in the headspace of the wool of day-old Dohne Merino lambs born in the 2007 lambing season consisted of only 9 compounds, *viz* ethyl tetradecanoate (**C88**) (33%), 6,10,14-trimethyl-2-pentadecanone (**C93**) (9%), nonanal (**C27**) (9%), isopropyl tetradecanoate (**C92**) (5%), dodecanoic acid (**C69**) (5%), tetradecanal (**C73**) (5%), tetradecanoic acid (**C85**) (5%), pentadecanal (**C81**) (4%) and hexadecanoic acid (**C104**) (3%). In comparison, approximately 61% of the total quantity of VOCs present in the headspace of wool samples collected from the same lambs on Day 7 comprised 7 constituents: nonanal (**C27**) (27%), tetradecanal (**C73**) (9%), 6,10,14-trimethyl-2-pentadecanone (**C93**) (7%), heptanal (**C8**) (6%), dodecanoic acid (**C69**) (4%), octanal (**C19**) (4%) and dodecanal (**C57**) (4%).

Ignoring the presence in the wool of cholest-5-en-3β-ol (**C124**), which is practically non-volatile and ubiquitous in mammalian material, 62% of the total average peak area of the VOCs present in the headspace of the wool of day-old Dohne Merino lambs born in the 2010 lambing season comprised 4 constituents: ethyl tetradecanoate (**C88**) (29%), nonanal (**C27**)

(15%), isopropyl tetradecanoate (**C92**) (13%) and heptanal (**C8**) (5%). In the wool analyzed from the same lambs on Day 7, 50% of the VOCs present in the headspace of the wool samples comprised 7 compounds: nonanal (**C27**) (17%), 6,10,14-trimethyl-2-pentadecanone (**C93**) (8%), tetradecanal (**C73**) (7%), hexanal (**C4**) (6%), heptanal (**C8**) (4%), decanoic acid (**C53**) (4%) and octanal (**C19**) (4%). On Day 100, 61% of the VOCs present in the headspace of these lambs comprised 9 compounds: tetradecanal (**C73**) (14%), nonanal (**C27**) (9%), 6-methyl-2-heptanone (**C14**) (9%), heptanal (**C8**) (6%), hexanal (**C4**) (5%), dodecanal (**C57**) (5%), tridecanal (**C67**) (5%), (*Z*)-9-hexadecenoic acid (**C103**) (4%) and octanal (**C19**) (4%). During the lambing seasons of 2007 and 2010 the majority of the compounds present in the olfactory signature of the lambs were aldehydes.

The majority of the VOCs (53% in 2007, 60% in 2010) showed an increase in concentration as the lambs grew older. The majority of these compounds were saturated and unsaturated, branched and unbranched aldehydes. In 2007 and 2010, the concentrations of, respectively, 46% and 37% of the VOCs decreased as the lambs grew older. The concentrations of the 2 esters **C88** and **C92** were always lower in the older lambs. The relevant information is summarized in Table 2.

Table 2. Temporal changes in the percentage peak areas of VOCs from the cranial wool collected on Days 1 and 7 in 2007 and Days 1, 7 and 100 in 2010

Higher average relative concentrations		Lower average relative concentrations	
C48	Nonan-4-olide	**C4**	Hexanal
C60	(*5E*)-6,10-Dimethyl-5,9-undecadien-2-one	**C6**	4-Methyl-1-pentanol
C65	2-Tridecanone	**C8**	Heptanal
C69	Dodecanoic acid	**C14**	6-Methyl-2-heptanone
C72	Hexadecane	**C15**	1-Heptanol
C85	Tetradecanoic acid	**C19**	Octanal
C88	Ethyl tetradecanoate	**C23**	(*E*)-2-Octenal
C92	Isopropyl tetradecanoate	**C26**	2-Nonanone
C93	6,10,14-Trimethyl-2-pentadecanone	**C30**	(*E*)-2-Nonenal
C99	2-Heptadecanone	**C33**	1-Nonanol
C104	Hexadecanoic acid	**C34**	2-Decanone
		C44	1-Tridecene
		C45	Undecanal
		C46	Tridecane
		C50	(E)-2-Undecenal
		C54	2-Dodecanone
		C57	Dodecanal
		C62	(E)-2-Dodecenal
		C66	1-Pentadecene

C67	Tridecanal
C73	Tetradecanal
C78	(E)-2-Tetradecenal

Some compounds were only identified in wool collected on one of the wool sampling occasions. For example, 3-methyl-2-undecanone (**C47**), 6,10-dimethyl-2-undecanone (**C56**), and the two unidentified compounds (**C59**) and (**C96**) were present only in wool collected on Day 1 in 2007. 2-Ethyl-3-methyl-pyrrolidine-2,5-dione (**C38**) was only detected on Day 1 in 2010 and (*Z,Z)*-9,12-octadecadienoic acid (**C110**), 1-icosanol (**C114**) and icosanoic acid (**C115**) were only detected in samples collected on Day 100 in 2010. Although these compounds could possibly be contaminants, they belong to the compound classes that are typically present in the collected wool samples, and they are therefore probably secreted by the lambs and reach detectable levels only in older lambs. Of the large number of VOCs belonging to the wide variety of compound classes identified as wool VOCs from the headspace analyses of wool collected from ten lambs in 2007, and from six lambs born in 2010, the majority were present in all the wool samples investigated during both lambing seasons, albeit in varying concentrations. Certain constituents were always present in high concentrations, and in total accounted for the larger part of the wool volatiles.

Statistics

The PCA biplots depicted in Figures 4a and 4b were constructed to obtain insight into the multivariate character of the results. These biplots provide optimal two-dimensional representations of the data matrices under discussion. Only 63 of the wool VOCs were present in the wool samples collected in 2007 and in 2010, for comparison purposes, only these 63 were used to construct the biplots. Not all 20 samples and 87 variables are equally well represented in the biplot and axis predictivity (Gardner-Lubbe et al. 2008). Sample predictivities and axis predictivities provide detailed information about how accurately each data 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 Figure 4a, only the 11 axes with predictivities higher than 0.800 are displayed. The quality of display for the PCA biplot in Figure 4a is 58%, a value reflecting the proportion of the variation in the data accounted for in the first 2 dimensions of the two-dimensional display (Gower and Hand, 1996). The other 42% is explained in the remaining

18 dimensions. The 11 constituents with predictivities higher than 0.800 are, in order of decreasing predictivity: 8-methylnonanal (**C31**), decanal (**C35**), undecanal (**C45**), 1-hexadecanol (**C98**), hexadecanal (**C90**), heptadecane (**C80**), 2-pentadecanone (**C79**), 1-tetradecanol (**C75**), 2-2,6,10,14-tetramethylhexadecane (**C91**), tetradecanone (**C71**), and nonanal (**C27**). According to the biplot (Figure 4a), there are two groups of highly correlated high molecular weight constituents: the first comprises 2-tetradecanone (**C71**), hexadecanal (**C90**) and 1-hexadecanol (**C98**), and the second comprises 1-tetradecanol (**C75**), 2-pentadecanone (**C79**), heptadecane (**C80**) and 2,6,10,14-tetramethylhexadecane (**C91**). The wool collected from day-old lambs contains higher concentrations of these constituents than the wool from week-old lambs. The more volatile, lower molecular weight aldehydes nonanal (**C27**), 8-methylnonanal (**C31**), decanal (**C35**) and undecanal (**C45**) also display mutual correlation.

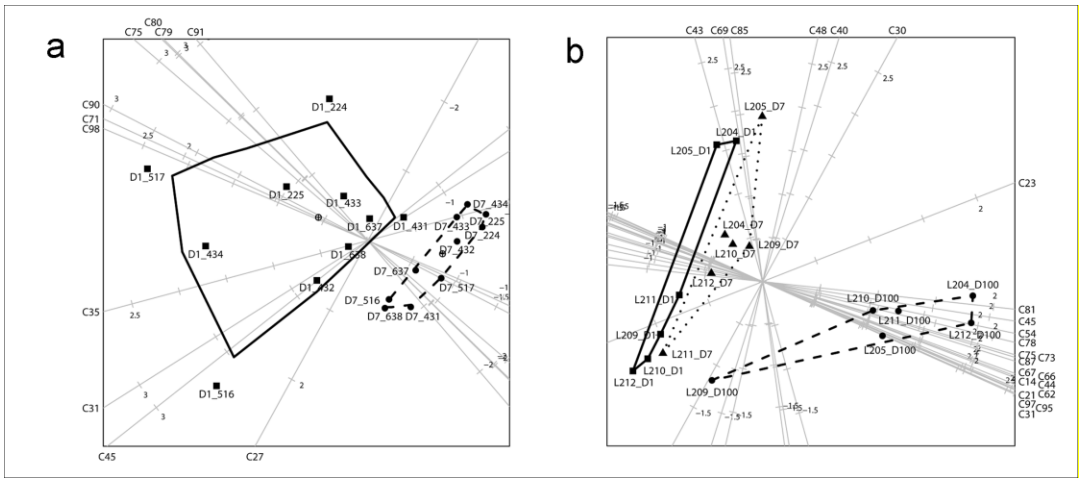

Figure 4 (a) PCA biplot for the headspace VOCs of cranial wool collected from lambs during the lambing season of 2007 on Day 1 (black) and Day 7 (dashed line); and (b) PCA biplot for the headspace VOCs of cranial wool collected from lambs during the lambing season of 2010 on Day 1 (unbroken line), Day 7 (dashed line) and Day 100 (dotted line).

The PCA biplot constructed from the data captured in 2010 is depicted in Figure 4b. The quality of display for this PCA biplot is 66% and only the 23 constituents with predictivities higher than 0.800 are displayed. The biplot indicates that the following constituents are highly correlated: **C81, C45, C54, C78**, **C75, C73, C87**, **C67**, **C66**, **C14**, **C44**, **C21**, **C62**, **C97**, **C95**, and **C31**. Wool collected on Day 1 had the lowest concentration of these highly correlated compounds. The concentrations of these compounds increased with the age of the lambs, and they were present in the highest concentrations in wool collected on Day 100. Furthermore, the concentration of (*E*)-2-octenal (**C23**) also increased as the lambs grew older, but this constituent it is not highly correlated with the last-mentioned group of

constituents. Constituents **C43**, **C69**, **C85, C48**, **C40** and **C30** are also correlated, and appear to be more important during the earlier stages of a lamb's life. As expected, there is more similarity between the VOC profiles of wool collected in 2010 from the same lambs on Days 1 and 7 than between the wool collected on Days 1 and 100.

Some compounds were only identified in wool collected on one of the wool sampling occasions. For example, 3-methyl-2-undecanone (**C47**), 6,10-dimethyl-2-undecanone (**C56**), and the two unidentified compounds (**C59**) and (**C96**) were present only in wool collected on Day 1 in 2007. 2-Ethyl-3-methyl-pyrrolidine-2,5-dione (**C38**) was only detected on Day 1 in 2010 and (*Z,Z)*-9,12-octadecadienoic acid (**C110**), 1-icosanol (**C114**) and icosanoic acid (**C115**) were only detected in samples collected on Day 100 in 2010. Although these compounds could possibly be contaminants, they belong to the compound classes that are typically present in the collected wool samples; they are therefore probably secreted by the lambs and reach detectable levels only in older lambs. Of the large number of VOCs belonging to the wide variety of compound classes identified as wool VOCs from the headspace analyses of wool collected from ten lambs in 2007, and from six lambs born in 2010, the majority was present in all the wool samples investigated during both lambing seasons, albeit in varying concentrations. Certain constituents were always present in high concentrations, and in total accounted for the larger part of the wool volatiles. Only 4 constituents, 8-methylnonanal (**C31**), undecanal (**C45**), 1-tetradecanol (**C75**) and 1-hexadecanol (**C98**), showed good predictivities in the lambing seasons of both 2007 and 2010.

Analysis of distance (AoD) (Gower et al. 2011) of the quantitative data obtained in 2010 was not a primary object of this investigation, and the sample size (number of lambs) was also too small for a rigorous statistical analysis. Nevertheless, the AoD plots depicted in Figure 5 showed that temporal changes in the odor of the experimental lambs took place in a coherent manner. Collecting wool samples from twins thus had the unforeseen advantage that AoD plots could be constructed and provided information on the intra-twin similarity of the odor profiles of twins.

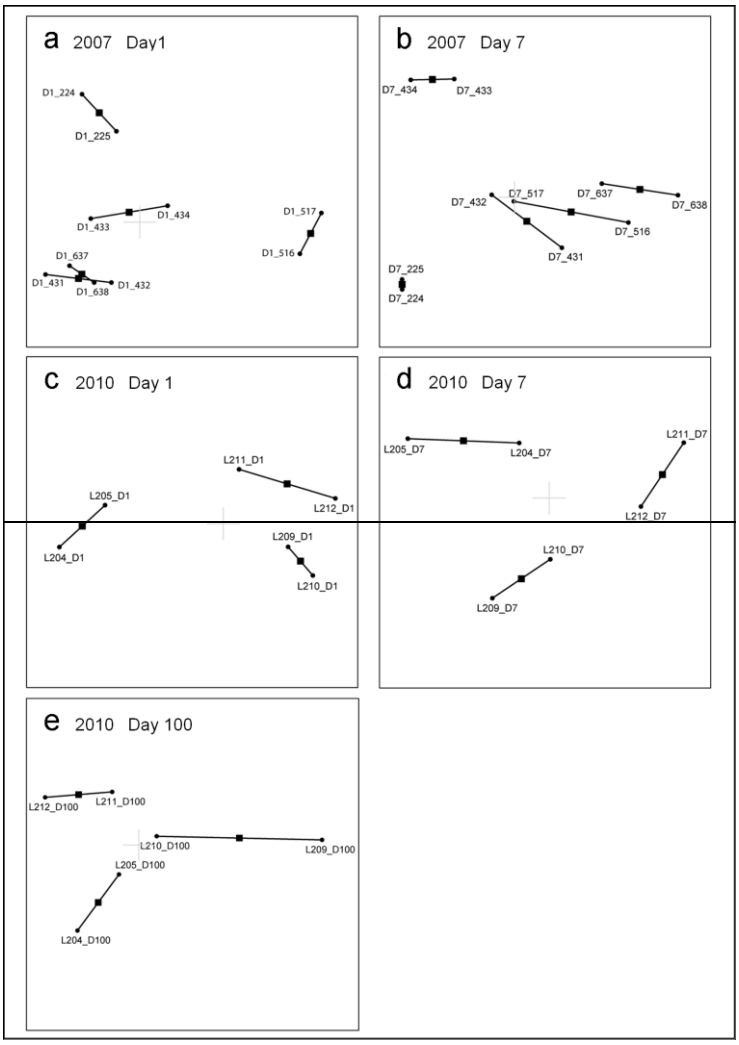

Figure 5 Analysis of distance (AoD) biplots (Gower et al. 2011) of data on the quantitative composition of the volatile organic compounds (VOCs) from cranial wool samples collected from Dohne Merino lambs on Days 1, 7, and 100 during the 2007 and 2010 lambing seasons, demonstrating the similarity of the odor profiles of twin lambs relative to those of other lambs from the small flock of lambs available for the experiment. Pairs of twins were considered as separate groups. Group means are indicated with solid squares, group members with solid circles and twin lambs are connected with black bars. Biplot axes representing the compounds have been suppressed.

Discussion

There is general consensus between sheep farmers and ethologists that the strong bond formed between a ewe and her lamb within the first few hours after its birth is disrupted if they are separated for a few days. However, the duration of the separation that results in irreversible disruption of the bond has not yet been established with reasonable accuracy, but apparently it is influenced by the duration of contact between ewe and lamb before they are separated, as well as the duration of the separation of ewe and lamb afterwards (Lévy et al. 1991; Keller et al. 2005). It is also not known whether ewes are simply not capable of

retaining the olfactory image of their lambs for more than a few days, or whether there is some other logical explanation for this phenomenon.

The present investigation into the characteristics of the presumed neonatal recognition cue of sheep showed that the largest majority of the VOCs listed in Table 1 were present in cranial wool collected during the lambing season of 2007 as well as that of 2010, albeit in different relative concentrations. If it is taken into consideration that there are certain limitations regarding the sensitivity of the analytical instrumentation that was used, it is even possible that all of the VOCs could have been present in both seasons, but some of them were not detected because they were present in concentrations below the detection threshold of the analytical techniques. Despite the higher rate of evaporation of low-boiling compounds, the more volatile compounds were mostly present in higher concentrations in wool collected from older lambs. This could be construed as evidence in favor of the cue being produced by the lamb and not by the ewe, but this possibility still has to be investigated in more detail.

Some of the compounds listed in Table 1 have previously been identified in lanolin (Schlossman and McCarthy, 1979; Motiuk, 1979a, 1979b, 1980), the wool of sheep (Lisovac and Shooter, 2003), or in the inguinal gland of ewes (Rietdorf, 2002). The occurrence, identification and probable function of many of the branched and unbranched long-chain aliphatic compounds listed in the table, or compounds belonging to the same compound classes, have been discussed in considerable detail in reference books or review articles (e.g., Albone 1984; Burger 2005). In the current context, it might be more appropriate to consider the occurrence of these compounds as skin volatiles in other mammals, for example in humans. Unfortunately, only human skin volatiles have been investigated in sufficient detail for such a comparison. More than 400 compounds have already been isolated and identified from human skin extracts (Dormont et al. 2013). However, using headspace analysis, only 20 to 90 compounds from human odors have been detected at naturally occurring body temperature. Of the 25 chemical compounds most often reported in these studies, only 8 were also identified in the cranial wool of lambs in the present study, *viz*. undecane, hexadecane, hexanal, octanal, nonanal, decanal, undecanal, and 6,10-dimethyl-5,9-undecadiene-2-one. Members of some of the other compound classes present in lamb's wool, such as alcohols, ketones, carboxylic acids and esters, have also been reported in the volatile profile of human skin (Dormont et al. 2013). 2-Pentylfuran is found in alcoholic beverages and in many foods, including coffee, potatoes, tomatoes, roasted filberts and soybean oil, and is sold as a flavouring agent. 2-Pentylfuran is found in the breath of patients with *Aspergillus fumigatus* infections (Chambers et al. 2009), it is a plant growth promoter (Zou et al. 2010), and it has

been identified in human breast milk (Stafford et al. 1976). If present in sheep's milk, it could have been picked up by the cranial wool of the lambs of suckling lambs. It is difficult to rationalize the presence of the nitrogen-containing compounds, **C22**, **C29**, **C37**, **C38**, and **C39**, in the wool samples. However, these compounds were identified in the cranial wool of twins that were housed with their dams in a shed, and although the wool samples were collected from lambs before their earmarks were treated with an disinfectant, it is possible that these compounds could be ingredients and/or metabolites of the constituents of the disinfectant spray that were picked up from the air by the wool of the lambs before the samples were collected.

Regarding the qualitative and quantitative composition of their cranial wool VOCs, twin lambs remained grouped together, although not as closely on Day 100 as on Days 1 and 7. This is confirmed by the AoD plots depicted in Figure 5, which show that in 2010 as well as in 2007, twin lambs possessed odor profiles that were more similar to each other than to those of other randomly selected non-twin lambs in the flock, at least during the first 100 days of their lives. Nevertheless, each twin still had its own unique olfactory signature.

As illustrated in the PCA biplots depicted in Figures 4a and 4b, complete separation was observed between the wool samples collected from the lambs on Days 1 and 7 in 2007 as well between those collected on Days 1, 7 and 100 in 2010. The fact that there is no overlapping of the VOC content of the wool samples collected from lambs at various stages during the first 100 days of their lives indicates that a complete change in the VOC profile of the wool and thus in the odor of the lambs took place as the lambs grew older. Clearly, the neonatal recognition cue of sheep does not have a fixed and permanent composition, but continuously changes as lambs grow older. It is interesting that the odor profiles of individual lambs in the flock change in a parallel manner so that the flock of lambs remains grouped closely together, as does the flock as a whole. If perpetuated throughout their lives, this trend could contribute to cohesion in a flock of sheep. It is interesting that many sheep breeders and farmers have observed that twin lambs mostly prefer to graze together and are often found together when they are sheared or have to undergo veterinary treatment.

We conclude that in order to preserve the exclusive olfactory attachment to her lamb, a ewe has to adjust or adapt her olfactory system to the temporal changes in the odor of her lamb. Thus, if the recognition of a lamb is based mainly on the available olfactory information, she has to continuously monitor the odor of her lamb in order to keep up with its changing odor profile. Temporal changes in the odor of the lambs, rather than an inability to

retain an olfactory image of their lambs for more than a few days, appear to be the main reason for ewes' frequent sniffing at their lambs.

The failure of attempts at fostering lambs using mixtures of synthetic analogs of the wool VOCs of a ewe's own lamb (Burger et al. 2011b) could be ascribed to, *inter alia*, co-evaporation of the solvent and some of the highly volatile constituents of the synthetic mixture, and to the dissemination of the synthetic VOC from cotton fleece jackets instead of lamb's wool. The temporal changes that take place in the odor of neonatal lambs now introduce another complicating factor into the execution of bioassays with synthetic analogs of the natural VOCs, in as far as the outcome of such experiments also will depend also on whether the collection of a wool sample, the quantitative analysis of the wool VOCs, the formulation of a corresponding synthetic mixture, and the fostering experiment could be carried out within a quite limited time span.

One question that still has to be answered is whether the neonatal recognition cue of sheep is produced by the lamb, or whether it is a maternal label. Despite their higher rate of evaporation, the low-boiling VOCs were present in higher concentrations in the wool of older lambs than in wool collected from young lambs. If the cue is a maternal label, the concentration of the more volatile compounds would be expected to evaporate as lambs grow older. Furthermore, exploratory experiments have shown that the integrity of the recognition cue is compromised when cue-impregnated cotton fleece jackets are exposed to a moderate breeze at temperatures around 25 °C for periods ranging from 2 to 5 hours (unpublished results). These observations could be construed as evidence in favor of the cue being constantly replenished by the lamb. However, it is not yet known whether all of the identified wool VOCs, or only some of the highly volatile compounds are essential constituents of the cue. It is also possible that autoxidation of, for example, the aldehydes, instead of selective evaporation of any highly volatile compounds, could be responsible for the loss of attractiveness of jackets exposed to the atmosphere. These possibilities still have to be investigated.

Poindron et al. (2010) have shown that amniotic fluid is important in experienced ewes for the establishment of maternal responsiveness, and that it also carries some chemosensory information facilitating exclusive bonding. We have found that the amniotic fluid in which a lamb is born contains most of the compounds identified in its cranial wool, albeit in totally different relative quantitative concentrations (unpublished results), an observation that apparently supports the hypothesis that the cue is produced by the lamb. Finally, it is also possible that bacterial metabolism of residual amniotic fluid or compounds produced by skin

glands could play a role in the production of the neonatal recognition cue in sheep, as it does in some other animals. The role of microbial ecology in kin recognition and other aspects of animal behavior have recently been reviewed by Archie and Theis (2011). This aspect will receive our urgent attention in the near future.

Funding

This work was funded by the National Research Foundation, Pretoria, South Africa and Stellenbosch University.

Acknowledgements

We thank the personnel of the experimental farm of Stellenbosch University, Mariendahl, for making experimental sheep available for this research and for invaluable advice and assistance.

References

Albone ES. 1984. Mammalian semiochemistry.Wiley, Chichester.

Aldrich C, Gardner S, Le Roux NJ. 2004. Monitoring of metallurgical process plants using biplots. J Am Inst Chem Eng. 50:2167–2186.

Alexander G, Shillito EE. 1977. The importance of odor, appearance and voice in maternal recognition of the young in Merino sheep (Ovis aries). Appl Anim Ethol. 3:127–135.

Alexander G, Stevens D. 1981. Recognition of washed lambs by Merino ewes. Appl Anim Ethol. 7:77–86.

Archie EA, Theis KR. 2011. Animal behaviour meets microbial ecology. Anim Behav. 82:425–436.

Burger BV. 2005. Mammalian Semiochemicals.Topics in Curr Chem. 240:231–278.

Burger BV, Marx B, le Roux M, Herbert SA, Amakali KT. 2011a. Development of second-generation sample enrichment probe (SEP) for improved sorptive analysis of volatile organic compounds. J Chromatogr A. 1218:1567–1575.

Burger BV, Viviers MZ, le Roux, NJ, Morris J, Bekker JPI, le Roux M. 2011b. Olfactory Cue Mediated Neonatal Recognition in Sheep, *Ovis aries*. J Chem Ecol 37:1150–1163.

Chambers ST, Syhre M, Murdoch DR, McCartin F, Epton MJ. 2009. Detection of 2-pentylfuran in the breath of patients with *Aspergillus fumigatus*. Med Mycol. 47:468–76.

Dormont L, Bessière J-M, Cohuet C. 2013. Human skin volatiles: A review. J Chem Ecol. 39: 569-578

Gardner-Lubbe S, le Roux NJ, Gower JC. 2008. Measures of fit in principal component and canonical variate analyses. J Appl Statist. 35:947–965.

Gower J, Hand, DJ. 1996. Biplots. Chapman Hall, London.

Gower JC, Lubbe S, Le Roux NJ. 2011. Understanding biplots (Sections 5.8–5.9).Wiley, Chichester.

Keller M, Meurisse M, Lévy F. 2005. Mapping of brain networks involved in consolidation of lamb recognition memory. Neuroscience 133:359–369.

Kowalski BR, Bender CF. 1972. Pattern recognition. A powerful approach to interpreting chemical data. J Am Chem Soc. 94:5632–5639.

Lévy F, Gervais R, Kindermann U, Litterio M, Poindron P, Porter R. 1991. Effects of post-partum separatiobn of maternal responsiveness and selectivity in parturient ewes. Appl Anim Behav Sci. 31 101-110

Lévy F, Keller M, Poindron P. 2004. Olfactory regulation of maternal behavior in mammals. Horm Behav. 46:284–302.

Lindsay D. 1988. Breeding the Flock: Modern Research and Reproduction in Sheep. Inkata Press, Melbourne:.

Lisovac AM, Shooter D. 2003. Volatiles from sheep wool and the modification of wool odors. Small Ruminant Res. 49:115–124.

Lynch JJ, Hinch GN, Adams DB. 1992. The behaviour of sheep. Biological principles and implications for production. C·A·B International and CSIRO Australia, Melbourne.

Morgan PD, Boundy CAP, Arnold GW, Lindsay DR. 1975. The roles played by the senses of the ewe in the location and recognition of lambs. Appl Anim Ethol. 1:139–150.

Motiuk K. 1979a. Wool wax acids: a review. J Am Oil Chem Soc. 56:91–97.

Motiuk K. 1979b. Wool wax acids: a review. J Am Oil Chem Soc. 56:651–658.

Motiuk K. 1980. Wool wax acids: a review. J Am Oil Chem Soc. 57:145–146.

Poindron P, Carrick M.J. 1976. Hearing recognition of the lamb by its mother. Anim Behav. 24:600–602.

Poindron P, Otal J, Ferreira G. Keller M, Guesdon V, Nowak R, Lévy F. 2010. Amniotic fluid is important for the maintenance of maternal responsiveness and the establishment of maternal selectivity in sheep. Animal 4:2057–2064.

Rietdorf M. 2002. Identifizierung und Synthese flüchtiger Substanzen aus Säugetieren. PhD Thesis, Universität von Hamburg.

Schlossman ML, McCarthy JP. 1979. Lanolin and derivatives chemistry: relationship to allergic contact dermatitis. Contact Dermatitis 5:65–72.

Shillito EE, Alexander G. 1975. Mutual recognition amongst ewes and lambs of four breeds of sheep (Ovis aries). Appl Anim Ethol. 1:151–165.

Stafford M, Horning MG, Zlatkis A. 1976. Profiles of volatile metabolites in body fluids. J Chromatogr. 126:495–502.

Terrazas A, Ferreira G, Lévy F, Nowak R, Serafin N, Orgeur P, Soto R, Poindron P. 1999. Do ewes recognize their lambs within the first day postpartum without the help of olfactory cues? Behav Proc. 47:19–29.

Wyat TD. 2010. Pheromones and signature mixtures: defining species-wide signals and variable cues for identity in both invertebrates and vertebrates. J Comp Physiol A. 196:685–700.

Zou C, Li Z, Yu D. 2010. Bacillus megaterium Strain XTBG34 promotes plant growth by producing 2-pentylfuran. J Microbiol. 48:460–466.