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Review article

Enhancing bull sexual behavior using estrus-specific molecules identified in cow urine

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ABSTRACT

Deficiencies in bull mating behavior have implications for bovine artificial insemination activities. The aim of this study was to identify the compounds present in fluids emitted by cows during estrus, which could enhance bull libido. Chemical analysis of urine samples from cows led to the characterization of molecules varying specifically at the preestrous and estrous stages. The synthetic counterpart molecules (1,2-dichloroethylene, squalene, coumarin, 2-butanone, oleic acid) were used to investigate the biological effects on male sexual behavior and sperm production. When presented to males, 2-butanone and oleic acid synthetic molecules significantly lowered mounting reaction time and ejaculation time (−33% and 21% after 2-butanone inhalation, respectively, $P < 0.05$). The "squalene +1,2-dichloroethylene" combination induced a 9% increase of sperm quantity ($P < 0.05$). This study suggests that the identified estrous-specific molecules could be part of the chemical signals involved in male and female mating behavior and may be used for a wide range of applications. The identification of these molecules may have implications for the cattle breeding industry.

1. Introduction

Production centers collecting semen for bovine artificial insemination (AI) are regularly confronted with bulls displaying reduced or complete absence of sexual behavior, in a temporary or permanent manner. This concerns both young individuals, which may be eliminated from the breeding program after a failed sexual function assessment, and adult bulls [1], for which the efficiency of semen collection has a high economic impact. Consequences of these disorders are numerous for semen collection centers and for breeding companies. Significant time losses associated with an increase in the risks taken by technicians to excite the bulls with low libido lead to a lower efficiency of workforce. The accompanying reduced number of semen doses has consequences for genetic improvement. Considering the impact of deficient mating behavior on the availability of bull semen, new and sustainable management solutions for bull semen production are critical.

In cattle, as in most of mammals, chemical communication has been described to act either singly or in combination with auditory, visual, or tactile stimuli during the process of reproduction [2]. Males in the presence of estrus females perform a courtship sequence, which includes smelling behavior (distant or close to the cow), licking, and flehmen. This courtship sequence, resulting in a complete sexual behavior with erection, mounting of the female, and

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copulation, supports a chemical information exchange between partners during natural estrus [3]. Several body fluids are thought to carry this chemical information. When estrous and nonestrous fluids were presented to males of different species, including bulls (saliva, vaginal fluids, milk, feces, serum [4,5]), dogs (vaginal mucus, urine, milk, plasma [6,7]), and rats (urine [8]), the ability to discriminate between the two states is compelling. In the past 10 years, investigation of estrous and nonestrous fluids from cows has led to the characterization of a number of estrus-specific compounds and their effect on bulls. Triethylamine, acetic acid, and 4-propyl phenol, identified in the saliva of estrus cows, have been shown to increase flehmen frequency and mounting behavior in bulls [5]. Acetic acid, 1-iodoundecane, and propionic acid, identified as estrus-specific compounds in feces, were shown to induce longer flehmen and increase the number of mounts when presented, alone or in mixture, to bulls [9]. n-Phthalate and 1-iodoundecane were identified as estrus-specific compounds in cow urine [10], but their biological activity has not been found.

On the basis of these findings, we hypothesized that chemical signals present during estrus may also be implicated in the sexual behavior of bulls. The objectives of this study were to identify chemical signals emitted by cows during the heat period and to test their effect on sexual bulls' behavior and sperm production.

2. Materials and methods

2.1. Reagents and chemicals

All chemicals and reagents used in this study (dichloromethane, ethanol, glycerol, 1,2-dichloroethylene (DCE), coumarin, squalene [SQ], oleic acid [OA], 2-butanone) were of analytical grade purity and purchased from Sigma–Aldrich.

2.2. Cows and cycle following

This study was conducted using 32 cows (*Bos taurus*, Prim'Holstein, and Montbéliarde breeds), housed the INRA-UEPAO (Institut National de la Recherche Agronomique - Unité Expérimentale de Physiologie Animale de l'Orfrasière, Nouzilly, France) and UNCEIA (Union Nationale des Coopératives d'Elevage et d'Inséminations Animales, Châteauvillain, France) experimental stations. Cows were fed a standard diet of straw and *ad libitum* water. Estrus was induced by synchronization with two PGF2α injections spaced 10 days apart. Stages of the cycle were determined by immunoassay of plasma progesterone (Ovucheck plasma kit for bovine; BioVet) and LH (LH Detect kit, ReproPharm) concentration every six hours and behavioral observations (twice a day), during the interval from the second PGF2α injection to the end of estrus. Five collection sessions in which five, six, or eight cows were followed, have been performed.

2.3. Urine collection

For each animal, urinary samples were collected in sterilized glass vials (Supelco) during midstream urination, three times a day, from 2 days after the second PGF2α injection during 1 week, corresponding to preestrous and estrous stages, then 11 days after the second PGF2α injection for the luteal stage. Samples collected from the beginning of the procedure until first signs of estrus were categorized as "preestrus samples." Samples collected around the LH peak (when observed) from cows accepting mount were categorized as "estrus" (between 10 and 48 hours). Sampling was done during natural urinations (no manual bladder stimulations). Vaginal secretions were also collected when emitted. The collected samples were frozen within the hour and stored up to 3 months at −20 °C until analysis.

2.4. Extraction of urinary chemical compounds and gas chromatography–mass spectrometry (GC–MS) analysis

Thawed portions of urine (4 mL) and vaginal mucus (1 mL) were extracted twice by dichloromethane (v:v, purity > 99%) on ice. After a brief mix by vortexing and centrifugation (2500 rpm for 20 minutes at 4 °C), the upper organic phase was dried on an anhydrous sodium sulfate column (Sigma–Aldrich) to remove traces of water. The extract was concentrated to 100 μL under gentle nitrogen stream and stored at −20 °C until chemical analysis. Gas chromatography–mass spectrometry analyses were performed on a quadrupole mass analyzer DSQ II (Thermo Scientific) coupled to a FOCUS (Thermo Scientific) gas chromatograph. Separation of the extracted urinary compounds was carried out on an Equity-5 (30 m× 0.25 μm ID × 0.25 μm) capillary column (Supelco). Helium was used as the carrier gas at a flow rate of 1 mL/min. The conditions for the chromatographic analysis were as follows: the initial temperature 30 °C was maintained for 5 minutes, and then, it was raised to 280 °C at a rate of 5 °C/min, where it was maintained for 10 minutes. Samples (2 μL) were injected in splitless mode (0.5 minutes). The separated compounds were ionized by electron impact in positive ion mode, using electron energy of 70 eV. The injector, the transfer line, and the source temperature were set to 280 °C, 280 °C, and 180 °C, respectively. Gas chromatography profiles of the extracted compounds were compared for each cow during one estrus period using the GC–MS instrument, whereas the peak identities were established on the basis of retention time, mass spectra, and comparisons in the National Institute Search Technology (NIST) spectra data bank and when available with standard compounds (DCE, coumarin, SQ, OA, and 2-butanone).

2.5. Conditioning of estrus-specific molecules and used combination

The commercially available identified molecules (DCE, coumarin, SQ, OA, and 2-butanone) were solubilized, alone or in mixture in a nonpressurized aerosol dispenser (Hyteck Aroma-Zone, France). Each molecule was diluted at a final concentration of 25 pg/mL in glycerol–water solution (50/50, v:v). The concentration was chosen on the basis of the estimated quantity of the more abundant compound, coumarin. Quantity was deduced from peak area in the chromatograms of the GC–MS analysis. All solutions were