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Identification of urinary sex pheromones in female buffaloes and their influence on bull reproductive behaviour

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ABSTRACT

The present study was carried out to identify the urinary sex pheromones of buffaloes and their role in relation to bull reproductive behaviour. Urinary samples were collected from 12 buffalo heifers at four different phases of estrous cycles. Fourteen compounds were identified throughout the cycle, which included phenol, ketone, alkane, alcohol, amide, acid and aldehyde. Among the 14 chemical profiles, three compounds were only found on the day of estrus, viz. 1-chlorooctane, 4-methylphenol and 9-octadecenoic acid. Behavioural investigation clearly showed that bulls were attracted and exhibited repeated flehmen behaviour towards the 4-methyl phenol. The bulls displayed penile erection and mounting behaviour while exposed to 9-octadecenoic acid. However, the other compound, 1-chlorooctane, did not influence such sexual behaviours. The present results provide evidence that the estrus-specific urinary volatile compounds appear to be sex pheromones which initiate the bull's reproductive behaviour.

1. Introduction

Pheromone signals play a significant role in eliciting both behavioural and endocrine responses in conspecifics (Vandenbergh, 1999; Brennan and Keverne, 2004; Beynon and Hurst, 2004). In mammals, other than primates, males are attracted to females in estrus by pheromonal cues (Albone, 1984; Halpin, 1986; Johnston, 1990). Sources of such cues are urine, faeces, and vaginal secretions, as well as exocrine glandular products (Aron, 1979; Muller-Schwarze, 1983; Rivard and Klemm, 1989; Dominic, 1991; McClintock, 2002; Sankar and Archunan, 2008). In ungulates, estrus indicating pheromones have been reported in urine and vaginal secretion that initially lead to flehmen response and later to mounting behaviour (Crowell-Davis and Houpt, 1985; Rajanarayanan and Archunan, 2004; Sankar and Archunan, 2004).

Timing of pheromone release is often critical for a species to synchronize reproductive behaviour. Rasmussen et al. (1982, 1997) have established that during estrus, especially prior to ovulation, female Asian elephants release a urinary pheromone that elicits high frequencies of several types of chemosensory responses including flehmen in male elephants. Thus, the flehmen response is a discrete motor pattern exhibited specifically during the sensory evaluation of chemicals. The flehmen response formed the basis for a specific and quantitative bioassay in reference to estrus phase (Rasmussen et al., 1982). Subsequent to chemoresponses, sexual arousal behaviours may occur in males which include penile erection, mounting and copulation. Penile erection is commonly used as a measure of sexual arousal and sexual preference in men (Schiavi et al., 1993) and rats (Sachs, 1997), although it has been used to some extent to assess male sexual arousal in non-human primates (Nadler et al., 1994; Pomerantz, 1995).

One of major problems in detecting estrus in buffalo is the absence of any reliable method for estrus detection; estrus signs shown by buffalo are not clearly manifested hence it is commonly known as silent heat (Danell et al., 1984). Unlike in the cow and other ungulates, visual signs of estrus are not prominent in the buffalo, especially for example the discharge of vaginal mucus. Hence, there is need for a reliable method to detect estrus in buffalo. Our previous investigation demonstrated that the average numbers of all flehmen behaviour (2.03 ± 0.66) and repeated flehmen (1.05 ± 0.64) behaviour during estrus periods were significantly higher than those of diestrus periods of all (0.69 ± 0.25) and repeated flehmen (0.11 ± 0.10) (Rajanarayanan and Archunan, 2004).

Identification of urinary pheromones in buffaloes would pave a way to develop a biochemical kit for detecting estrus effectively. Therefore, the present investigation was carried out to examine the presence of volatile compounds in the urine of buffalo during various phases of the estrous cycle in order to detect the estrus-specific compounds, and to study bull behaviour in response to the identified compounds.

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2. Materials and methods

2.1. Animal and estrus phase confirmation

Urinary samples were collected from 12 healthy heifer murrah buffalo (*Bubalus bubalis*) approximately 20–30 months old at Government Livestock Farm, Orathanadu, Tanjore, Tamilnadu, India. The animals were housed in sheds and paddocks, fed with a standard diet straw and *ad libitum* water. The phases of the estrous cycle were carefully determined for six naturally occurring consecutive cycles and confirmed by trans-rectal palpation by assessing the morphological changes in the internal reproductive organs. Further, the estrus phase (day '0') was confirmed by fern pattern analysis from cervico mucus samples (Sukh Deo and Roy, 1971). The cervical mucus, collected by inserting a sterile swab into the cervical regions, was smeared between two glass slides and observed for the presence of fern like crystals.

2.2. Sample collection

The urine samples were collected during six consecutive estrous cycles (72 estrous cycles; days from −12 to +12; day '0' estrus); the samples were categorized as pre-estrus (days −4 to −2), estrus (day '0') post-estrus (day 2–4) and diestrus (days −11 to −8) (Rajanarayanan and Archunan 2004). The urine samples were collected during midstream urination from the heifers in pre-sterilized glass bottles. The samples were strained through cheesecloth or nylon mesh (60–120 μm) at the time of collection and immediately stored at −20 °C.

2.3. Sample preparation for GC–MS analysis

Dichloromethane (DCM) was used as solvent for GC–MS analysis. Urine sample (25 ml) was taken from each phase and mixed with 25 ml of DCM and filtered through a silica-gel column (60–120 mesh) 30 min at room temperature. The filtered extract was reduced to 1/5 of its original volume by cooling with liquid nitrogen to condense it.

2.4. GC–MS analysis

The sample was fractionated and chemical compounds were identified by gas chromatography–linked mass spectrometry (GC–MS; QP-5050, Schimadzu, Japan) (Rameshkumar et al., 2000; Achiraman and Archunan, 2006). Two microliters of extract were injected into the GC–MS system on a 30 m glass capillary column with a film thickness of 0.25 μm (30 × 0.2 mm i.d. coated with UCON HB 2000) using the following temperature programme: initial oven temperature of 40 °C for 4 min, increasing to 250 °C at 15 °C/min, and then held at 250 °C for 10 min. GC–MS was run under computer control at 70 eV. The solvent (i.e. DCM) peak was seen at 4.0 min during GC–MS analysis. The mass spectrometer was programmed for a mass scan of 33–300, which allowed for identification of compounds from C3 through C18. The compound identification was made by probability-based matching using the computer library (NICT 12) built within the system. The identified estrus-specific compounds were then compared with the standard compounds run under the same conditions, and confirmed on the basis of retention time shown in GC–MS.

2.5. Bull behaviour study

The bull's behaviour in response to synthetic compounds was carried out in the Government Livestock Farm, Orathanadu, Tanjore Dist., Tamilnadu, India. Twelve cows and 12 bulls (30–36 months old) of *B. bubalis* were used in the present study. The synthetic compounds (Aldrich Chemicals, St. Louis, USA) were procured and soaked with dichloromethane (DCM) on cotton wool, and applied manually onto the genital region particularly vulval region of non-estrous cows for behavioural assay. The solvent DCM was used for GC–MS analysis as well as dissolving the compounds in various concentrations, e.g. 0.5%, 1.0%, 2.0% and 5.0%. Since 1% concentration of estrus specific compounds were found to be effective in eliciting the flehmen and other behaviours penile erection and mounting, 5 ml of the same concentration (1%) was used throughout the experiment to assess the bioactivity. The bulls were allowed to sniff the genital regions of the non-estrous cows for a period of 15 min in a single exposure, and the reproductive behaviours such as flehmen behaviour, penile erection and mounting behaviour were exhibited by bulls in response to estrus specific compounds (1-chlorooctane, 4-methyl phenol and 9-octadecenoic acid). The other compounds like phenol (pre-estrus) and 1-chlorotetradecane (diestrus) were taken in order to compare with estrus specific compounds as they are specific in particular phase. The flehmen behaviour was recognized as: when the male sniffs the genital region of female and raises its neck, extends its chin and inhales with slightly opened mouth, tongue held in flat position and upper lip curled. The penile erection was included when the penis is protruded from the body during the bioassay. When the cow was mounted by a bull, it was considered as mounting. The numbers of mounts were counted when a female buffalo was mounted by bull.

The data for behavioural assay were compiled using the SPSS statistical software, 11.5th version, and subjected to the analysis of Duncan's multiple range test (DMRT). Descriptive statistics are given as mean ± SD (standard deviation) and was carried out with Microsoft® Excel 2007 program.

3. Results

The fern like crystals (typical fern pattern) was observed only on cervico mucus of day '0' and not found in other phases of the estrous cycle.

The gas chromatographic profiles of pre-estrus (Fig. 1), estrus (Fig. 2), post-estrus (Fig. 3) and diestrus (Fig. 4) phases of the estrous cycle were specified. Fourteen different volatile compounds (Table 1) from all the stages of the estrous cycle were identified in the urine samples, which included phenol, ketone, alkane, alcohol, amide, acid and aldehyde. Among the compounds 2-octanone, 5-methyl-2-(1-methylethyl)-cyclohexanol, 2-methyl-*N*-phenyl-2 propenamide, decanoic acid, 1-chlorotetradecane, *N*,*N*-bis (2-hydroxy ethyl) dodecanamide, tetradecanoic acid and hexadecanoic acid were present in all urine samples. By contrast, compounds such as phenol, 3-propyl phenol and 9-octadecenal were only found in the pre-estrus urine. Compounds, 1-chlorooctane, 4-methyl phenol and 9-octadecenoic acid were only present in the estrus urine samples. Gas chromatography analyses showed that the compounds fall in the retention time between 5 and 45 min.

Bull flehmen behaviour (Table 2) and other behaviours like penile erection (Table 2) and mounting (Table 2) towards the synthetic compounds were analysed. The flehmen behaviour of bull was greatly ($p < 0.05$) influenced by estrus-specific compound 4-methyl phenol as well as the mixture of the three estrus-specific compounds as compared to that of the diestrus compound. Penile erection and mounting were significantly ($p < 0.05$) higher in response to 9-octadecenoic acid compared to 1-chlorooctane and 4-methyl phenol. However, the flehmen behaviour, penile erection and mounting activity were found to be highly significant ($p < 0.05$) in bulls when exposed to the mixture of estrus-specific compounds compared to that of individual estrus-specific compounds and diestrus compounds.