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Different chemical fractions of fetal fluids account for their attractiveness at parturition and their repulsiveness during late-gestation in the ewe

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ABSTRACT

We have investigated whether the chemical components of fetal fluids (FFs), which elicit repulsion in late gestating ewes, are also those responsible for the attractiveness of fetal fluids at parturition. An aqueous fraction of FFs (A1), obtained after extraction with hexane, was tested for repulsion in late-pregnant ewes and for attraction at parturition. We also investigated if the repulsive and attractive characteristics of this A1 fraction were maintained after an additional extraction with dichloromethane (DCM, CH_2Cl_2) that produced two more fractions (aqueous/high polarity: A2 and dichloromethane/medium polarity: DCM). Thus, late-pregnant ewes were tested for repulsion of aqueous extracts of FFs (A1, A2 and DCM fractions) in a two-choice test of food preference, whereas parturient ewes were tested for attraction toward these same fractions in a two-choice test of licking warm spongy cloths. The A1 fraction was repulsive to late-pregnant ewes and attractive to parturient females. In contrast, neither the A2 nor the DCM fractions were repulsive to late-pregnant ewes, whereas both fractions were attractive to parturient ones. The discordance between the repulsive and attractive properties of the A2 and DCM fractions suggests that the attractiveness of FFs for parturient ewes and its repulsiveness for females outside the peripartum period depend on mixtures of substances that are at least partially different. Some compounds with high and medium polarity in the A2 and DCM fractions would act synergistically to generate the repulsiveness of FFs, whereas both high and medium polarity compounds can evoke attraction independently of each other.

1. Introduction

Birth fluids and placenta are potent chemosensory cues in many mammalian species and play an important role in the behavior of the mother and her young during the postpartum period [1–8]. Parturient ewes and goats lick their neonate very actively during the first postpartum hours [4]; in sheep this has been shown to depend on the attraction of the mother to birth fluids [9]. Indeed, the fetal fluids (FFs) and afterbirth materials are strongly repulsive to ewes through their whole reproductive cycle, but become highly attractive at parturition [10,11]. The compounds responsible for this phenomenon of repulsion–attraction have not been identified so far. In an attempt to characterize the chemical nature of the substances involved in the repulsiveness of amniotic fluid (AF) in non-pregnant ewes, Lévy [12] determined that after an extraction of this fluid with hexane, repulsive substances were contained only in the aqueous polar fraction and not in the low polar one. In addition, after a second extraction of the aqueous fraction with dichloromethane, none of the five fractions obtained showed a significant repulsive activity (see Fig. 1, [12]).

However, to our knowledge, whether the aqueous fraction obtained by hexane extraction or the fractions obtained after extraction by dichloromethane are attractive to parturient ewes has never been investigated. Therefore, it is not clear whether the chemosensory cues responsible for the attractiveness of AF to parturient ewes are the same as those responsible for repulsiveness observed in non-parturient ones. A comparison of the reactions of non-parturient and parturient ewes to these fractions should provide some indication. To assess this question, we studied 1) whether the aqueous fraction (A1) of fetal fluids (FFs) obtained after extraction with hexane was not only repulsive to late-pregnant ewes but also attractive to parturient ewes, and 2) whether this was also the case after performing an additional extraction on the A1 fraction with dichloromethane obtaining two more fractions (aqueous: A2 and dichloromethane:

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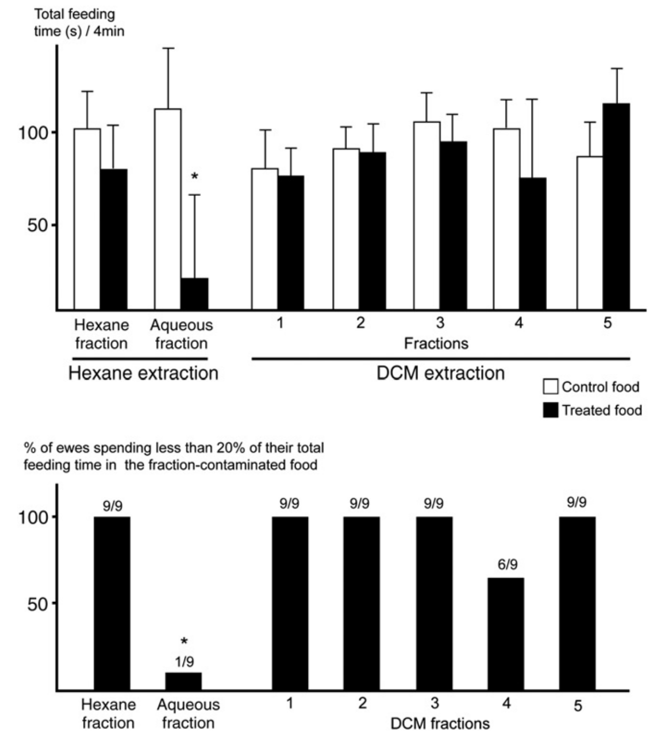

Fig. 1. Consumption of various fractions of amniotic fluids in a two-choice test of food preference between two troughs contaminated or not contaminated with the amniotic fluid extracts (dark and empty bars respectively). Fractions were obtained first from an extraction by hexane (left-hand part of the figure), and then another extraction procedure was carried out on the first aqueous fraction with dichloromethane (DCM), dividing it into five more fractions (right-hand part of the figure).
Adapted from Lévy, unpublished Master Thesis, 1981, with permission of the author.

DCM). Differing from Lévy's initial study [12], we used a mixture of allantoic and amniotic fluids, to emulate the natural conditions that occur when parturient ewes lick their neonates, which is when they become attracted to a mix of these two fetal fluids.

2. Materials and methods

2.1. Experimental design

Different fractions of FFs were tested in double choice tests using different ewes for repulsion and attraction tests. During late pregnancy, we assessed the repulsion of the ewes to the experimental fractions with a two-choice test between food mixed with the fraction to be tested or with the control substance compound, according to the procedure described by Lévy [10]. On the other hand, at parturition, attraction was measured by a two-choice test between experimental and control fractions placed on warm cloths, since it is not required to increase the ewes' interest with food at that time.

2.2. Animals

We used multiparous ideal ewes from the facilities of the National Agricultural Research Institute (INIA) “La Estanzuela”, Colonia, Uruguay. Reproduction was synchronized at ovulation with vaginal sponges (Sintex®) and an injection of pregnant mare stimulating hormone (250 UI, im, Novormon®) at sponge withdrawal, followed by controlled mating with rams. Parturitions were induced by the administration of 15.0 mg of dexamethasone (2.0 mg/mL, im, Disperdex, Dispert, Montevideo), on day 144 of gestation [13]. Deliveries occurred within a 24 h period, starting in average 36 h after the dexamethasone injection. It has already been demonstrated that the endocrine events of parturition induced by dexamethasone are similar to those of spontaneous parturition and that this procedure does not interfere with the olfactory preference of ewes [14], the establishment of maternal behavior, or the length of the sensitive period [13,15].

During most of the gestation period, ewes were maintained outside on green pasture with access to alfalfa and maize. One week before the injection of dexamethasone, the ewes were habituated daily to stay indoors in individual pens of 2.0×2.0 m for 3 h. During this period, two identical troughs of maize were placed on a tray, 10 cm apart, in front of the ewes, to habituate the ewes to the food and troughs in the individual pens. Females remained permanently in individual pens from the time when the dexamethasone injection was applied until the tests were performed during late gestation and parturition. Different ewes were tested in both periods.

This study was approved by the Ethical Committee of the Facultad de Ciencias of the Universidad de la República, Uruguay.

2.3. Collection of FFs and solvent partition of the various fractions

FFs were collected in a first wave of parturitions ($n = 10$) by puncturing the amniotic and allantoidal vesicles when they became accessible at the beginning of the parturition process. A pool of FFs (about half of each fluid) was filtered, aliquoted and stored at −20 °C until subjected to purification procedures or testing. We verified in a preliminary study that this pool of FFs was repulsive to non-parturient ewes and attractive to parturient ones (results not shown; also, below, see Validation of two-choice test procedure with warm cloths).

Before solvent extraction, the FFs were thawed, filtered through gauze and centrifuged (5000 rpm; 20 min; 4 °C). The supernatant was then fractionated by liquid–liquid partitioning, first with hexane and then with dichloromethane. While hexane removes lipophilic compounds, such as fats and hydrocarbons, dichloromethane extracts medium-polarity compounds such as steroids. Solvent extractions were performed sequentially in a 6 L separation funnel. First, 5.5 L of FFs was treated with three successive extractions by 0.5 L of hexane each time, obtaining 4.0 L of an aqueous fraction (A1). This fraction was divided in two halves. One half (2.0 L) was mixed with carboxymethyl cellulose (CMC) to mimic the viscosity of FFs, obtaining the A1 fraction used in the behavioral tests. The other half was further treated with three successive extractions by 0.5 L of dichloromethane each time, yielding a second aqueous fraction that was added with CMC to obtain fraction A2. The remaining dichloromethane fraction was evaporated under vacuum and the solid residue was re-suspended in a solution of CMC in water to obtain the DCM fraction.

As a prerequisite, we verified that the CMC and the residues of the solvents used for extraction did not influence the outcome of the tests. In addition, we took into account the fact that the aqueous fractions A1 and A2 had been saturated with organic solvents during the extracting process. Therefore the water to be used for the control fractions in the behavioral tests was also saturated in the same way. First water was saturated with hexane; the hexane was then extracted and half of this water was mixed with CMC, in the same proportion as the experimental fractions, to produce a neutral fraction of CMC that served as the control for A1. The remaining half of water was saturated with DCM, which was then extracted and the water mixed with CMC; this fraction was used as control for A2 and DCM. The aqueous/CMC substance in itself was neither repulsive nor attractive to late-pregnant and parturient ewes in the preliminary observations in which the validation of attraction for raw FFs was carried out ($n = 10$; results not shown).

2.4. Behavioral tests

2.4.1. Late-pregnancy: food preference tests

Late-pregnancy tests were performed on day 145 of gestation, 12 to 24 h before parturition. Food and water were removed from the pen 2 h before the tests, to standardize the physiological state of ewes and increase their hunger. The principle of the test rested on the ability of the tested fraction to prevent the ewes from feeding on food they were very fond of, maize pellets in this case [10].

Two plastic troughs were placed in front of the ewe on a tray, 10 cm apart, both containing 40.0 g of maize pellets; in one case they were damped with 20.0 mL of the experimental fraction, and in the other with the same volume of the control fraction, both at about 37 °C. The test lasted 3 min, starting when the ewe approached its nose at less than 5 cm from any of the two troughs. During this time, the latency to feed and the cumulative time spent feeding from each trough were recorded with stopwatches by an observer. At the end of the test, the remaining food was weighed to calculate the amount consumed from each trough. The troughs were carefully cleaned with water and dried thoroughly between tests. Different sets of troughs were used for each tested material to avoid risks of chemosensory contamination between the different fractions.

2.4.2. Parturition: warm cloth preference test

Parturition tests were performed just when the amniotic sac was broken, when the lamb's legs were visible, or immediately after the expulsion of the lamb. In this last case, the lambs were removed immediately at birth to prevent any contact with the mothers and were returned to their mothers at the end of the test. All lambs were accepted by their dams when returned.

Before testing, food and water were removed from the pen to reduce possible distractions of the ewe to be tested, and her attraction toward FFs was verified by placing a tray with FFs in front of her snout that elicited their licking behavior. All ewes displayed attraction toward FFs.

The preference for the different fractions at parturition was assessed with a new method not involving the presence of food, as the presence of attractive material was a sufficient incentive for the mother to perform the test (see Validation of two-choice test procedure with warm cloths below). This test consisted of placing two rubber bottles, 10 cm apart, in front of the ewe. These bottles were filled with hot water (around 60 °C) and covered with a removable cotton cloth. Ten milliliters of the experimental fraction was applied with a syringe on one of the cloths, and the same volume of the control fraction was applied on the other cloth (both fractions at about 37 °C). The preference test began when the ewe sniffed one of the cloths (i.e. snout less than 5 cm away from the cloth); then, the latencies to lick as well as the cumulative time spent licking each cloth were recorded during 3 min.

2.5. Inclusion criteria

To be included in the repulsion analysis a ewe had to eat during at least 5 s and, in addition, consume more than 5% of the total food placed in the troughs. For the attraction test the ewe had to sniff the experimental warm wet cloth at least once.

2.6. Validation of the two-choice test with warm cloths

At parturition it is not required to increase the interest of the ewes toward FFs; in addition, food is an attractive stimulus per se, that may mask the reactions of the ewes toward the fractions of FFs. Therefore, we designed a new test in which the attraction of the ewes was assessed by their licking of two water bags covered with a white cotton cloth, mimicking a lamb's coat, one impregnated with an experimental fraction and the other with the control fraction.

To assess whether this procedure was a valid method to detect attraction, parturient ewes were tested by two methods. The first method consisted of offering ewes ($n=13$) the choice between two troughs containing food mixed either with 20.0 mL of FFs or with the same volume of an aqueous solution of CMC as control, according to the procedure described by Levy et al. [10]. In the second method 10 different ewes were offered a choice between two warm cloths, one wet with 10.0 mL of FFs and the other with the same volume of an aqueous solution of CMC as control. As expected, at parturition, the time spent feeding from the food contaminated with FFs was significantly higher than the time spent feeding from the control trough [FFs: 60.0 s (25.0) vs. 15.0 s (15.0), median (median absolute deviation − MAD); $T_{13}=8.5$, $p=0.016$]. This was also the case for the test using a cloth wrapped around a hot water bag, in which ewes licked the cloth with FFs for a significantly longer period of time than the control cloth [142.5 s (22.5) vs. 0.0 s (0.0), median (MAD); $T_{10}=0.0$, $p=0.005$]. These results show that the two-choice test with warm cloths is a sensible and valid method to test attraction to FFs. In addition, this method avoids any interference of a possible attraction to food and has the advantage that it does not require the ewes to be trained prior to testing.

2.7. Experimental procedure

The preference for the FF fractions was evaluated as follows:

Late-pregnancy Each ewe ($N=18$) was tested three times for her choice between food contaminated with A1, A2 or DCM, or their respective controls. The tests were separated by a period of 3 min, and the order of the tests, as well as the locations (left or right) of the troughs, were counterbalanced. No differences were found between the three successive tests in the total amount of food eaten by the ewes in each test (results not shown). Thirteen of the 18 ewes tested were included in the analysis as they fulfilled the inclusion criteria mentioned above.

Parturition Each ewe ($N=18$) was tested three times for her preference of warm cloths treated with A1, A2 or DCM, or their respective controls. Each test was separated by a 3-min interval. The order of the tests and the location of the bags were counterbalanced, as described in the aforementioned procedure. Eleven of the 18 ewes tested for preference were included in the analysis as they met the inclusion criteria.

2.8. Statistical analysis

Data are expressed as medians and their median absolute deviation (MAD). Food intake is expressed as a percentage of the food intake per trough (food consumed (g) in a trough/total content of food (g) in the trough × 100).

As most variables did not follow a normal distribution (Kolmogorov–Smirnov test) nor had homogeneous variances (Cochran C test), the data were analyzed by nonparametric tests. Comparisons between two dependent groups were performed using the Wilcoxon matched pair test, and to compare more than two dependent groups we used the Friedman analysis of variance by ranks [22]. Significance was set at $\alpha=0.05$ and bilateral probabilities were used.

3. Results

3.1. Late pregnancy

The latency to feed from the A1-treated food was higher than the latency to feed from the control trough (Table 1). In addition, ewes ate a lower percentage of food (Table 2) and spent less time feeding from the food contaminated with A1 than from the control food ($T_{13}=6.0$, $p=0.006$; see Fig. 2A). In contrast, concerning the A2 fraction, neither the latency to eat (Table 1), the percentage of food ingested (Table 2), nor the time spent feeding ($T_{13}=28.0$, $p=0.39$; Fig. 2A) differed significantly between the A2 and control troughs. In the case of the DCM fraction, the latency to eat food contaminated

Table 1
Latencies (s) taken by late-pregnant ewes (upper part of table) to feed in a two-choice test between troughs contaminated with carboxymethyl cellulose (control) or with one of three fractions of fetal fluids (fraction) or by peri-parturient ewes (lower part of table) to lick warm cloths contaminated with carboxymethyl cellulose or with these same extracts.

Latencies (s)	Tested fraction	Control	Fraction	T	p
Repulsion in late pregnancy	A1	3.0 (2.0)	146.0 (34.0)	**4.0**	**0.04**
	A2	10.0 (10.0)	34.0 (32.0)	*38.0*	*0.60*
	DCM	3.0 (2.0)	56.0 (25.0)	**14.5**	**0.03**
	F_r	2.21	3.24		
	p	0.33	0.20		
Attraction at parturition	A1	180.0 (0.0)	10.0 (9.0)	**0.0**	**0.01**
	A2	180.0 (0.0)	7.0 (6.0)	**2.0**	**0.01**
	DCM	180.0 (0.0)	10.0 (10.0)	**5.0**	**0.02**
	F_r	0.86	0.20		
	p	0.65	0.91		

Latencies are expressed as median (s) and their median absolute deviations (MAD), A1: aqueous fraction resulting from extraction of fetal fluids with hexane, A2: aqueous fraction resulting from extraction of A1 with dichloromethane, DCM: DCM fraction. T and p values that represent significant differences are signalized with bold font, while non significant differences in cursive.

with this fraction was significantly higher than the latency to eat control food (Table 1). However, the time spent feeding from the trough containing the DCM fraction did not differ from the time feeding in the control trough ($T_{13}=26.0$, $p=0.17$, Fig. 2A), even though ewes tended to ingest a lower percentage of food from the trough with the DCM fraction than from the control trough (Table 2; $p=0.08$).

When comparing the A1, A2 and DCM fractions, the latencies to feed did not differ significantly among fractions (Table 1), whereas the time spent feeding actually did ($F_{r(13,2)}=6.78$, $p=0.04$; Fig. 2A). Thus, the feeding time from the trough with the A1 fraction was significantly lower than the feeding time from the trough with the A2 fraction ($T_{13}=4.0$, $p=0.006$). It also tended to be lower than the time of feeding in the DCM trough ($T_{13}=11.0$, $p=0.09$). Finally, feeding time in the DCM trough did not differ from that in the A2 trough ($T_{13}=28.0$, $p=0.39$).

3.2. Parturition

Parturient ewes displayed significantly shorter latencies to lick any of the three experimental fractions A1, A2 and DCM than to lick their respective controls (Table 1) and they also licked them for a significantly longer period of time than control cloths ($T_{11}=0.0$, $p=0.012$; $T_{11}=0.0$, $p=0.005$; $T_{11}=0.0$, $p=0.005$ respectively; Fig. 2B).

On the other hand, the latency to lick (Table 1) and the time spent licking ($F_{r(11,2)}=1.16$, $p=0.56$, Fig. 2B) did not differ significantly between the A1, A2 and DCM fractions.

Table 2
Percentage of food consumed by late-pregnant ewes (food consumed (g)/total food contained in trough (g)×100) in a two-choice test between troughs contaminated with carboxymethyl cellulose (control) or with one of three fractions of fetal fluids (fraction).

Percentage of food intake	Tested fraction	Control	Fraction	T	p
	A1	100.0 (0.0)	6.7 (6.7)	**6.0**	**0.006**
	A2	97.5 (2.5)	97.1 (2.9)**	*28.0*	*0.38*
	DCM	100.0 (0.0)	38.7 (38.7)*	*1.78*	*0.08*
	F_r	0.77	**10.7**		
	P	0.68	**0.005**		

Data are expressed as medians and their median absolute deviation (MAD), A1: aqueous fraction resulting from extraction of fetal fluids with hexane, A2: aqueous fraction resulting from extraction of A1 with dichloromethane, DCM: DCM fraction. T and p values that represent significant differences are signalized with bold font, while non significant differences in cursive.
** $p<0.005$, A1 vs. A2; Wilcoxon matched pair test.
* $p<0.05$, A1 vs. DCM; Wilcoxon matched pair test.

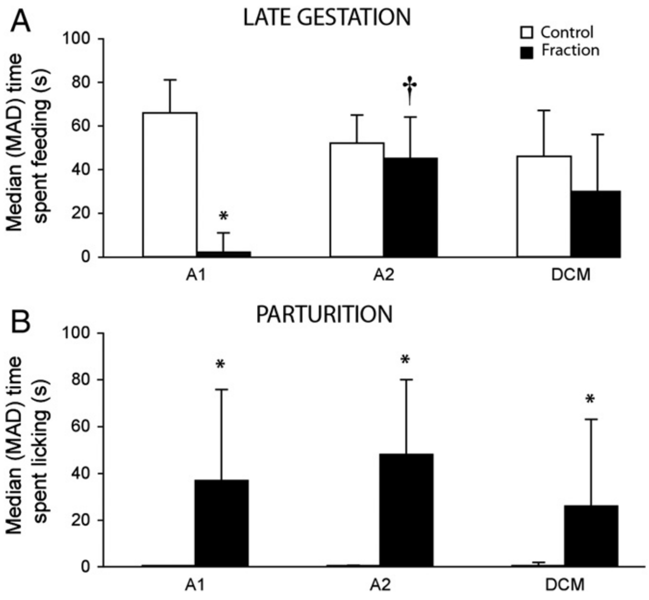

Fig. 2. Behavioral preference of late-pregnant and parturient ewes in a two-choice test when given the choice between a control substance (carboxymethyl cellulose) — empty bars and different fractions of fetal fluids — dark bars, obtained first by an extraction with hexane (A1) and then with dichloromethane (A2 and DCM). Panel A: Total time of food intake (s) of late-pregnant ewes. Panel B: Total time spent by parturient ewes licking a warm cloth. Data are expressed as median and their median absolute deviations (MAD). *$p<0.05$, **$p<0.01$ between fraction and control, †$p<0.01$ between A1 and A2, Wilcoxon matched pair test.

4. Discussion

Our results show that the aqueous fraction of FFs obtained after hexane extraction was active, whereas the two fractions resulting from DCM extraction had partially or completely lost their repulsive characteristic. These results are congruent with those obtained by Lévy [12] for AF outside of the peripartum period (Fig. 1). In contrast with the loss of repulsion, the DCM extraction did not lead to a loss of attraction for parturient ewes. Thus, parturient females exhibited a clear attraction for the A1, A2 and DCM fractions while showing no interest toward the control cloths. As a whole, the dissociation between the repulsive and attractive characters of the FF fractions studied indicates that the actual compounds responsible for repulsiveness are different from those accounting for attractiveness.

There are several possible explanations for the loss of repulsiveness of the A2 and DCM fractions. First, it could result from the separation of compounds of high and medium polarity, which act synergistically within the A1 fraction to exert their biological activity. The necessity of a synergic action by several chemosensory compounds has been reported in other instances, including in sheep [16–19].

Taking into account the significantly higher latency of late-pregnant ewes to feed from the DCM fraction than from the control one, it is also possible that the DCM fraction maintained part of the repulsive character of the A1 fraction, but the presence of other high-polarity component(s) contained in the A2 fraction is needed to enhance the repulsiveness of some components present in DCM fraction. A similar mechanism has been reported regarding the aversion of sheep toward canine feces, which depends on the presence of various compounds. In this case, the repulsive nature of some fatty acids is enhanced by a small proportion of neutral compounds such as aldehydes, alcohols and methyl-ketones [20]. In the present study, no attempts were made to chemically characterize the fractions, but it would be worthwhile to investigate whether compounds such as free fatty acids, that remained in the A1 fraction after hexane extraction are the main compounds responsible for the fraction's repulsiveness. Indeed, as FFs contain excretion products from the fetus, such compounds could have similar properties to the fatty acids in the feces of other mammalian species. Other excretion products with higher polarity such as short-chain acids or alcohols that could enhance the repulsive effects of free fatty acids may have remained in the aqueous fraction A2 after the extraction with DCM. Finally, it cannot be excluded that the DCM extraction led to partial or complete denaturing of some repulsive compound(s), thus contributing also to the decreased repulsiveness of the A2 and DCM fractions.

Whatever the reason for the loss of repulsiveness after DCM extraction, no parallel impairing of attraction in parturient ewes was observed for these fractions, thus supporting the hypothesis that at least some of the compounds involved in repulsiveness differ from those involved in attraction. However, it could be argued that the different activity of these fractions for attraction and repulsion was due to the fact that different methods were used to test attraction at parturition and repulsion in late-pregnant females. When parturient ewes were tested, they had the choice between the experimental and the control cloth that presumably was of no interest to them, whereas in late-pregnant ewes, the control trough contained food, and thus presented at least some interest. Consequently, the nature of the test could have enhanced the contrast between control and experimental fractions when testing for attraction. Nonetheless, this is not likely to be the case in the light of the results reported by Lévy et al. [10] in parturient ewes tested for attraction toward AF, using a two-choice test with food. In their study, ewes consumed very little from the control trough and spent more than 80% of their feeding time in the trough containing food contaminated with AF [10].

As a whole, our results suggest that the repulsive characteristic of the FFs is due to a mixture of high and medium polarity compounds that need to act together to be active. On the other hand, attraction may imply both high and medium polarity components that can be partitioned in biologically active substances between the DCM and A2 fractions. In addition, these high and medium polarity compounds appear to keep their attractive properties even when separated. The crude fractionation used in the present study prevents us from speculating which specific compounds, or even classes of compounds, may be responsible for the repulsion/attraction activities. Whereas highly lipophilic compounds, which would be extracted by hexane, can be excluded, the array of medium to highly polar physiologically active compounds is too vast to speculate on which one could be responsible. Low molecular weight compounds such as steroids, fatty acids, oligosaccharides or phospholipids are all possible candidates, but even peptides or small soluble proteins may be important, either as active compounds per se, or as carriers of the active molecules. The coupling of a finer fractionation method, such as one based on size exclusion or polarity-based chromatography, with a simpler bioassay, would undoubtedly facilitate a closer approximation to the active compounds.

The capacity of the parturient mother to be attracted toward these compounds does not depend on complete desensitization to any repulsive compound, since some aversive substances such as feces [21] or bovine AF stay repulsive even at parturition (Poindron, personal observations; [14]). Finally, looking for the differences in composition between sheep FFs and of other species whose FFs are not attractive to parturient ewes (e.g.; cows' amniotic fluid [14]) may be a promising way to clarify what types of compounds are responsible for AF attraction in sheep.

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