COMPONENTS IN BOVINE URINE WHICH CHANGE IN
CONCENTRATION DURING THE ESTROUS
CYCLE: POSSIBLE PHEROMONES

by
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During the last thirty years it has become increasingly apparent that pheromones play a major role in animal communication. Of economic interest to agriculturalists is the role pheromones play in signaling the state of estrus. The ability to efficiently detect estrus is becoming increasingly important, especially in the management of dairy and beef herds, where artificial insemination is used extensively. The understanding of pheromones may eventually provide a solution to this problem.

The purpose of this study was to identify one or more compounds in the urine of cattle which undergo a change in concentration during the estrous cycle.

In order to accomplish the objectives of this study,
urine was obtained from cows exhibiting estrus and again 7
days post estrus. The urine was extracted with ether and
the extract analyzed by gas chromatography. The area under
each chromatographic peak was determined by integration.
Areas were analyzed for variance between estrus and
di-estrus. An attempt was made to identify each of the
compounds represented by those peaks which proved to have
statistically different concentrations. Two significant
peaks were identified by coinjection with standards and
found to represent propionic acid and iso-caproic acid.
Three other statistically significant peaks were analyzed
by mass spectrometry but concentrations were too low to
determine the identities.

Results indicate that propionic acid, iso-caproic acid
and two of the unidentified compounds decrease in
concentration during estrus. The third unidentified
compound was found to increase during estrus. Studies have
not yet been done to show pheromonal properties, however,
the first step to allow such studies has now been
accomplished. Five compounds have been found which change
in concentration during estrus. Because the changes
reflect similar concentration changes in pheromones, it is
believed that they may eventually prove to be pheromonal.

(68 pages)
DEDICATION

I dedicate this work to my wife Debbie, who makes life joyful and pleasant in all situations.
ACKNOWLEDGMENTS

Grateful appreciation is extended to the Chemistry-Biochemistry Department of Utah State University for providing equipment and laboratory space for this project. I especially thank Dr. Janusz Pawliszyn, and Dr. Stephen Bialkowski for technical assistance and a general education in chromatography.

I express gratitude to my major professor Dr. Jay W. Call and all those who served on my committee for their patience and time. Especially to Dr. Doyle Johnson who originated the idea for the project and then closely advised me, while letting me learn from my own mistakes during the course of the project.

I also thank all of my family and friends for helping me and for sharing this experience with me.

Scott K. Johnson
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STATEMENT OF THE PROBLEM

Research in pheromones has revealed that in the urine or vaginal mucous of many species there are one or more compounds which change in concentration according to the stage of the estrous cycle. In most cases the compounds are found to rise during sexual receptivity. Based on this evidence, one is led to believe that such may be the case in bovine. Certainly, some studies support the theory that pheromones serve to signal sexual receptivity to the bull.

It is believed that elucidation of the compounds which undergo such cyclic concentration changes would be useful to the agricultural industry for several reasons.

First of all, pheromones have been shown to change in concentration during the estrus cycle. Therefore, it is believed that by identifying compounds which change in concentration during the estrus cycle, an initial step might be taken toward identification of bovine signalling pheromones.

Secondly, one of the major losses of revenue to livestock producers can be traced to the inefficient detection of animals in estrus. This is especially the case in the dairy and beef industries where the male is no longer used in the herd due to the advances of artificial insemination. It is believed that by identifying compounds which change in concentration at the onset of estrus, a technique might eventually be developed to use this
concentration change to detect estrus in an everyday situation.

Finally, by identifying compounds undergoing a cyclic concentration gradient, further understanding into reproductive processes may be gained.

Therefore, the objective of this study is to identify one or more compounds which change in concentration during the estrous cycle.
REVIEW OF LITERATURE

General Introduction

Based on the evidence of research performed in recent years, scientists are realizing that pheromones play an important role in our everyday lives. If pheromones play a major part in our lives, why are they not better understood? Within the last thirty years pheromone research has made tremendous advances. Even now, however, there is disagreement as to whether or not the very term "pheromones" can be applied outside of the insect kingdom (Vandenbergh, 1983). Part of the reason for the crevice in knowledge is due to the fact that the field of pheromone research is caught in that elusive area between biology and chemistry. This area encompasses such a vast amount of information that it has not yet been extensively investigated by enough researchers.

Use of the term "Pheromone", taken from the Greek 'pherien', to transfer, and 'hormon', to excite, was first proposed in 1959 by Karlson and Lüscher. Pheromones, or "carriers of excitation" (Nalbandov, 1976) have been defined as substances which are secreted externally by one animal and elicit a reaction from another receiving individual by either oral or olfactory means (Karlson and Lüscher, 1959; Hafez, 1972; Hadley, 1984). Pheromones are usually considered to function between individuals of the
same specie (Hadley, 1984). Therefore, much like audition, and vision, olfaction and even taste are means of communication among animals.

Though most early pheromone research focused on insects (Karlson and Lüscher, 1959), the existence of a chemical signal in mammals has been recognized for many years. One of the earliest studies of a pheromone-like substance was by the stablemaster of a king. Oibares simply found that when presented with vaginal secretions from an estrous mare, a stallion displayed signs of arousal (Claus and Karlson, 1983). Though pheromones were known to be present in mammals, the comparable ease of insect pheromone research and the economical impact that understanding insect pheromones can provide through pest control have acted to accelerate research of pheromones in insects. Thus, volumes have been printed concerning the specific pheromones produced by many species of insects. Studies have been performed to determine the means of production of pheromones as well as the means of reception. In many cases the chemical constituents of a pheromone have been reported. For example; an oviposition deterring pheromone was recently isolated in the European cherry fruit fly, \textit{Rhagoletis cerasi} L. The compound was spectroscopically found to be \text{N[15(Beta-glucopyranosyl) oxy-8-hydroxy-palmitoyl] taurine} (Hurter et al., 1987).

Once the composition of a pheromone in insects is
elucidated, it may then be synthesized and used in the field. For example, pheromones have served as lures for traps or as deterrents to ward off insects. Many of the advanced techniques used in pheromone research were developed for studies of insect pheromones and one must be aware that these studies have laid the foundation for the accomplishments which have been made in mammalian pheromone research.

There are two defined types of pheromones, each causing a different class of response. The two types include priming pheromones and signalling pheromones (also known as releasing pheromones) (Hadley, 1984).

**Priming Pheromones**

Priming pheromones initiate a chain of physiological events by means of stimulation or inhibition. Through these events the endocrine, reproductive and possibly other systems are altered (Wilson and Bossert, 1963). The changes incited by the priming pheromones are slow in comparison to those brought on by signalling pheromones (Hadley, 1984). It is theorized that priming pheromones act on the pituitary to stimulate or inhibit release of gonadotropins (Hadley, 1984). It is further suggested that they act on the nervous afferents to the central nervous system (Hadley, 1984). Once received, priming pheromones may take hours, days, weeks or months to elicit a full response and
once the alterations in behavior and physiology are made, the recipient is slow to return to the previous state.

There is evidence of many different modes of action and varied end results of priming pheromones. It has been found that in the house mouse (Mus musculus) alone there are at least three functions of the priming pheromones secreted by the male mouse. These include: 1. acceleration of maturation, 2. pregnancy blockage, and 3. induction of estrus (Izard, 1981). A fourth reproduction related function of priming pheromones which does not occur in mice is the synchronization of estrus. These four reproductive functions of priming pheromones will be considered in this treatise.

Sexual Maturation. One major reproductive function of priming pheromones is the role they play in prepuberal maturation. It has been evidenced in several species that the presence of a mature male or urine from a mature male of the species will cause juvenile conspecific (of same species) females to reach puberty earlier. This has been shown in several rodent species including the mouse (Mus musculus) (Vandenbergh, 1969) the rat (Rattus norvegicus) (Vandenbergh, 1976), the meadow vole (Microtus pennsylvanicus) (Baddaloo and Clulow, 1981) and the prairie deermouse (Peromyscus maniculatus) (Lombardi and Whitsett, 1980).

There is strong evidence that pheromones play a role
in the age of menarche in women. Prepuberal females reared among mature females are believed to reach menarche at a later age than those females reared among mature men (Burger and Gochfeld, 1985).

The advantage of accelerated maturity is of economical interest to our agricultural industry. Research has shown that by raising gilts in the presence of boars, the days to first estrus are significantly reduced (Brooks and Cole, 1970; Hughes and Cole, 1976). Further studies strengthen the argument that this is pheromone moderated by achieving the same results when raising gilts in a pen where a mature boar was previously housed (Kirkwood and Hughes, 1980). Today both these practices are being implemented by pork producers and result in substantial reproductive gains and thus economic gains.

Evidence suggests that other farm animals will respond to adult males with accelerated maturity. Sheep for example experience fewer days to puberty when the ewe lambs are raised with an adult ram (Dyrmundsson and Lees, 1972). Cattle also respond in like manner. After spraying bull urine in the nostrils of heifers for eight weeks, Izard and Vandenbergh (1982) showed a significant increase in the number of animals that attained puberty.

On the other side of maturation acceleration is evidence that priming pheromones produced by mature females slow down the maturation of other young females. This has
been shown primarily in the mouse (Vandenbergh, 1973) but there are indications that it occurs in other species such as in women as mentioned previously (Burger and Gochfeld, 1985).

**Pregnancy Block.** The second phenomenon related to priming pheromones and reproduction is known to occur only in a few rodents. In a landmark study which first turned the scientific community's attention to the presence of pheromones in mammals, Bruce (1959) found that implantation was inhibited in newly inseminated female mice when exposed to an adult male from a different strain. This was later determined to be the result of an androgen dependent pheromone (Dominic, 1964; Bruce 1965). Similar reactions are known to occur in the deermouse (Bronson and Eleftheriou, 1963), the field vole (Clulow and Clarke, 1968) and the meadow vole (Clulow and Langford, 1971).

**Estrus Induction.** Closely related to the priming pheromone function of estrus synchronization is estrus induction and suppression. When grouped closely together, it was found that female mice became anestrous (Whitten, 1956) under the influence of a priming pheromone (Bruce, 1965). It has been further shown that exposing these same females to a mature male resulted in resumption of estrous activity (Whitten, 1956) associated with an increase in plasma levels of follicle stimulating hormone (FSH) (Bronson and Desjardins, 1969). This has also been shown in
prairie deermice (Bronson and Marsden, 1964).

Research reveals that in sheep (Thompson and Schinckel, 1952) and goats (Ott et al., 1980) estrus occurs earlier in the breeding season when the females are exposed to adult males. Knight et al. (1983) found that by applying rams wool to the nostrils of anestrus ewes, ovulation could be induced to occur earlier in the season. It is known that the presence of a male goat will result in earlier estrus in the females (Smith et al., 1984). Interestingly, it was discovered that the presence of a mature male goat has the same effect on female sheep (Knight et al., 1983). Thus, perhaps both species produce the same or at least related pheromones.

Research has shown that the presence of a boar can induce post-partum estrus in a sow (Izard, 1981). A product used widely in the swine industry to take advantage of this reaction consists of a synthetic pheromone. Used in the form of an aerosol spray this product may significantly reduce the time to first post partum estrus (Hillyer, 1976).

In 1956 it was demonstrated that the post partum interval to first estrus in cattle can be greatly reduced by the presence of an adult bull (Petropavlovskii and Rykova, 1958). This too may be due to a male pheromone, however the results have not been duplicated since.
Estrus Synchronization. It has been noted that in many species, synchrony of estrus comes about through means that cannot be explained except through actions of pheromones. The rat models this effect very well. McClintock found in 1978 that when asynchronous rats were housed such that the only possible means of communication was through connecting airways, their estrous cycles eventually synchronized. This, however, is not the case in mice. Similar observations have been made concerning bitches housed together in a kennel; they also may eventually gain some degree of synchrony (Personal Observation).

Humans themselves are not immune to this function of priming pheromones. In fact it has been shown that when women are housed together, within three to four months their menstrual cycles become synchronized (McClintock, 1981; Quadagno et al., 1979).

Of greater interest to this thesis is the fact that cows too will auto-synchronize the estrous cycle given enough time in the company of other sexually active cows (Weston and Ulberg, 1976). Izard (1981) demonstrated that this effect is due to pheromones, when they exposed heifers being treated with prostaglandin F₂α to urine and cervical mucous from cows in estrus and thereby gained a significant increase in synchronization.
Signalling Pheromones

The second type of pheromone is the signalling pheromone. This type of pheromone causes an immediate change in the behavior of the receiving animal and allows that animal to return to its previous state as soon as the pheromone dissipates (Wilson and Bossert, 1963). Signalling pheromones evoke a rapid response in comparison to priming pheromones (Hadley, 1984). Unlike the priming pheromones, it is suggested that the main arena of focus of signalling pheromones is on the central nervous system afferents and not on the pituitary. Upon sensing the presence of a signalling pheromone, an animal may be sexually stimulated or may cower in fear, depending on the function of the pheromone. It is the signalling type of pheromone which will be treated by this thesis.

There are several different functions of signalling pheromones, not all of which are directly related to reproduction. For example, one function of a signalling pheromone is to warn or deter other animals of the same or different species. For example, the 16-androstenones produced in boar saliva may act as an olfactory threat to other males (Beaver, 1982). It was also found that when presented with plastic gloves contaminated with the serous secretions from the anal sacs of mature dogs, juvenile dogs recoiled in fear (Donovan, 1967). This too may be a result of pheromones. Identification is another function of
signalling pheromones. Many animals are known to identify their young by smell. Olfaction is said to play a major role in mother-young interactions (Leon, 1983).

The most commonly accepted function of pheromones in mammals is the function of communicating the sexual status of an individual. In most cases the female communicates receptivity during estrus. However, in a few cases the male is known to send pheromonal sexual signals.

**Male Signalling Pheromones.** One of the best known examples of a male signalling sexual status to the female is found in the boar. During the courtship, the boar will often chase the sow around the pen while rhythmically champing its jaws. This produces a thick smelly froth at the mouth of the boar. It has been found that pheromones consisting of 5-alpha-androstenone and 3-alpha-androstenone are produced in the submaxillary glands of the boar (Beaver, 1982). These are believed to be responsible for the stance which the sow eventually takes, allowing the boar to mount. Though application of hand pressure by the herdsman to the back of a sow in estrus will often result in the lordosis stance, it has been found that spraying an aerosol containing the 16-androstanes will improve the response markedly (Beaver, 1982).

Other cases where the female is attracted by the pheromones of the male include the goat (Shelton, 1980), the black-tailed deer (Brownlee et al., 1968), and the
pronghorn antelope (Müller-Schwarze et al., 1974).

Female Signalling Pheromones. Most other sexual status signalling pheromones originate with the female and are assumed to be present during estrus. Much of the research related to this area involves the removal of fluids or tissues containing supposed pheromones and then determining their ability to attract the opposite sex. It has been found that the urine or vaginal secretions from females in estrus of several species have a strong attraction to the male of the species. This includes the male in mice (Dixon and Mackintosh, 1971; Mugford and Norwell, 1970; Bronson, 1971; Whitten and Champlin, 1973; Bronson, 1979), dogs, cats, wolves, coyotes, wild cats (Stoddart, 1976), columbian ground squirrels (Harris and Murie, 1984) rhesus monkeys (Curtis et al., 1971; Michael and Keverne, 1968), baboons (Michael et al., 1972), humans (Michael et al., 1972), elephants (Rasmussen et al., 1982), hamsters (Steel, 1984; Johnston, 1974), swine (Signoret, 1970), horses (Waring et al., 1975), sheep (Banks, 1964; Lindsay, 1965), goats (Ladewig and Hart, 1980) and cattle (Donovan, 1967).

Comparison of sexual signalling pheromones across species lines suggests that at least in some cases they are functional. By exposing male and female college students to different odors including one treatment containing the boar pheromone 5-alpha-androst-16-en-3-one, Filsinger et al. (1984) found that there was a definite sex by treatment
interaction in the students response to stimuli and in their self concepts.

Methods and Results of Previous Research

The most common method of pheromone research to date is the simple bio-assay. A researcher will take a fluid from a female in estrus and tease a male to determine if it elicits a sexual response. This was the method of determining the existence of female signalling pheromones in most of the species listed previously. For example, in the columbian ground squirrel, Harris and Murie (1984) took vaginal swabs from females and presented them to males. Subsequent activity indicated that the male was able to distinguish the estrous from post-estrus swabs. Similar research was conducted in most of the other species.

The next common step in mammalian pheromone research is to find a solvent which will extract the pheromone and then run a bio-assay on the solvent similar to that described above. For example in the elephant, urine from females in estrus was collected and extracted using either diethyl ether, dicloromethane or chloroform. The extract was then concentrated to 0.5 ml and reabsorbed in acetone. This was mixed with non-estrous urine and the sample was poured on the ground shortly before allowing the bull to enter. Upon entering, the bull quickly located the sample and exhibited great interest while increasing the frequency of flehmen
responses and experiencing some penile erections. No such interest was shown in response to the controls consisting of non-estrous, non-treated urine (Rasmussen et al., 1982).

Similar research has been conducted with urine in several species. In most cases it was found that the urinary compound of interest was soluble in ether. This includes the Asian elephant (Rasmussen et al., 1982), the rhesus monkey (Michael et al., 1971), women (Michael et al., 1974), the bitch (Goodwin et al., 1979), and the mouse (Shwende et al., 1984).

Often the next step in analyzing the pheromone activity of a specie is to separate it chromatographically from the rest of the sample. Curtis et al. (1971) performed this successfully by extracting rhesus monkey urine with ether and washing it with 5 ml. N/100 sodium hydroxide. The solution was then eluted through DEAE-cellulose and collected into fractions. These fractions were tested for biological activity by presenting them to male conspecifics and observing for increased sexual excitation. The active portion was then further tested (Curtis et al., 1971). In a separate study, Tenax GC® was used in a precolumn prior to gas chromatography of mouse urine volatiles (Schwende et al., 1984).

In a few mammalian species pheromone-like compounds have actually been identified. In most cases this has been accomplished through gas chromatography. Upon analyzing a
set of samples and determining which peaks are significantly different between estrus and non-estrus, the chemical corresponding with that peak is identified. One method used to perform this is to coinject a set of standards with the urinary extract to see if there is a separation on the chromatogram (Curtis et al., 1971). Another identification method which may or may not be done along with coinjection of standards is mass spectrometry (Curtis et al., 1971; Schwende et al., 1984). In mice, several pheromone like constituents have been found. Using gas chromatography, Schwende, et al. (1984) found that in the estrous mouse the following were secreted in the urine at significantly enhanced levels: n-pentyl acetate, cis-2-penten-1-yl acetate, p-toluidine, 2-heptanone, and 3 unsaturated ketones.

Hamsters have been studied extensively in this manner and the primary constituent found has been dimethyl disulfide which alone appeared to trigger sexual activity in the male (Singer et al. 1976).

In vaginal secretions of bitches the primary constituent indicative of estrus was methyl p-hydroxy benzoate which was found to attract males of the species (Goodwin, et al. 1979).

In the monkey, Michael and associates did some of the early vaginal secretion work. They found the active constituents of the receptive female to be acetic,
propionic, iso-butyric, butyric, iso-valeric and iso-caproic acids. A mixture of these compounds from the laboratory shelf when applied to a non-estrous female triggered mating behavior in the male. (Michael et al., 1971). Subsequently Curtis et al. (1973) found, that p-hydroxy-phenyl-propionic acid was another primary stimulator of the males. When applied to non-estrous females mating attempts increased from 6 to 52 attempts during 12 trials of 1 hour each.

In humans, results were very similar to those found in the monkey. Chemical assay differed mainly in higher levels of acetic and propionic acids during the follicular phase (Michael et al., 1974).

In the work of both Michael et al. (1971) in the rhesus monkey and Schwende et al. (1984) in the mouse it was found that the volatile compounds were actually present during non-estrus. However, the concentrations were "elevated" during estrus. Schwende computed the areas of the peaks through integration and then did a quantitative statistical analysis to determine statistical significance of the peaks (Schwende et al., 1984).

Identification of pheromonal compounds and their functions is accelerating as more research is done in the area. Consider the overview (TABLE 1) of results of
### TABLE 1. RESULTS OF PHEROMONE RESEARCH IN VARIOUS SPECIES

<table>
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<tr>
<th>Species</th>
<th>Function</th>
<th>Chemical Compound</th>
<th>Reference</th>
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<tbody>
<tr>
<td>Black-tailed Deer</td>
<td>Attract Females</td>
<td>Cis-4-hydroxy dodec-6-enoic acid lactone</td>
<td>Brownlee et al. 1968</td>
</tr>
<tr>
<td>Dog</td>
<td>Stimulate mounting by males</td>
<td>Para-methyl hydroxybenzoate</td>
<td>Goodwin et al. 1979</td>
</tr>
<tr>
<td>Domestic Boar</td>
<td>Stimulate lordosis in females</td>
<td>3-hydroxy-5-androst-16-ene</td>
<td>Patterson 1968</td>
</tr>
<tr>
<td>Golden Hamster</td>
<td>Stimulates sexual behavior of males</td>
<td>Dimethyl disulfide</td>
<td>Singer et al. 1976</td>
</tr>
<tr>
<td>Mongolian Gerbil</td>
<td>Stimulates investigation and marking</td>
<td>Phenylacetic acid</td>
<td>Thiessen et al. 1974</td>
</tr>
<tr>
<td>Mouse</td>
<td>Attracts males and stimulates sexual behavior</td>
<td>n-pentyl acetate cis-2-penten-1-yl acetate</td>
<td>Schwende et al. 1984</td>
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<tr>
<td></td>
<td></td>
<td>p-toluidine 2-heptanone t-5-heptene-2-one t-4-heptene-2-one 3-heptene-2-one</td>
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<tr>
<td>Pronghorn</td>
<td>Attracts females</td>
<td>Isovaleric acid</td>
<td>Müller-Schwarze et al. 1974</td>
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<tr>
<td>Rhesus Monkey</td>
<td>Stimulates male sexual behavior</td>
<td>Acetic acid</td>
<td>Michael et al. 1971</td>
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<td>Propionic acid</td>
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<td>Isocaproic acid</td>
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<td></td>
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<td>p-hydroxy-phenyl-propionic acid</td>
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<td>Women</td>
<td>Unknown</td>
<td>Similar to Rhesus Monkey but more</td>
<td>Michael et al. 1974</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Acetic acid and Propionic acids</td>
<td></td>
</tr>
</tbody>
</table>
pheromone research in various species.

Upon considering the structures of the pheromones identified in the various species, few similarities are found. Within species there are similarities. For example, in the mouse there are 4 seven carbon ketones as well as 2 variations of pentyl acetate. This is in contrast to the dimethyl disulfide found in the hamster. The most common interspecies relationship is the small volatile fatty acids common to the primates and the pronghorn.

Little has been reported concerning origin and method of production of the various pheromones. It has been shown that the vaginal microflora may play a part in the production of the volatile fatty acid pheromones of primates (Michael et al., 1972). Later, Michael and Bonsall (1977) found that a collection of many types of naturally occurring bacteria from the vagina are required in order to produce the pheromones.

It is known that various reactions occur to alter the ambient environment of the vagina during the follicular phase. For example, in many species the superficial cells of the vaginal epithelium undergo cornification (Benson, 1976; Nalbandov, 1976) and glycogen storage increases in the cells of the epithelium (Benson, 1976). In most species, the pH of the vagina drops (Nalbandov, 1976). Many species experience a rise in the basal body temperature at ovulation (Johnson and Ulberg, 1965). In
bovine the amount of cervico-vaginal mucous increases dramatically during estrus and undergoes significant changes (Johnson and Ulberg, 1965; White, 1972). All of these and other changes in vaginal conditions may in fact combine to induce the vaginal microflora to increase the production of pheromones.

Though pheromones in primates may have bacterial origins in the vagina, this does not explain all odor differences during estrus in other species. In the cow it was noted that dogs could detect a difference in odor of cows during estrus when presented with several body fluids, including blood, milk, naturally voided urine as well as urine voided via catheter (Kiddy et al., 1984). In these cases, it is not likely that the pheromones are produced by bacteria.

Bovine Signalling Pheromones

Research into existence and application of pheromones in cattle has been slow, in spite of the fact that pheromones could be of some interest to the dairy and beef industries due to the possible economic impact. One reason for the relatively small volume of research could be the confusion of early results. The first reported studies relating to bovine pheromones were performed by Hart and associates in 1946. Taking the first step, they applied vaginal mucous from estrus cows to the genital membranes of
non-estrus cows. According to their findings, the bull was attracted to the cow through olfaction. Since that time it has been reported that pheromones, at best, are secondary in the attraction of bulls. Almquist and Hale (1956) based this decision on the observation that when collecting semen, bulls respond equally well to teaser bulls, steers, or cows. One must bear in mind, however, that these bulls were conditioned to seek the reward of sexual experience regardless of sex or status of the animal being mounted. Therefore, conclusions concerning pheromones based on these observation may be misleading. Further investigation revealed that, when blindfolded, bulls are less likely to identify a cow in estrus (Hale, 1966). This observation strengthened the earlier finding that pheromones may not be the primary means of communicating sexual receptivity.

Still, the role of pheromones can not be completely excused. In 1967 Donovan showed that fecal and vaginal secretions from cows in estrus attract bulls. It was further demonstrated that urine from estrous cows, when applied to non-estrous cows will evoke sexual interest from bulls (Sambraus and Waring, 1975). In later work cervico-vaginal mucous from estrous cows was placed on glass dishes and presented to bulls. The bulls responded with a great deal of interest by sniffing and producing the flehmen reaction. When the mucous was placed on a dummy, the bulls made attempts to mount and ejaculate. No such
reactions occurred when using mucous from di-estrous cows (Paleologou, 1977).

A recent study performed by Blaschke and associates (1984) investigated the roles of vision, olfaction, and audition in aiding a bull in identifying estrous cows. Conclusions suggest that sight is primary in identifying the cow in estrus. Olfaction and tactile stimuli then serve to stimulate the libido of the bull. Audition was found to have very little effect in the bull's ability to identify the receptive cows.

Further research has demonstrated that estrous cows definitely emit an odor which is not present during non-estrus. Furthermore, the odor is consistent from cow to cow. In 1978 Kiddy et al. found that trained dogs could pick out samples of vaginal mucous and urine from estrous cows when presented along side non-estrous samples. They went on to find that the dogs could actually pick out the estrous cows. Like the dog, it was found that operant conditioned rats could also detect estrous cows through olfaction (Ladewig and Hart, 1981). Later, using the same methods, it was shown that dogs could differentiate between estrous and non-estrous samples when presented with the milk (Hawk et al., 1984; Kiddy et al., 1984), vulva-vestibule swabs, vaginal mucous, naturally voided urine, bladder urine, or blood plasma (Kiddy et al., 1984).

Some investigation has been done concerning the timing
at which the odor becomes present in relation to the
estrous cycle. Using dogs to test the odors of vaginal
fluids it was found that a distinct odor existed during
estrus. On days 4 and 5 before estrus the dogs did not
respond. On days 3 to 1 before estrus the dogs began to
respond until at estrus the response was very consistent.
Then on day 1 post estrus the odor appeared to be
undetectable (Kiddy and Mitchell, 1981). In an effort to
use bulls' flehmen reactions to identify the timing of
pheromones, it was determined that there was an increase in
repeated reactions from about day 2 pre-estrus up through
estrus. By day 1 post estrus the number of repeated
flehemens was dramatically reduced and by day 2 post estrus
the responses were comparable to the rest of the di-estrus
period (Hradecky et al., 1983).

At present there has been no report of the solvent in
which the bovine pheromones are soluble nor has there been
any report of an attempt to identify the compounds. The
author is aware, however of a few unpublished and
unsuccessful attempts to accomplish these objectives. In
1980 Klemm and associates performed a study in which they
examined the vomeronasal cavity of bulls and gathered data
concerning means of pheromone sensation. Mentioned in the
study was the eventual goal of determining the chemical
constitution of the pheromones.
Current Pheromone Theory

Recent theory in the field of pheromones suggests that pheromones are not as simple as previously believed (Albone, 1984). In fact, mammalian pheromones are very complicated in comparison to insect pheromones (Duvall et al., 1986). The most recent school of thought supports the hypothesis that mammals key on an "Odor Image" (Albone, 1984). That is, rather than one or a few compounds playing a role in chemical signalling, perhaps dozens of compounds work to exact an effect. According to Crump and co-workers (1984) it was found that by chromatographically separating samples from black-tailed deer, the biological activity was lost before fractions were narrowed down to single compound entities. This suggests that the compounds must be present together in order to produce the overall odor image.

Semiochemistry has advanced to the point that three possible classifications of pheromones in mammals are suggested according to their interactions with co-constituents (Müller-Schwarze et al. 1986). The first possibility is that the compound is "inhibitory". That is, "The total effect is less than the sum of the component effects" (Müller-Schwarze et al., 1986). If an absolute meter could be used to measure the response of an animal to an odor stimulus, separate constituents of a sample might be presented to an individual for bio-assay, and the response recorded. If then the responses from all the
separated constituents were added together and compared to the response from a bioassay using all the compounds in the same sample, the complete sample effect would be less than the sum of the responses to the separated constituents.

The second approach is that the compound might be "additive". In this case "The total effect is the sum of all the component effects" (Müller-Schwarze et al., 1986). Thus, after recording and adding together all the responses of a subject to separate components of a sample, the outcome would equal the response if the subject were presented with a complete and intact sample.

Finally, it is suggested that a sample component might be "synergistic". That is, "The total effect is greater than the sum of the component effects" (Müller-Schwarze et al., 1986). An extreme case of this is called "Gestalt" and occurs when removal of any one component from a sample results in total loss of all biological activity (Müller-Schwarze et al., 1986). In the case of synergism of constituents, a single constituent may be presented for bio-assay and a response obtained. This response, however, would be mild compared to the response elicited by the intact sample.

Synthesis of an odor image may be extremely difficult, as compounds which appear in very low concentration may have an overwhelming effect on the overall odor (Müller-Schwarze et al., 1986). Thus, identification of
every pheromonal compound may be necessary in order to accomplish this task. However, the harnessing of pheromone potential may not require synthesis of the odor image. Elucidation of only a few significant compounds may prove to be a powerful tool in understanding and utilizing the potential of pheromones.

Literature Summary

The role of pheromones could have a strong economical impact on animal agriculture. Priming pheromones could serve to accelerate maturity, better synchronize estrus, and decrease the period between parturition and resumption of estrous activity. This study, however, deals with bovine signalling pheromones. Some confidence can be maintained that they do exist. The focus of this study is to identify one or more compounds which undergo a change in concentration during estrus as opposed to non-estrus. The economic potential to the agricultural industry could eventually be great as it could lead to a means by which detection of estrus might be more accurately performed.
MATERIALS AND METHODS

Eleven registered holsteins from the Caine Research Dairy at Utah State University were used in this study. All cows were in a normal estrous cycle. Urine samples were taken from cows diagnosed in standing estrus. Each cow was used as its own control by again taking a sample from that same cow 7 days after the onset of estrus. This timing was decided upon in order to allow the corpus luteum to more fully mature but to avoid the surge in estrogen often present at day 10 post estrus.

Upon selection of a cow, a mid stream sample of naturally voided urine was collected into a 1 liter graduated cylinder. Urination was stimulated by gently massaging the vulva and perineal area along with application of a fine mist of warm water on the same area. The mist of water also served to clean fecal material and other debris from the exterior of the vulva. Once collected, the sample was measured and transferred to a clean 1000 ml flask with a ground glass stopper. The urine was permitted to cool to room temperature. Five hundred ml of the urine was placed in a beaker and the rest was discarded.

In order for any short chain volatile fatty acids to be extracted by the ether they would have to be in their undissociated form. This was achieved by lowering the pH of the urine from an average of 8.0 to 1.5. The formula to
determine ideal pH was based on acetic acid which has a $K_a$ of $1.8 \times 10^{-5}$ and is the strongest of the short chain volatile fatty acids and therefore the least likely to be in its undissociated form. The determination was based on 99.9% of the acetic acid becoming undissociated. The urine was titrated to a pH of 1.5 using concentrated sulfuric acid.

Prior to titration, a small drop of octyl alcohol was added to the urine to control the foaming caused by the release of carbon dioxide when the sulfuric acid reacted with the sodium bicarbonate naturally found in the urine of many ruminants.

Once the pH was lowered the process of extraction began. The acidified urine was poured into a 500 ml separatory funnel and 50 ml of anhydrous ethyl ether was added. After adding the ether a magnetic stirring bar was used to mix the solution while it was gently swirled for five minutes. The funnel was vented as needed thus causing the loss of some of the ether. After swirling, the solution was allowed to sit for a minute and then the urine was separated from the remaining ether. The urine was then poured back into the funnel and 25 ml of ether was added. Again the solution was swirled for 5 minutes and separated. Four more washings with 25 ml of ether per washing were performed and then the urine was discarded. All of the ether extracts were placed in a 100 ml flask with a ground
glass stopper and stored at 6°C. By injecting samples into the gas chromatograph, it was determined that the concentration of the sixth washing was very low when compared to the first washing. Thus, confidence can be maintained that the extraction procedure was efficient.

Upon removal from storage, the flasks were uncapped and the solution was concentrated by blowing a light stream of air through a pasteur pipette onto the surface of the extract until the volume was such that it fit easily into a 25 ml test tube. By evaporating the solution in this manner the temperature dropped to 0°C. The advantage of concentration by this method as opposed to warming the solution is that due to the very low temperature, the compounds of interest are less likely to be lost by evaporation. The solution was transferred to a test tube and any precipitate was washed into the test tube with three washings of 4 ml of ether each. The air stream was again applied to the surface of the ether solution and the volume was allowed to drop sufficiently to fit into a 10 ml graduated cylinder. As the volume decreased, a precipitate often resulted. The solution was centrifuged at 5,000 rpm for five minutes and the liquid phase funneled into a 10 ml graduated cylinder. Once again the air stream was applied to the solution. The precipitate remaining in the test tube was washed again with 4 ml of ether and centrifuged. The ether was added to the cylinder and two more washings were
performed in like manner. After all washings were collected in the graduated cylinder, the volume was lowered by the evaporative method mentioned above to about 6.5 ml and the solution transferred to a small screw top vial.

Two chromatograms were obtained on each sample using a Hewlett Packard 5890 gas chromatograph connected to a Hewlett Packard 3850 integrator by the 1 V cable. Flow rate was 20 ml per minute using helium as the carrier gas. The oven was temperature programmed to start at 70° C and at 5 minutes began to climb at a rate of 5° C per minute until it reached 150° C where it stayed until 45 minutes post injection. At 45 minutes the temperature climbed immediately to 190° C. Both the injection port and the detector were maintained at 200° C. A 6 foot long glass column with a 1/4 inch outer diameter and a 2 mm inner diameter was used. Packing material was Graphpac-GC® and the liquid phase was phosphoric Acid and Carbowax®. Mesh was 60/80 and percent load 0.3 and 0.1. Detection was accomplished with a flame ionization detector. For each sample, 3 μl were injected using a 5 μl syringe which had been kept on ice in order to facilitate aspiration of the ether solution.

Once the chromatogram was run, data from the integrated area of the peaks was corrected for variability in processing using the following formula:

\[ A_C = \frac{A_O}{(6.5/f)} \]
Given that:

\[ A_C = \text{The corrected area} \]
\[ A_0 = \text{the actual integrated area} \]
\[ f = \text{final volume of extract after concentration} \]
\[ 6.5 = \text{standard final extract volume after concentration} \]

The integrated areas of the peaks from the two subsample chromatograms were entered for each cow and each treatment into the spreadsheet and averaged. Each peak was inspected by analysis of variance using the average of the subsamples to determine any differences between estrous and non-estrous groups. The Random Block Design was used as the statistical model (Dowdy and Wearden, 1985). Use of the random block design is indicated when an extraneous source of variability is to be controlled. When using different treatments of animals, this can best be done by sampling the animal under treatment and then sampling the same animal without treatment. The animal is then considered a "block". In this case the inter-cow variability was eliminated from the analysis by making the cows act as blocks by sampling the same cow at estrus and again during di-estrus. Examples of inter-cow variability can be seen in figures 2, 3, 4, 5 and 6.

Due to baseline drift and electronic noise some peaks were lost or distorted, resulting in missing data. The random block design requires that there be no missing data.
Therefore, on those peaks where both subsamples from the same cow were unusable, resulting in a missing data point, the block (cow) was removed from both treatments of the analysis. If only one subsample was missing, the intact subsample was used to replace the missing data.

In addition to an analysis of the peaks, secondary data were also analyzed for statistical variance between groups. This included the original pH, the amount of acid required in the titration, and specific gravity.

Care was taken in identification of peaks. Out of a possible 40 peaks, only 22 peaks were analyzed. Though attempts were made to analyze the others, it was found that they were often either too small to be accurately measured, or they were not well enough separated from the other peaks to ensure proper identification and quantification, and were therefore discarded. It is recognized that exclusion of a peak from analysis does not suggest that the compound is not significant. The peaks that were used were clearly separated from all other peaks and had distinct shapes and retention times so as to allow confident identification. Retention times of each peak were ranked in chronological order of each run. These were then graphed and regressed against the order in which they were run (See figure 1). In most cases the peak fit a linear model with the $R^2$ values ranging from 1.5% to 98.1%. In each case, those observations which exceeded 2 standard deviations from the
Figure 1. Example of use of regression to identify peaks. Note: This graph represents the actual recorded retention times (Y axis) against the sequence in which the samples were run (X axis). Notice how this compound came off a little earlier with each run. The average retention time for this peak was 31.8 minutes. Peaks 1 and 6 are greater than 2 standard deviations from the expected mean according the regression line. Therefore they are considered unusual observations and were double checked to assure that they did correspond with this compound.
expected mean at any point were inspected more closely to insure that the correct peak had been used. The measure of 2 standard deviations was used because of it's standard meaning in statistics. The slope of the line, caused by column fatigue, varied from -.118 to .041.

Once a peak was identified as being statistically different in concentration between groups, the chemical identity of the compound creating the peak was determined. This was accomplished by coinjection of dilute standards along with the various samples to show that additional peaks did not appear in the area.

Some peaks appeared in regions where it could not be determined which standard would be appropriate for coinjection. An attempt was made to identify these peaks using an LKB 2091 Gas Chromatograph-Mass Spectrometer; conditions similar to those used in the previously described gas chromatography were employed. Each peak was scanned for mass spectra and the atomic mass recorded.
RESULTS

Several observations were made during the processing of the urine preparatory for gas chromatography. For example, it was noted that upon cooling the urine after collection, a very sweet and pleasant smell comparable to that of spearmint could be detected. Likewise in some samples an acrid odor was noted. No attempt was made to correlate these findings with the treatments.

It was noted that when extracting the urine with ether, the organic extract took on a rich golden color. The odor was foreign to ether and the residue was sticky when the ether was evaporated off. As the number of washings increased this effect was lessened until the final washing appeared clear and nearly colorless.

As the extract was concentrated it became very dark brown. A light sliver-like precipitate fell out during the concentration. A portion of this was reabsorbed in ethyl alcohol and tested on the gas chromatograph. No peaks resulted.

No statistical difference between treatments was found in amounts of acid required for titration, nor in specific gravity. Likewise no statistically significant variation was found in the pH of the urine. The average pH of both groups was 8.24 and ranged from 7.54 to 8.75 except for one estrous cow, the urine of which was at pH = 6.73. This cow
demonstrated the presence of acetic acid in proportions exceeding 5.5 times the average amount. Her specimen also had a large amount of yellow precipitate fall out in the aqueous phase during separation. In every other way she appeared normal.

The chromatographic peaks appeared well separated in all areas except for the interval between 20 and 26 minutes during which separation was poor and some peaks were difficult to identify. Most of the peaks consisting of the volatile fatty acids appeared sharp and narrow and were easily discerned. With progression of time, a drift in retention times occurred (figure 1). With some peaks the drift was as great as 3 minutes. This, however, is a common occurrence due to column fatigue and did not interfere with identification of peaks.

Due to the highly volatile nature of ether, the solvent peak came off very early on the chromatogram and was quite wide, often covering as much as 5 minutes of the output. This, of course, obscured any urinary constituents which might have come off during that time.

Though most variability resulted between cows, there was some variation between subsamples of the same cow and treatment (see figures 2,3,4,5,and 6). This is most likely due to inaccuracy of the detector and error in injection amount. For this reason two sub-samples were used in order to help eliminate error.
Figure 2. Graph of concentration of propionic acid in all samples analyzed. Y axis is concentration (area under the peak) in pico amps/second. X axis is each subsample used.

Figure 3. Graph of concentration of iso-caproic acid in all samples analyzed. Y axis is concentration (area under the peak) in pico amps/second. X axis is each subsample used.
Figure 4. Graph of concentration of Compound C in all samples analyzed. Y axis is concentration (area under the peak) in pico amps/second. X axis is each subsample used.

Figure 5. Graph of concentration of Compound E in all samples analyzed. Y axis is concentration (area under the peak) in pico amps/second. X axis is each subsample used.
Figure 6. Graph of concentration of Compound D in all samples analyzed. Y axis is concentration (area under the peak) in pico amps/second. X axis is each subsample used.

After correction for processing error, averages within groups were determined and compared. Table 2 shows the results of the 21 peaks statistically analyzed.

These peaks were analyzed for statistically significant variation between groups using the model mentioned. The results are seen in Table 3.

Based on the values given in Table 3, a statistical difference between treatments has been found at retention times 14.83, 27.27, 31.80, 36.45, and 62.82.

The retention times of 8 volatile fatty acids were determined by injection under conditions similar to those used for chromatography of the urine samples (See Table 4.
<table>
<thead>
<tr>
<th>Rt</th>
<th>ID</th>
<th>Estrus Average</th>
<th>Non-Estrus Average</th>
<th>Diff.</th>
</tr>
</thead>
<tbody>
<tr>
<td>9.6</td>
<td>Acetic Acid</td>
<td>925800</td>
<td>651748</td>
<td>274052</td>
</tr>
<tr>
<td>14.8</td>
<td>Propionic Acid</td>
<td>156469</td>
<td>189519</td>
<td>-33049</td>
</tr>
<tr>
<td>18.0</td>
<td>Iso-Butyric Acid</td>
<td>86778</td>
<td>77923</td>
<td>8855</td>
</tr>
<tr>
<td>19.8</td>
<td>Butyric Acid</td>
<td>535417</td>
<td>610189</td>
<td>-74771</td>
</tr>
<tr>
<td>21.9</td>
<td></td>
<td>91877</td>
<td>87720</td>
<td>4156</td>
</tr>
<tr>
<td>22.5</td>
<td></td>
<td>98207</td>
<td>150500</td>
<td>-52292</td>
</tr>
<tr>
<td>23.5</td>
<td></td>
<td>207330</td>
<td>189389</td>
<td>17941</td>
</tr>
<tr>
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<td>Iso-Valeric Acid</td>
<td>113997</td>
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</tr>
<tr>
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<td>Valeric Acid</td>
<td>289084</td>
<td>322736</td>
<td>-33651</td>
</tr>
<tr>
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<td>Compound C</td>
<td>46136</td>
<td>92267</td>
<td>-46131</td>
</tr>
<tr>
<td>28.9</td>
<td></td>
<td>1491251</td>
<td>53601</td>
<td>1437650</td>
</tr>
<tr>
<td>31.8</td>
<td>Compound D</td>
<td>414301</td>
<td>337385</td>
<td>76915</td>
</tr>
<tr>
<td>33.4</td>
<td></td>
<td>249626</td>
<td>264153</td>
<td>-14526</td>
</tr>
<tr>
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<td>Iso-Caproic Acid</td>
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<td>529251</td>
<td>-153439</td>
</tr>
<tr>
<td>39.9</td>
<td>Caproic Acid</td>
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<td>-64659</td>
</tr>
<tr>
<td>46.4</td>
<td></td>
<td>4753399</td>
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<tr>
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<td></td>
<td>5775295</td>
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<td>557571</td>
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<td>Compound E</td>
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<td>-694965</td>
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<td>71.1</td>
<td></td>
<td>2410588</td>
<td>2741647</td>
<td>-331058</td>
</tr>
</tbody>
</table>

Note: The identification of those peaks determined by coinjection are given. A diamond (♦) is shown beside those peaks which are statistically significantly different. The values are given in units of pico amps/second as recorded by the automatic integrator. A negative difference indicates that the average for the non-estrus was higher than the average for the estrus at the indicated retention time.
TABLE 3. STATISTICAL RESULTS OF ALL PEAKS ANALYZED FOR VARIANCE BETWEEN ESTRUS AND DI-ESTRUS

<table>
<thead>
<tr>
<th>Rt(^a)</th>
<th>ID</th>
<th>&quot;F&quot;</th>
<th>df(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>9.6</td>
<td>Acetic Acid</td>
<td>1.11</td>
<td>07</td>
</tr>
<tr>
<td>14.8</td>
<td>Propionic Acid</td>
<td>6.75*</td>
<td>10</td>
</tr>
<tr>
<td>18.0</td>
<td>Iso-Butyric Acid</td>
<td>0.00</td>
<td>10</td>
</tr>
<tr>
<td>19.8</td>
<td>Butyric Acid</td>
<td>2.68</td>
<td>10</td>
</tr>
<tr>
<td>21.9</td>
<td></td>
<td>0.31</td>
<td>08</td>
</tr>
<tr>
<td>22.5</td>
<td></td>
<td>4.53</td>
<td>06</td>
</tr>
<tr>
<td>23.5</td>
<td></td>
<td>0.30</td>
<td>07</td>
</tr>
<tr>
<td>24.8</td>
<td>Iso-Valeric Acid</td>
<td>2.16</td>
<td>08</td>
</tr>
<tr>
<td>25.6</td>
<td>Valeric Acid</td>
<td>1.08</td>
<td>07</td>
</tr>
<tr>
<td>27.3</td>
<td>Compound C</td>
<td>6.48*</td>
<td>08</td>
</tr>
<tr>
<td>28.9</td>
<td></td>
<td>0.98</td>
<td>08</td>
</tr>
<tr>
<td>31.8</td>
<td>Compound D</td>
<td>9.04**</td>
<td>10</td>
</tr>
<tr>
<td>33.4</td>
<td></td>
<td>0.08</td>
<td>10</td>
</tr>
<tr>
<td>36.5</td>
<td>Iso-Caproic Acid</td>
<td>9.44**</td>
<td>09</td>
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<tr>
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<td>46.4</td>
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<td>08</td>
</tr>
<tr>
<td>55.5</td>
<td></td>
<td>0.17</td>
<td>10</td>
</tr>
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<td>58.7</td>
<td></td>
<td>2.75</td>
<td>09</td>
</tr>
<tr>
<td>62.8</td>
<td>Compound E</td>
<td>16.07****</td>
<td>10</td>
</tr>
<tr>
<td>71.1</td>
<td></td>
<td>1.80</td>
<td>08</td>
</tr>
</tbody>
</table>

Level at which statistically significant

* .050
** .025
*** .010
**** .005

NOTE: \(^a\) Rt indicates the retention time. \(^b\) df refers to degrees of freedom. Those compounds marked with a diamond (♦) were found to have statistically significant differences in concentration at the indicated levels.
TABLE 4. RETENTION TIMES OF VOLATILE FATTY ACID STANDARDS

<table>
<thead>
<tr>
<th>Acid</th>
<th>Retention Time (min)</th>
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<td>Iso-valeric acid</td>
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<tr>
<td>Iso-caproic acid</td>
<td>36.4</td>
</tr>
<tr>
<td>Caproic acid</td>
<td>39.9</td>
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</tbody>
</table>

a Retention time given in minutes post-injection.

and figure 7). Based on these retention times and by co-injecting the standards with actual samples, it was determined that two of the statistically significant peaks correspond to volatile fatty acids (figure 7), including,

14.83 - Propionic Acid

and

36.45 - Iso-Caproic acid.

Because the compounds eluted at retention times 27.27, 31.80 and 62.82 were not among these eight volatile fatty acids, an attempt was made to determine their identities by atomic mass spectrometry. After repeated trials it was determined that these compounds appear in such low concentrations that their identity could not be determined using conventional methods. For clarity, these compounds will be referred to as Compound C, Compound D and Compound E throughout the rest of this treatise with retention times of 27.3, 31.8 and 62.8, respectively.
Figure 7. Sample chromatogram compared to standards. Note: Peaks b, g, i, j and k are statistically significant. At 27 minutes the baseline rises due to change in attenuation of the integrator. At 45 minutes attenuation is again changed and the chart speed is reduced from .5 Cm/minute to .2 Cm/minute.

<table>
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<tr>
<th>Peak</th>
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<th>Compound</th>
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<th>Compound</th>
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</tr>
<tr>
<td>* b</td>
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<td>Propionic Acid</td>
<td>h</td>
<td>39.91</td>
<td>Caproic Acid</td>
</tr>
<tr>
<td>c</td>
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<td>* i</td>
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<td>d</td>
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<td>* j</td>
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<tr>
<td>e</td>
<td>23.49</td>
<td>Iso-Valeric Acid</td>
<td>* k</td>
<td>62.82</td>
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</tr>
<tr>
<td>f</td>
<td>23.55</td>
<td>Valeric Acid</td>
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</tr>
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</table>
It should be noted that in the cases where a statistically significant difference was found, the estrus cows did not always represent the group with the higher levels of the compound identified (Table 2). In fact it was found that propionic acid, iso-caproic acid, Compound C and Compound E were present in greater amounts in the samples collected 7 days post estrus. Only Compound D at retention time 31.80 was found to be at statistically significant higher levels during estrus.

In addition to the eleven cows used in the study, 2 cows were sampled which had been injected with 500 µg cloprostenol\(^1\) (synthetic prostaglandin) 3 to 4 days prior to the observation of estrus. Upon comparison of the means of chromatographic peaks of these cows to the means of those cows sampled under a normal estrus cycle, it was determined that several peaks appeared inconsistent with the norm (Table 5). Based on these preliminary findings it appears that treatment with prostaglandin may affect the concentration gradient established under an uninterrupted cycle. Further research, however, is warranted before final conclusions can be made.

\(^1\) - Estrumate\(^\text{®}\), Haver, BayVet Division, Miles Laboratories, Inc., Shawnee, KS 66201
<table>
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<tr>
<th>Rt</th>
<th>ID</th>
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<th>Estrus Non-Treated</th>
<th>Di-Estrus Treated</th>
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DISCUSSION

Up to the present time, research in bovine signalling pheromones has been focused in the area of the bio-assay. It has been shown that dogs can detect a variation in odor in the cow during estrus by means of vaginal swabs (Kiddy et al., 1978), milk (Hawk et al., 1984; Kiddy et al., 1984), blood or urine, naturally voided or through catheter (Kiddy et al., 1984). Studies have shown that bulls respond to estrous odors (Hart et al., 1946; Donovan, 1967; Sambraus and Waring, 1975; Paleologou, 1977; Blaschke et al., 1984; Hradecky et al., 1983). Little doubt can yet be maintained that there is a difference in odor during the estrous cycle. The next step in understanding and utilizing the potential of these odorous variations is to identify the compounds involved.

Based on the results of this study, five peaks have been identified as being statistically different between estrus and non-estrus. Two of these peaks represent propionic acid and iso-caproic acid. The remaining peaks are not yet identified. This, however, does not negate the valid estrus-state-dependent differences in concentration of these unidentified peaks. Though the concentration of these compounds was too low for the mass spectrometer, one must realize that the detector for the mass spectrometer is less sensitive than the flame ionization detector used to determine the relative concentrations. Thus, it can yet be
maintained that Compound C, Compound D and Compound E do undergo changes in concentration based on stage of the estrous cycle.

Due to the fact that all 5 of these compounds experience changes in concentration based on the status of the estrous cycle, it might be suggested that they are pheromonal in nature. However, no study has been made to determine if such is the case. It is assumed that pheromones undergo a change in concentration during estrus in order to signal receptivity, as is the case in the mouse (Schwend et al., 1984), rhesus monkey (Michael et al., 1971) and other species (see Table 1). Therefore, based on the fact that the compounds identified in this study undergo changes similar to pheromones in other species, it is suggested that these compounds may well be pheromonal in nature.

The fact that 4 of these compounds were found to be lower in concentration during estrus and higher in concentration during di-estrus is of great interest as this phenomenon has not here-to-fore been reported to occur in any other species. Though this change in concentration was not expected, it is not without explanation or reason. Four possible explanations are given herein, all of which have a biologically predicated basis.

First of all is the possibility that these compounds signal unreceptivity. That is, they may act to repel the
bull during unreceptive periods, rather than as an attractant during estrus. Though only attractants have been previously reported in a sexual signalling situation, a sexual repellant could still be considered a pheromone. As mentioned previously, pheromones are known to act as deterrents outside of the sexual context. Mature dogs, for example, produce an odor from the anal sacs which serve to repel juvenile conspecifics (Donovan, 1967). Though the situations differ, this demonstrates that the neuroendocrine pathways can exist for such a phenomenon to occur.

A second plausible explanation for the low levels of these compounds during estrus could be that they may be precursors to pheromones or other compounds produced at elevated levels during estrus. Thus, these compounds would be depleted faster during estrus than during the non-estrus period. If such is the case, these compounds may or may not have pheromonal properties.

A third explanation of this phenomenon is supported by the theory of odor imaging (Albone, 1984). As stated, this theory suggests that many different compounds combine to develop a distinct odor. In such a case, slight changes in any one compound may serve to alter the odor sufficiently to cue the receptive animal to the state of estrus of the sender. One must be aware, that when one of these compounds decreases in concentration, not only is it no longer as
easily detected, but the odors of other compounds may become more apparent as they may be increasing in concentration relative to these components. Those odors that are no longer masked may serve to evoke a response. In this case, as propionic acid, iso-caproic acid, Compound C and Compound E decrease and Compound D increases during estrus, the overall odor of the urine may change in such a way as to signal the estrous state to the recipient.

A fourth explanation of the decrease in concentration could be that these compounds may actually be by-products of bio-chemical processes which take place under non-estrus conditions. Perhaps the higher level of progesterone during non-estrus causes the release of these compounds through unknown pathways. Once again, if such is the case, then these compounds may or may not be pheromonal in nature.

In light of the unknown origins of these compounds as well as most pheromones, the nature of pheromones bears discussing. A question yet to be answered is whether or not reactions to sexual signalling pheromones are learned responses. Certainly if the response is learned then pheromones may in fact be nothing more than an incidental by-product or precursor in other genetically programmed bio-chemical pathways. Some data supports the theory that a bull learns to cue on smells. In his study to determine the role of olfaction in identifying estrous cows, Blaschke et al. (1984) found that mature bulls, accustomed to
association with cows were more inclined to detect estrus through odors than immature bulls or bulls raised separate from the herd. This suggests a learned response.

However, an inherent ability to detect pheromones can not be entirely dismissed. It has been observed that immature and unexperienced bulls will follow a cow closely for 2 to 3 days before she reaches standing estrus (personal observation). As mentioned previously, this coincides with possible timing of the presence of pheromones in cattle. If these young bulls have not been in situations where they might be conditioned to notice odor changes, and if they are, in fact, cuing on pheromones then the ability to react to the odor stimulus is not a learned response and the pathway of origin of the pheromone may be more complex.

It is interesting that two of the statistically significant compounds found in this study are short chain volatile fatty acids. This perhaps strengthens the argument that they may be pheromonal due to the relationship with the pheromones found in other species. Not only have short chain volatile fatty acids been shown to be pheromones in primates (Michael et al., 1971), but iso-valeric acid specifically was shown to have pheromonal characteristics in the pronghorn antelope, another ruminant (Müller-Schwarze et al. 1974).
Due to concern that the concentration of the urine at the time of collection might have a bearing on the outcome, the specific gravity of each sample was determined and analyzed for significant variance between groups. No difference was found. Concern continued, however, because of low confidence in the use of specific gravity as a measure of concentration. The matter is now settled due to the fact that of the 5 compounds found in this study, 4 are found to appear at low concentrations during estrus and 1 compound is elevated during estrus. If urine concentration had exerted an effect on the final outcome, then the effect would have been all in one direction. That is, any variability in concentration of any compound would have been all lower at estrus or all higher at estrus, but not both.
CONCLUDING REMARKS

That propionic acid, iso-caproic acid and Compounds C, D and E are pheromones has yet to be determined. Still, the results of this study offer significant additions to the field of animal agriculture. If these compounds are pheromonal, then the first step may have now been taken to launch the progression of bovine pheromone understanding. If, however, these compounds are not pheromonal, they may still eventually serve to aid in detection of estrus in an everyday situation. Possibly of greater importance is the fact that further understanding into reproductive processes may be gained through the accomplishments of this study.


Donovan, A. C. 1967. Some clinical observations on sexual


Lindsay, D. R. 1965. The importance of olfactory stimuli in the mating behavior of the ram. Animal Behav. 13:75-78.


