**Techniques in Mammalian Semiochemistry I. A Novel and Robust Bioassay for the Neonatal Recognition Cue of Sheep *Ovis aries***

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**Index of Electronic Supplementary Material**

The results of our previous research into the neonatal recognition (semiochemical / pheromone) communication in sheep has been reported in:

1. Olfactory cue mediated neonatal recognition in sheep, *Ovis aries*.

B.V. Burger, M. Z. Viviers, N. J. le Roux, J Morris, J.P.I. Bekker, Maritha le Roux.

J. Chem. Ecol. **37:**1150–1163 (2011). DOI 10.1007/s10886-011-0020-72.

2. Temporal changes in the neonatal recognition cue of Dohne Merino lambs (*Ovis aries*).

M.Z. Viviers, B.V. Burger, N.J. le Roux, J. Morris, M. le Roux.

Chemical Senses 39: 249-262. 2014. DOI:10.1093/chemse/bjt075

3. Characterization of amniotic fluid of Dohne merino ewes (*Ovis aries*) and its possible role in neonatal recognition.

M.Z. Viviers, J.P.I. Bekker, B.V. Burger, N.J. le Roux, J.Morris and M. le Roux. 2015.

Z. Naturforschung C 70:115–128. DOI 10.1515/znc-2014-4120

**Video 1**. Recorded: 20-03-2007

Ewe rejecting one of her twin lambs⎯a phenomenon that is relatively prevalent in twin-bearing ewes. This video prompted our research into the semiochemical basis of this strange behaviour.

Video 2. Recorded: 05-04-2009

Ewe rejecting her own lamb wearing a cotton fleece jacket that had been worn overnight by an alien lamb (another ewe’s lamb). This experiment proved that the alien lamb’s recognition cue had been transferred to the jacket.

**Videos 3 – 8**. Recorded: 28-03-2012

Clean cotton fleece jackets were left overnight on the experimental ewes’ own lambs. During the night the recognition cue was transferred by evaporation and diffusion of its volatile constituents to the jackets. These ‘O’ (own) jackets were presented to the respective ewes for olfactory comparison with ‘A’ (alien) jackets that had been worn by the lambs of other ewes and were used as controls. In the bioassays, the jackets were draped over tufts of grass. From the perspective of a ewe, the silhouette of a reclining lamb is presumably visible as a white line against a darker background. As the ewes mostly approached the jackets at a fast pace, they were apparently deceived into mistaking the jackets for their sleeping lambs. All of them spent much more time sniffing at the jackets of their own lambs than at the jackets that had been worn by alien lambs.

**Videos 9 – 14.** Recorded: 29-03-2012

The ‘O’ jackets were stored overnight at –23°C, then pinned to a wire mesh fence and exposed to wind (average air speed 2.5 m/s) at 25°C for 5 h. The jackets were then presented to the same ewes, as on the previous day, for olfactory appraisal. The experimental ewes did not recognize the recognition cues of their respective lambs. Apparently the essential constituents of the recognition cues have evaporated from the jackets.

Videos **15 – 16**. Recorded: 15-03-2016 and 17-03-2016

To circumvent the time-consuming transfer of the lamb-ewe recognition cue to jackets and to avoid any possible adulteration of the recognition cue, the wool from the flanks and backs of experimental lambs was harvested and presented to their mothers in tulle pouches.

During a severe and long-lasting drought there was no grass in the pastures at Mariendahl upon which the jackets or wool pouches could be displayed on grass tufts during this part of the study. The bioassays thus had to be carried out on a dirt road, using a shorter approach than previously to the cue-carrying samples. The pouches were supported on a few dry sticks and some straw, placed on a rain water diverting ridge on the road. The ewes were mostly walked relatively slowly towards the pouches, which could have allowed them sufficient time to realize that a small and flat pouch could not be the lost lamb. Mostly they also had to be walked repeatedly around the pouches to get them within sniffing range of the wool samples.

During the lambing seasons of 2016 and 2017, many investigations were carried out into the longevity of the recognition cue by comparing the response of experimental ewes to their lambs’ wool before (pouch ‘B’) and after aeration of the wool (pouch’ A’). A pouch (‘C’) containing wool from an alien lamb was used as control. As we found that some ewes seem to lose interest towards the end of a protracted bioassay, these samples were presented to their mothers in the reverse order, *i.e*., first the more difficult ‘A’ sample and then the ‘B’ sample (in the same video).

Two of the videos recorded during the 2016 lambing season were selected to illustrate the procedures that we had to use under the conditions prevailing during those years and also illustrate some of the typical differences in the behavior of the experimental ewes we used in these experiments. The ‘B’ samples in both videos had been aerated for 20 min.

In video 15, ewe 163 immediately started sniffing at the pouches and spent a total of 35 s and 2 s at the ‘A’ and ‘C’ pouches, respectively, during the first stage of the test, whereas she spent 36 s and < 2 s at the ‘B’ and ‘C’ pouches, respectively, during the final stage of the test. Apparently, the recognition cue was still detectable in the ‘A’ sample.

This test was repeated two days later under similar conditions. The general behavior of ewe 24 in Video 16 was quite different from that of ewe no. 163 in Video 15. It took the handler quite a long time to elicit the first sign of interest in the wool samples from this ewe. Nevertheless, ewe no. 24 spent a total of 25 s and 12 s at the ‘A’ and ‘B’ pouches, respectively, and only approximately 4 s at the ‘C’ pouch.

Although ewe no. 163 spent some time looking around in all directions for her missing lamb, she appeared to be convinced of a relationship existing between her lamb and the ‘A’ and ‘B’ pouches. Ewe no. 24, on the other hand, left the impression that she believed her lamb to be somewhere far away in the distance.

In as much as the ewes very clearly accepted the wool in the ‘B’ and ‘A’ pouches as that of their own lambs, these tests could be considered successful. However, for the more exacting differentiation between wool containing the fresh recognition cue and wool that has been stripped from some of its essential constituents, results of tests carried out under these less than optimal conditions are not acceptable.