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BIOPHYSICAL ANALYSIS OF EMANATED PHEROMONAL ODOR CHANGES IN COWS USING ELECTRONIC NOSE TECHNOLOGY

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The objective of the study was to investigate the possibility of applying the Electronic Nose (EN) technology to analyze and detect emanated pheromonal odor changes during estrus cycle in cows. In comparison to gas chromatography (GC) headspace samples analysis and blood hormonal (estradiol and progesterone) analysis using Elisa and RIA. Also to establish a protocol to appoint the proper time of artificial insemination (AI) in cows. The study was conducted on 54 Holstein-Friesian cows. Out of sensors of the EN used in this study, sensor#2 showed the highest response to all measured perineal samples, which adhered perfectly to plasma hormone (E_2) levels, and GC analysis of the perineal samples which showed progressive change in acetaldehyde as estrus was approached.

Keywords: COWS, ESTRUS, PHEROMONES, ELECTRONIC NOSE (EN), ACETALDEHYDE

Poor estrus detection is a major problem for the AI industry because missed estruses represent lost opportunities for use of semen from genetically superior bulls, since the most fertile semen and the best inseminator in the world can't overcome the problems of inseminating cows at the wrong time [6]. Consequently, a basic understanding of the bovine estrous cycle can increase the effectiveness of reproductive management [9]. It is a tremendous task to detect standing estrus in a cow herd, and nothing can substitute for visually observing the cattle. Several estrus-detection aids are commercially available, but these are just aids. The more time spent with the cattle, the better [11]. Estrus detection aids are heat expectancy charts, pressure-sensitive mount detectors, tail chalk, detector animals, and electronic aids. They may be used to help identify cows that are in estrus but may otherwise go unnoticed [10]. However, none of these techniques has yielded consistent, reproducible data, and estrus detection still relies primarily on the observation of estrous behavior or on the results of a milk progesterone assay, neither of which afford high fertility rates and both of which are labor intensive. The production of a simple, reliable protocol to aid the herdsperson is still not available [11].

In mammals, sexual behaviours of males and females are induced and their hormonal status may be also changed via the stimulation of vomeronasal organ. Vomeronasal organ is primarily responsible for mediating responses to some, but by no means all, pheromone-like signals [4]. Pheromones are chemical substances that are released by animals in order to stimulate modifications in the neuroendocrine system of receiving individuals thus producing a physiological and behavioral response [7]. A pheromonal function has been proposed for the skin glands of the bovine perineum. These glands are specialized sebaceous glands that are located on either side of the vulva, and undergo morphological changes at estrus [1].

The main task in odor recognition is to create a model as similar to the human and animal model as possible. EN are being developed as a system for the automated detection and classification of odors, vapors, and gases. EN is represented as a combination of two components: sensing system and pattern recognition system [8]. Recent advances in artificial olfaction technology have allowed us to monitor perineal odor through estrus [6].

Thus, the objectives of the present study are to: (1) investigate the possibility of applying the EN technology for detecting emanated pheromonal odor changes associated with estrus in cows; (2) assess estrus cycle in cow using the EN technology as compared to conventional methods (i.e., behavioral observations, rectal palpation, and hormonal analysis); (3) establish a protocol to appoint the proper time of artificial insemination in cows.

Materials and methods

Animals. This study was carried out on 4 healthy Holstein-Friesian dairy cow groups, at the Alexandria Agriculture Farm (Alexandria, Egypt). All cows were housed in a free-stall system and given rations to meet their maintenance and production requirements. Group A cows (n=15) and group C cows (n=9) were observed at their natural midluteal phase of the estrus cycle. While estrus was induced in group B cows (n=15) and group C cows (n=9) by a single intramuscular injection in day 0 with a prostaglandin F2α (PGF2α) — 500 μg cloprostenol in 2 ml of *Estrumate*™ (*Malinkrodt Veterinary Ltd.*, Middlesex, UK), under the supervision of a resident veterinarian. Day of estrus was assessed on basis of progesterone (P_4, pg/ml) and estradiol (E_2, ng/ml) levels, behavioral observations; Cows were observed 3 times/day throughout the study period and daily rectal palpation for presence of *corpus luteum*.

Methods. *Plasma Hormone Assays.* Blood samples were obtained from the coccygeal vein of each cow in the morning after perineal swaping from the day of PGF2α injection for 9 days (group B) and for 8 days (group D). Each sample was capped, labeled and identified on basis of cow no. and the date of sampling. The samples were centrifuged for ×9 for 10 min and plasma supernatant was decanted and frozen only once at –20 °C. Latter the plasma samples assayed for progesterone (P_4) and estradiol (E_2) (modified from *DRG diagnostic*, Marburg, Germany).

Sample collection. Samples were collected from the perineal region (area around the vulva) [1]. The area was washed with clean tap water and soft brush in order to minimize contaminate fecal odor [6]. The area was dried with soft tissues and left to dry for 10 min. Samples were then taken from a dorsal lateral perineal site using 3 cotton swabs/cow. Samples were collected in pairs of the same tubes: one for the EN and the other for the GC.

Electronic Nose (EN). First-tube samples were analyzed using a commercially available portable E-Nose (PEN3, *Airsense Analytics GmbH*, Schwerin, Germany) with an array of 10 different metal-oxide sensors that measure independently and register continuously relative changes in conductance due to a vapor or odor during an experiment. Odors in the headspace (i.e., the space over the cotton swabs) of each sealed tube was carried by the carrier gas (e.g., dry air), and the difference in the sensor output was recorded. The software interacts with the user by displaying the correct time points to connect and disconnect the sample to the E-Nose inlet. All measurements were repeated twice and results files containing sensors patterns for every experiment were saved for subsequent analysis.

Gas chromatography. Second tube samples of both studied groups were analyzed using GC (*Auto System XL*, *Perkin Elmer*, USA) at the Pahrmaceutical Analytical Chemistry Department, Faculty of Pharmacy, Alexandria University. The headspace of each sealed tube was aspired and injected immediately into the GC, where oven temperature was kept at 29 °C for 2 min. and then programmed to increase to 56 °C at 5 °C increments. The injector and detector temperatures were set at 180 and 200 °C, respectively. H_2 and N_2 were set at 45 cm/s flow rates and areas under peaks were calculated using the driving software. Reference standard of acetaldehyde was prepared and measured to calculate its concentration in each sample [12].

Data Analysis. All measurements by the E-Nose were analyzed using the Principle Component Analysis (PCA) technique. The greatest variance by any projection of the data comes to lie on the first coordinate, which is called the principal component #1; and the second greatest variance on the second coordinate, which is called the principle component #2. PCA is theoretically the optimum transform for given data in least square terms. Moreover, sensors responses of each individual cows were averaged and compared to investigate

their relative sensitivity for monitoring changes in pheromonal odors in the midluteal phase and in the estrus cycle using ANOVA followed by a Fisher's PLSD *post-hoc* test. Differences were considered to be significant at $P<0.05$.

Results and discussion

Changes in perineal odor if correctly approached could form the basis of a new method for estrus detection. In the present study perineal odor of cyclic cows was monitored. Moreover estrus was identified using GC for acetaldehyde behavioral observations, and plasma assays for P_4 and E_2.

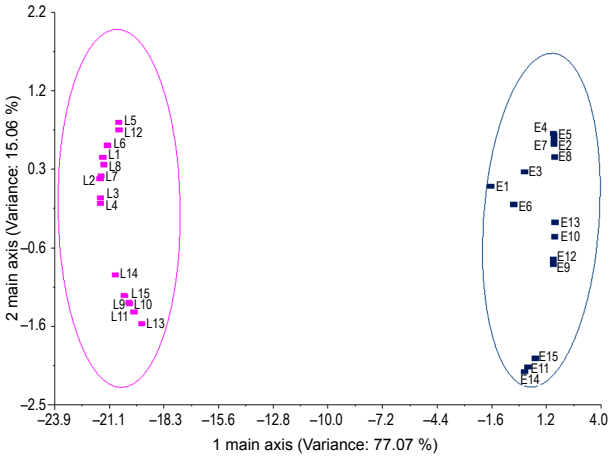

Fig. 1. Principal component analysis of perineal odor sample, group A: 15 cows each sampled on the day of estrus (E) and in the midluteal (L). Showing there is difference between the tow clusters of differentiation inspit of there was one of the two needles was congested

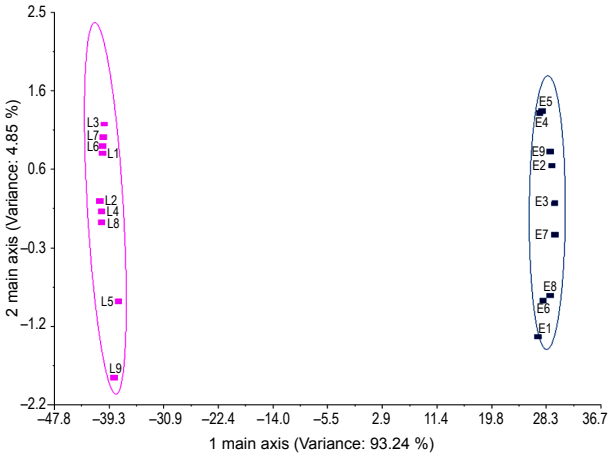

Fig. 2. Principal component analysis of perineal odor sample, group C: 9 cows each sampled on the day of estrus (E) and in the midluteal (L). Showing that there is great difference between the tow clusters more than showed in fig. 1

Experiment I. *Group A: 15 non synchronized Holstein Friesian cows* and *group C: 9 non synchronized Holstein Friesian cows.* By PCA, perineal odor data indicated difference between cows (group A) in the midluteal phase (L) and cows in estrus (E) (fig. 1). Only one of the ten sensors was responding (sensor #2) since one of the 2 needles was congested which affected on the chamber flow, thus repeating the experiment with group C was necessary to make sure that the measurements not affected by the needle congestion. In group C by repetition there was three sensors responding (#2, 6 and 8). By PCA the perineal odor data indicated

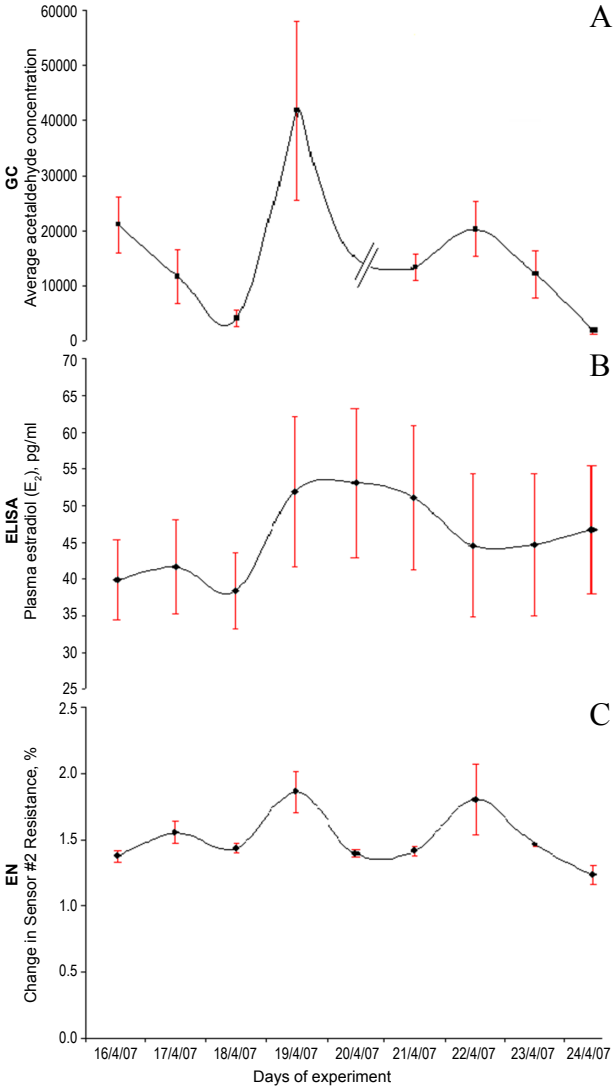

Fig. 3. The relationship between A) headspace gas chromatograms (GC) from perineal swabs, B) plasma estradiol concentration through estrus and C) the response of one of 10 sensors (#2) of electronic nose (EN) to perineal odor through estrus. Data are representing as means (±SEM) for 15 cows (group B). Estrus was induced using a single intramuscular injection of PGF2α when midluteal (day 14 or 15).

greater differences than in group A (fig. 2), however in both group A and C didn't show on which day the odor changes occur.

Experiment II. *Group B (15 synchronized Holstein Friesian cows)* and *group D (9 synchronized Holstein Friesian cows).* In group B only 1 sensor showed change in resistance through experiment, this was interpreted to represent a change in perineal odor although there was congestion in one needle which affected chamber flow. It is clear from fig. 3 GC results (except 1st day of GC) coinside with response of sensor #2 and the 1st 4 days of the plasma E_2 profile. In group D: with repetition in this group, out of the 10 sensors, three sensors showed change in resistance through experiment (#2, 6 and 8), which was interpreted to represent a change in perineal odor. The SEM bars of plasma estradiol were large due to the individual variation among the cows in spite of using automatic method in E_2 kit measurements (ELISA) [2, 3].

These data are in line with observations by [6], how showed strong correlation between concentrations of circulating steroid hormones and signals from bovine perineal swabs that were measured with an EN. The EN sensors responded to changes in volatile substances with changes in resistance. Molecules causing such a response would be likely to be detectable as an odor signal. To date, no group has carried out gas GC of the volatile constituents of the perineum. In light of the studies by [5] acetaldehyde possibly is released from a range of body fluids and may provide a marker for estrus. We used pure acetaldehyde spikes as authentic odor to determine acet-

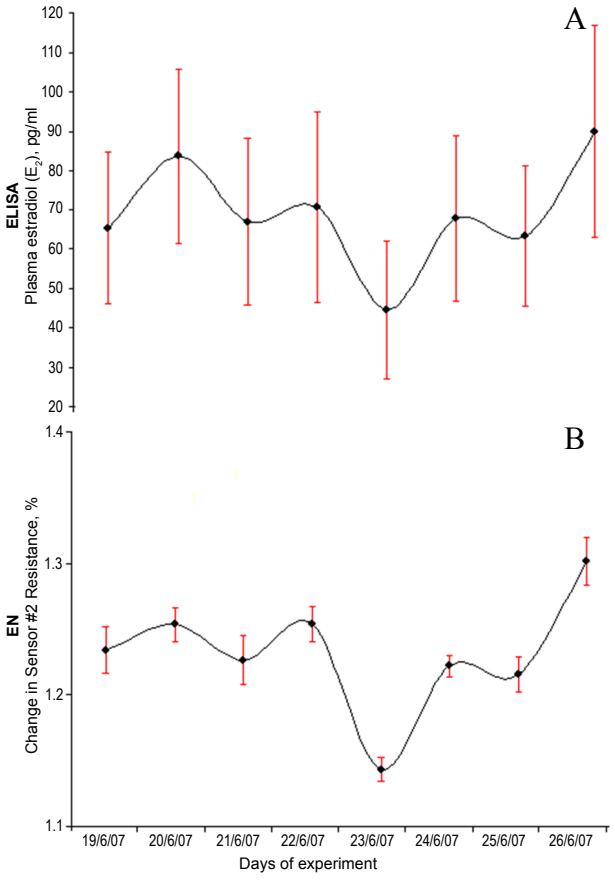

Fig. 4. The relationship between A) the response of 1 of 10 sensors (#2) to perineal odor through estrus and B) plasma estradiol concentration through estrus. Data are representing as means (±SEM) of 9 cows (group D). Estrus was induced using a single intramuscular injection of cloprostenol when midluteal (day 14 or 15). EN — Electronic Nose; E_2 — estradiol.

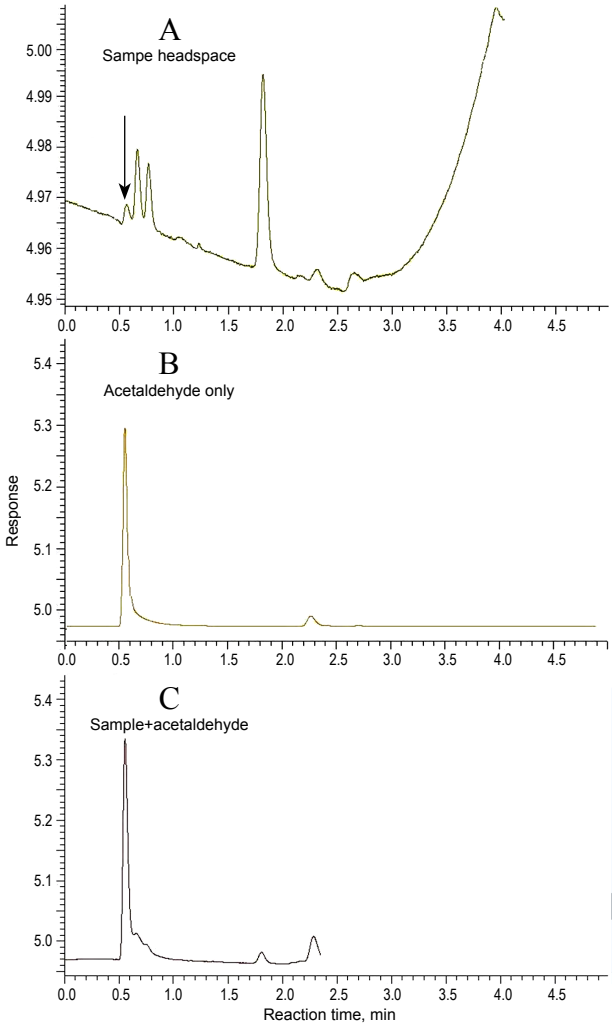

Fig. 5. Headspace gas chromatograms from perineal swabs showing coelution of acetaldehyde standard at the same time of retention as the 0.5 min. peak. (A) Sample headspace, (B) headspace from authentic acetalhyde solution, (C) sample was spiked with authentic acetaldehyde.

aldehyde in perineal head space to predict estrus. A peak that eluted at about 0.56 min occurred in all samples across estrus cycle (fig. 5a). This peak had the same retention time as that of authentic acetaldehyde (fig. 5b).

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