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I am submitting herewith a thesis written by Thomas Eugene Bianconi entitled "Change in Concentration of Volatile Fatty Acids in Urine and Vaginal Secretions of Ovariectomized Heifers During Artificially Induced Estrus." I recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Animal Science.

R L Murphree

R. L. Murphree, Major Professor

We have read this thesis
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A A Chamberlain

Robert R. Shrode

Accepted for the Council:

L Evans Rich

Vice Chancellor
Graduate Studies and Research

CHANGE IN CONCENTRATION OF VOLATILE FATTY ACIDS IN URINE AND VAGINAL SECRETIONS OF OVARIECTOMIZED HEIFERS DURING ARTIFICALLY INDUCED ESTRUS

A Thesis

Presented for the

Master of Science

Degree

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ABSTRACT

Physiological estrus was induced in two ovariectomized heifers (a Hereford and an Angus) by sequential daily injections of progesterone and estradiol. Vaginal mucus and urine were collected daily from both females. A method for extraction of the short chain fatty acids and removal of substances which interfered with chromatographic analysis was developed. The concentrations of acetic, propionic, butyric, isovaleric, and valeric acids in urine and mucus were determined.

Concentrations of all acids were higher in urine than in mucus in both animals. The concentrations in both urine and mucus were higher in the Angus female than in the Hereford female.

Bulls exposed to an ether extract of urine collected from the Angus female one day after injection of estrogen exhibited sexual excitation. The males were not stimulated by urine extracts from the Hereford female.

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CHAPTER I

INTRODUCTION

It has been known for some time that an estrous female emits a substance which attracts and provokes the mating response in her male counterpart. It has been recognized also by beekeepers that when one bee becomes provoked to the point of stinging, the entire hive seems to erupt, with menacing workers ready to sting the intruder. These seemingly unrelated phenomena have in common the fact that one individual exerts an influence over another. It is now known that such actions are provoked by chemicals excreted by many animals. Such chemicals are called pheromones. This study reports preliminary research into what substance(s) cause the male bovine to show an interest in the female of the species. Specific analysis of short-chained fatty acids in urine and vaginal mucus samples taken from ovariectomized heifers given hormone injections was the focal point of this study.

CHAPTER II

LITERATURE REVIEW

The term pheromone was proposed by Karlson and Butenandt (1959) and has since been widely accepted to describe chemical substances involved in animal communication. Jacobson (1972) states that the term pheromone comes from Greek words, pherein, meaning to carry, and horman, meaning to excite or stimulate. Pheromones are chemical agents produced and released to the outside by one member of the species that function to integrate members of the same species by provoking behavioral and physiological responses (Silverman, 1977).

Much work has been done on the effect of pheromones in insects. Olfactory sex pheromones have been found in more than 30 species of moths, butterflies, flies, beetles and cockroaches, and synthetic analogues have become important commercially for insect pest control (Michael and Keverne, 1968).

A substance isolated from the virgin female Japanese beetles has been shown to attract males of the species in field bioassays (Tumlinson, et al., 1977). A trail following pheromone was isolated and identified in the leaf cutting ant by Tumlinson, et al., 1971.

In the mammal, prominence, diversity and frequent occurrence of scent glands in many species suggest a widespread use of chemical communication (Blum, et al., 1970). Most mammalian species possess excellent olfactory capabilities upon which they depend for detecting and recognizing faint traces of scent, according to Gleason and Reynierse (1969). In the absence of male stimulation, juvenile female

stimulation reduces the rate of sexual maturation in female mice (Vandenbergh, 1973). It has been shown also that females maintained in groups show a higher incidence of anestrus or pseudopregnancy than when caged individually; this suggests the possible presence of an inhibitory pheromone in the female. Researchers have found that pseudopregnancy can be prevented by removal of olfactory bulbs from the females, thereby showing the importance of olfaction in pheromone transmission (Michael and Keverne, 1968).

It has been shown also that the male mouse produces a pheromone that both induces and accelerates the attainment of estrus so that estrus is synchromized when the pheromone acts simultaneously on a group of female mice (Bronson, et al., 1968).

The importance of the olfactory system has been clearly shown by many experiments. Female mice which had the olfactory lobes removed demonstrated a significant reduction in rate of ovulation (Zarrow et al., 1970). Many animals rely on olfactory messages and the use of specialized scent glands for territorial marking, self marking and marking of females by males (Michael and Keverne, 1968). Peters and Mech (1974) suggested that olfactory stimulation was involved in the territorial markings of wolf packs.

The marmoset monkey produces an odorous secretion which combined with the urine is applied to all the items of the monkey's environment (Epple, 1973). Michael and Keverne (1968) presented evidence that sexual excitation and activity in male rhesus monkeys is mediated in certain cases by hormone dependent, olfactory-like acting pheromones, possibly of vaginal origin. They demonstrated further that sexual

behavior of rhesus males undergoes rhythmic variations in relation to the menstrual cycles of the female with which they are paired. The results are consistent in that ovariectomized rhesus females treated with estrogen produced substances with pheromonal properties and that these substances increases sexual activity and motivation of males by way of the olfactory sense (Michael, et al., 1971).

Evidence that cervical mucus of cows contains olfactory stimulating properties was demonstrated by Akhlebininskii and Ishutov (1975). Their work was with dogs of the Eastern European Shepherd breed which were tested for their ability to discriminate in a neutral environment between cervical mucus samples and smears taken from the skin of cows. The authors concluded that pheromones exist in the cow and that they reflect critical endocrine patterns during the estrous cycle.

Another team of researchers demonstrated that an olfactory response was caused by a chemical agent produced and released from specific areas in yet another species. Muller-Schwarze (1969) extracted the tarsal scent from the hair tuft of tarsal organs excised from male black tailed deer. He reported that this extract significantly increased the sniff-and-lick response of the tarsal organs of the black tailed deer.

Using chromatographic procedures, Michael et al., (1971) reported that the pheromonal compound in female rhesus monkeys was composed of simple aliphatic acids. Subsequently, Bonsall and Michael (1971) demonstrated that the production of these acids depends upon the bacterial content of the vagina and suggested that the gonadal hormones may regulate acid production by determining the availability of substrate

or by control of vaginal pH. The fact that the acids occur naturally in vaginal secretions and that synthetic mixtures of authentic acids possess equally powerful sex attractant properties, together, provide strong evidence that the attractant properties of estrogen-stimulated vaginal secretions depend at least in part on their acid content. This view is strengthened by the absence of acids, or their presence in low concentrations, in the secretions of ovariectomized, untreated females (Michael and Keverne, 1968).

Michael and Saayman (1968) demonstrated that estrogen administered to ovariectomized female rhesus monkeys produced varying results depending on site of administration. Subcutaneous versus intravaginal routes of administration were examined. Greater enhancement of overall sexual activity of the pair with greater stimulation of the female's invitations occurred with subcutaneous injections. Contrastingly, intravaginal administration gave increased male interest, suggesting a localized effect of estrogen on pheromone or aliphatic acid release.

Curtis et al., (1971), also demonstrated olfactory communication to be present in the rhesus monkey. Vaginal secretions of ovariectomized estrogen treated female rhesus monkeys produced increased mounting activity and ejaculatory behavior in male monkeys. These behavioral effects were blocked in the males when they were temporarily anosmic. They further demonstrated that this "copulin" chemical of the vaginal secretions could be extracted with ether. Due to the low concentrations, gas chromatography and mass spectrometry was used to isolate and identify chemical constituents of the vaginal secretions. They were found to contain aliphatic acids, specifically, acetic,

propionic, isobutyric, butyric and isovaleric acids. Identification of the volatile aliphatic acids as naturally occurring constituents of vaginal secretions in the rhesus monkey and demonstrations that synthetic mixtures of authentic acids possess powerful sex attractant properties provide strong evidence that attractant properties of estrogen-stimulated vaginal secretions depend in part on the aliphatic acid content.

Michael and Keverne (1970) further demonstrated the olfactory response of males to estrogen treated ovariectomized monkeys. No response was shown when males were anosmic, thus demonstrating definite olfactory activity.

Short-chained fatty acids very similar to those found in the vagina of the rhesus monkey were found in vaginal secretions of the human female. These facts suggest a possible hormonal relationship between the short-chained aliphatic acids and the female sex steroids (Waltman et al., 1973; Michael et al., 1974).

Michael et al., (1974) demonstrated in young women that volatile fatty acids are natural constituents of vaginal secretions. They further demonstrated that the concentration of volatile fatty acids increased during the late follicular phase of the menstrual cycle and declined during the luteal phase. Women on oral contraceptives produced smaller amounts of volatile acids and did not show rhythmic changes in acid content during the menstrual cycle. This implies hormone related control of aliphatic acid release. The occurrence of fatty acids in vaginal fluids was similar in monkeys and women. The ratios of the fatty acids were similar for the two species (the rhesus monkey

and women) except for higher levels of acetic and propionic acids in the human samples.

Keith et al.,(1975) reported that systematic differences appeared to exist in the odorous secretions of pre- and post-coital vaginal secretions of the human. They suggested that short-chained aliphatic acids in the vaginal secretions may play a pheromone role in primates including humans.

McClintock (1971) demonstrated that human females closely associated may exhibit menstrual cycle synchronization. The synchronization over a six month period in a group of young women demonstrated the possibility of olfactory stimulation. This study produced statistically significant tendencies towards increased synchronization of menstrual cycles.

Fitz (1975) isolated and identified short chained volatile fatty acids from cervical mucus of estrous and estrogen treated ovariectomized cows. He demonstrated the volatile fatty acids to be acetic, propionic, buturic, valeric and isovaleric acids. The present study was conducted to determine the relationship between cervical mucus and urine concentrations of these fatty acids.

CHAPTER III

EXPERIMENTAL PROCEDURE

Cows from the University of Tennessee, Knoxville, dairy herd and two ovariectomized heifers (one Angus and one Hereford) were the subject animals. The heifers were ovariectomized on June 18, 1975 at approximately 18 months of age.

Mucus was collected from the estrous dairy cows by inserting a gloved hand into the rectum and grasping the cervix. A gentle massaging action expelled mucus into a plastic bag. Samples were then placed in a freezer (-10C) within 15 minutes of collection. These samples were used only in establishing the most satisfactory extraction procedure.

Both mucus and urine were collected from the ovariectomized heifers. During the period of collections the females received daily injections of progesterone followed by injections of estradial benzoate. The injection schedule is shown in Table I. The intent was to simulate progesterone and estrogen changes during the late diestrus and estrous phase of the cycle of intact females.

The estrogen solution was prepared by dissolving 5 mg estradiol benzoate in 1 ml benzyl alcohol and adding 9 ml corn oil. The solution required slight heating to ensure that all hormone was in solution. The progesterone solution was prepared by dissolving 500 mg progesterone in 2 ml benzyl alcohol and adding 18 ml corn oil and heating slightly.

To collect the secretions from the ovariectomized heifers, two super sized "Tampex" tampons were tied together and then inserted

TABLE I

SCHEDULE FOR HORMONE TREATMENT

DAY	TIME	HORMONE[a]	AMOUNT
Day 1	No Treatment		
Day 2	1000 Hrs.	Progesterone	0.5 ml
	2200 Hrs.	Progesterone	0.5 ml
Day 3	1000 Hrs.	Progesterone	0.5 ml
	2200 Hrs.	Progesterone	0.5 ml
Day 4	1000 Hrs.	Progesterone	0.5 ml
	2200 Hrs.	Progesterone	0.5 ml
Day 5	1000 Hrs.	Progesterone	0.5 ml
	2200 Hrs.	Progesterone	0.25 ml
Day 6	1000 Hrs.	Progesterone	0.25 ml
	2200 Hrs.	Progesterone	0.25 ml
Day 7	No Treatment		
Day 8	1000 Hrs.	Estrogen	1.0 ml
	2200 Hrs.	Estrogen	1.0 ml

[a]The hormone concentration of progesterone was 25 mg/ml and the concentration of estrogen was .5 mg/ml

into the vagina of each heifer. KY™ Jelly was used to lubricate the 1 1/2" speculum, vulva, and vagina to aid in insertion (some bleeding was noted early in the collection period). The tampon bundle was replaced with a fresh, clean bundle every six hours throughout the experiment, care being taken to catch any adherant mucus that was withdrawn with the tampon bundle. The tampons were immediately placed in clean, screw-top jars and frozen (-10C).

The tampons were purchased locally, the brand and size were kept constant throughout the experiment. Each tampon was removed from its wrapping and placed in a stainless steel collar for washing. A standard refluxing apparatus was used to wash the tampons. All tampons were washed with deionized water, isopropyl alcohol and ether in that order for 2 hours each. The tampons were left in the stainless steel collars and placed in an oven at 40C for 24 hours. The dry tampons were removed, tied in bundles of two with cotton string (refluxed in the same manner) and placed in clean screw-top jars.

The ovariectomized animals were conditioned to urinate at time of mucus collection, and the urine was collected in screw-top jars. The urine and tampons were frozen within ten minutes of collection.

Urine was collected by use of a urinary (Folley) catheter during one of the collection trials. The heifers were placed in stanchions, catheters inserted, and the hormone regime outlined in Table I administered. The urine was collected in 8000 ml flasks kept cool by ice. The flasks were transferred at six hour intervals to storage at -10C. No urine was collected from the dairy herd cows.

All samples required preparation before analysis by gas chromatography could be performed. This preparation involved refining the samples to extract the substances desired (volatile fatty acids) and quantitating the concentrations.

The extraction process for the urine was as follows. The urine was thawed and 10 ml aliquots of whole urine removed. To the 10 ml sample was added 2 ml of 25% phosphoric acid to deproteninize the sample, and 10 ul of the internal standard (2-ethylbutyric acid .470 g/1 pH 3.6) was then added to the solution. The urine was then spun in a refrigerated centrifuge at 12,000 rpm for 10 minutes and the supernatant decanted. At this point, the samples were frozen for later analysis. When the samples were to be analyzed on the gas chromatograph (G.C.) they were again centrifuged at 12,000 rpm for 10 minutes prior to injection of the supernatant in the G. C.

The urine from the collection sequence in which the heifers were stanchioned and urine collected via catheter required processing also. The urine from each animal for the second, fourth, fifth and ninth days of the collection cycle was processed by placing 100 ml of urine in a separatory flask and 50 ml of anesthesia grade ether added. The flask was sealed and shaken vigorously and allowed to stand for 30 minutes. The ether by-layer was drawn off and 25 ml of mineral oil added; this mixture was agitated and stored at 5C.

Nine different extraction procedures were attempted in an effort to produce mucus samples of consistently high purity so as to allow G. C. analysis. The initial extraction procedure was that used by Fitz (1975). It is described as Extraction Procedure Number 1.

Eight modifications of this process are also listed with the last process (process number 9) being the one used for analysis of the mucus samples in this study. Each modification was replicated several times using mucus collected from dairy cows and by tampons from the ovariectomized heifers.

Extraction Procedure Number 1

The tampon bundle was separated and washed three times with 10 ml of anesthesia grade ether. A glass rod was used to squeeze the ether from the tampons. This process was repeated three times. To the ether wash was added 3 ml of 0.1 N NaOH, enough to raise the pH to approximately 10, and 40 ml of isopropyl alcohol. The solution was spun in a refrigerated centrifuge at 12,000 rpm for 20 minutes. Evaporation to near dryness was accomplished in a water bath using the centrifuge vial with transfer to a small test tube to complete the drying of the last few ml of liquid. Upon attaining dryness, the tubes were sealed with parafilm to await G. C. injection. The samples were prepared for G. C. injection by reacidifying the salt crystals using a 9:1 ether/formic acid solution. One hundred ul of ether/formic acid were injected through the parafilm covering and a second covering was placed over the injection opening. The reacidified solution was agitated vigorously on a vortex mixer before being placed in an ice bath for 15 minutes prior to injection

Extraction Procedure Number 2

Cold separated tampon bundles were twice washed with 20 ml of deionized water. To the wash water was added 10 ul hexanoic acid

(.01%) as an internal standard. Sufficient .01 N NaOH was added to bring pH up to 9.0 (at least 3 ml always being used). The now alkaline wash was centrifuged at 15,000 rpm for ten minutes at 10C. The decantant was boiled to dryness with the last few ml being transferred to a small test tube for completion of drying. The remaining crystals were re-acidified with 100 ul of 1.0% formic acid (triple distilled formic acid in deionized water), agitated on a vortex mixer and placed in an ice bath to await injection into the G. C.

Extraction Procedure Number 3

This procedure was the same as Number 2 except that 1.0 ml of 25% phosphoric acid was added prior to centrifugation. The decantant was frozen and recentrifuged to remove precipitated protein. The liquid was drawn off and heated to dryness. The crystals that were formed were reacidified with 100 ul of 1% formic acid (triple distilled formic acid in deionized water), agitated on a vortex mixer and placed in an ice bath to await injection into the G. C.

Extraction Procedure Number 4

The cold tampons were twice washed with 20 ml of deionized water, using a glass rod to squeeze out the water. Ten ul of .470 g/l 2-ethylbutyric acid was added as an internal standard. Sufficient .01 N NaOH was added to bring the pH to approximately 10.0, using at least 3 ml. The alkaline solution was centrifuged at 15,000 rpm for ten minutes. The decantant was evaporated to dryness with the last few ml being transferred to small test tube. To the remaining salt crystals and residue was added .025 ml of 10% formic acid, 10 ml

anesthesia grade diethyl ether and 20 ml of 2-propanol. The solution was agitated gently on a vortex mixer and allowed to stand in an ice bath for ten minutes before centrifugation at 15,000 rpm for ten minutes in a refrigerated centrifuge. The supernatent was removed and to it was added 10 ml of .01 N NaOH (if phase separation resulted, sufficient 2-propanol was added to break the phase). The solution was then dried in a water bath with the last few ml being transferred to a small test tube to complete the drying. The dry tubes were sealed with parafilm to await acidification with 100 ul of 1% formic acid, stored at -10C.

Extraction Procedure Number 5

The cold tampons were washed with 40 ml anesthesia grade ether and to the ether wash was added 100 ul of 2-ethylbutyric acid as an internal standard (.470 g/1). Enough 2-propanol (approximately 20 ml) was added to break any bylayer that may have formed; a minimum of 10 ml was always added.

The pH of the solution was determined by electronic pH meter and a minimum of 3 ml of .01 N NaOH added, or sufficient NaOH to bring the pH above 8.0. The now alkaline solution was centrifuged at 14,000 rpm at 0 C for 20 minutes. The supernatent was decanted and heated in a water bath at 97 C. The last few ml were transferred to a small test tube for completion of the drying process. To the thick layer formed on the bottom of the test tube or breaker was added 25 ml of hexane, the solution agitated and stirred with a glass rod, centrifuged at 14,000 rpm at 0 C for ten minutes, then decanted and evaporated to dryness in a water bath. If residue formed again, the hexane process

was repeated. The dry sample was then acidified with hexanoic acid and injected into the G. C. in 2 ul aliquots.

Extraction Procedure Number 6

This extraction procedure was the same as Number 5 to the point of hexane use for removal of residue layer. Diethyl ether (25 ml) was used instead of hexane. The diethyl ether solution was centrifuged at 14,000 rpm at 0 C for 10 minutes with the supernatant being decanted and evaporated to dryness in a water bath at 97C. The dry crystals were acidified with hexanoic acid prior to injection of 2 ul aliquots into the G. C.

Extraction Procedure Number 7

Each tampon was placed in 30 ml of deionized water and allowed to soak for 30 minutes at 10 C, and 200 ul of 2-ethyl butyric acid added as internal standard. A false bottom was placed in each tube prior to centrifugation at 15,000 rpm for 20 minutes at 0 C. The decantant was placed in an ice bath and to it was added 5 ml of NaOH (.01 N). The samples were evaporated to dryness in a hot water bath. Tubes containing the crystals were capped with pliofilm and stored at -10 C until acidified prior to injection into the G. C.

Extraction Procedure Number 8

Cold tampons were washed in 20 ml deionized water, and to the wash water was added 100 ul of the internal standard 2-ethyl butyric acid. Sufficient NaOH (.01 N) was added to bring the pH above 8.0, with a minimum of 3 ml of NaOH being used. The solution was centrifuged

at 15,000 rpm for ten minutes at 0 C, decanted and evaporated to dryness in a water bath. To the residue remaining was added 25 ml anesthesia grade ether. The mixture was then agitated on a vortex mixer for five minutes, decanted and centrifuged. The decantant was placed in a hot water bath and dried. The crystals remaining were acidified with hexanoic acid in preparation for G. C. injections.

Extraction Procedure Number 9

To the cold tampon bundle was added 25 ml deionized water. Then agitation with a glass rod accomplished thorough washing. The water was then centrifuged at 5,000 rpm for 30 minutes at 0 C. Ten ml of the supernatant was pipetted from the centrifuge tube, and to this was added 4.0 ml of 25% phosphoric acid and 10.0 ul 2-ethylbutyric acid as an internal standard. The sample was then frozen at -10 C to facilitate protein separation.

The thawed samples were centrifuged at 5,000 rpm for 30 minutes at 0 C and then decanted and a 5 ul aloquot injected into the G. C. Deplicate gas chromatograph injections of 5 ul each were made of each sample. A 10 ul injection of deionized water was made following each sample to prevent "bleeding" from previous injections.

Since, of all the methods studied, this procedure consistently yielded the most repeatable data when working with samples from dairy cows, samples to which known quantities of short chain fatty acids had been added and samples collected with tampons, it was used throughout the main phase of this study.

A Bendix Model 2600 gas chromatograph with a Model CRS 309 integrator was used to analyze the samples. A six feet by 2 mm glass

column packed with supelco 10% SP 1200/1% H_2PO_4 on 80/100 chromosorb WAW plugged with glass wool was used. The freshly packed column was cured for 24 hours at 120 C before use. The operational settings for the gas chromatograph (G. C.) were: oven temperature, 120 C; H_2 flow rate 4.0; air flor rate 2.0; carried gas flow rate 3.0; inlet temperature, 210 C; detector temperature, 200 C; a Henuation of 100 with integrator at the low 2X noise level.

Standards of acetic, propionic, butyric, isovaleric and valeric acids were prepared from stock solutions of these acids. Chromatographic data were statistically analyzed using the Statistical Analysis (SAS) Computer Software package. Data sets with missing values were deleted.

CHAPTER IV

RESULTS AND DISCUSSION

The mean concentrations of acids in urine and mucus from the two ovariectomized females are shown in Tables II and III. These means should not be construed as representive values for any given day of the experimental period but rather are presented to show the relative concentrations of the different acids. It should be noted that acid concentrations were consistently higher in urine than in mucus samples; furthermore the values were higher in the Angus than in the Hereford heifer.

The average daily concentrations of fatty acids in the urine for the Hereford and Angus respectively are shown in Tables IV and V and graphically on pages 24 and 25. Acetic acid was consistently higher than all other acids in urine and mucus samples. Generally there appears to be an increase of all acids on days six and seven followed by a decline to near baseline levels. There are subsequent increases in all acids occurring between days nine and twelve. There is no satisfactory explanation for increased levels of acids prior to day eight since the reproductive tract was under the influence of progesterone to that time. If estrogen was the hormone responsible for increased synthesis of the fatty acids there should have been no increase prior to day eight. The increase after day nine can be attributed to the estrogenic effect. Futhermore, comparisons of Figures 1 and 2 (urine) and Figures 3 and 4 (mucus) show that the fatty acid peak synthesis in the Hereford female preceded the peaks in the

TABLE II

MEANS OF VOLATILE FATTY ACIDS (IN MICRO MOLES PER MILLILITER) IN VAGINAL MUCUS OR URINE (HEREFORD FEMALE)

ACETIC URINE-MUCUS	NUMBER OF SAMPLES	MEAN CONCENTRATION (± STANDARD S. E.)
Acetic Urine	17	47.67 ± 32.55
Acetic Mucus	53	3.23 ± .97
Propionic Urine	17	53.99 ± 28.78
Propionic Mucus	50	.01 ± .01
Butyric Urine	15	2.15 ± 1.37
Butyric Mucus	48	0.03 ± .01
Isovaleric Urine	16	3.83 ± 1.87
Isovaleric Mucus	53	0.01 ± 0.01
Valeric Urine	17	13.46 ± 9.42
Valeric Mucus	51	.07 ± .04

TABLE III

MEANS OF VOLATILE FATTY ACIDS (IN MICRO MOLES PER MILLILITER) IN VAGINAL MUCUS OR URINE (ANGUS FEMALE)

ACETIC URINE-MUCUS	NUMBER OF SAMPLES	MEAN CONCENTRATION (± STANDARD S.E.)
Acetic Urine	28	120.39 ± 49.76
Acetic Mucus	52	1.76 ± .40
Propionic Urine	26	70.52 ± 28.56
Propionic Mucus	52	.09 ± .08
Butyric Urine	27	2.68 ± 1.58
Butyric Mucus	50	0.07 ± .04
Isovaleric Urine	27	5.78 ± 2.28
Isovaleric Mucus	51	0.01 ± .01
Valeric Urine	28	19.84 ± 18.26
Valeric Mucus	52	0.02 ± 0.01

TABLE IV

AVERAGE DAILY FATTY ACID VALUES IN URINE OF HEREFORD FEMALES

DAY OF COLLECTION	ACETIC	PROPIONIC	BUTYRIC	ISOVALERIC	VALERIC
1	-	-	-	-	-
2	2.845	0.740	0.001	0.515	0.002
3	-	-	-	-	-
4	-	-	-	-	-
5	-	-	-	-	-
6	16.455	13.758	0.001	0.800	1.844
7	-	-	-	-	-
8	37.837	47.509	0.0003	1.644	0.037
9	4.685	0.463	0.014	0.076	0.012
10	16.155	18.267	0.951	7.305	3.436
11	26.266	11.567	6.425	0.401	49.764
12	3.814	2.773	0.0004	0.137	0.011
13	147.054	183.164	3.658	10.215	17.663
14	-	-	-	-	-

TABLE V

AVERAGE DAILY FATTY ACID VALUES IN URINE OF ANGUS FEMALE

DAY OF COLLECTION	ACETIC	PROPIONIC	BUTYRIC	ISOVALERIC	VALERIC
1	-	-	-	-	-
2	151.961	104.692	0.000	1.883	0.079
3	0.371	0.000	0.001	0.029	0.001
4	3.346	15.445	0.043	0.079	0.037
5	514.674	88.088	0.056	13.212	1.702
6	39.953	42.776	0.045	2.247	5.824
7	290.307	197.804	0.095	20.614	4.034
8	26.539	9.231	0.018	0.907	0.176
9	1.117	0.815	0.0002	0.186	0.005
10	39.474	18.654	17.331	2.548	1.507
11	2.009	0.017	2.504	0.253	0.052
12	73.398	160.48	9.630	8.030	170.955
13	-	-	-	-	-
14	1.605	0.008	0.308	0.118	0.036

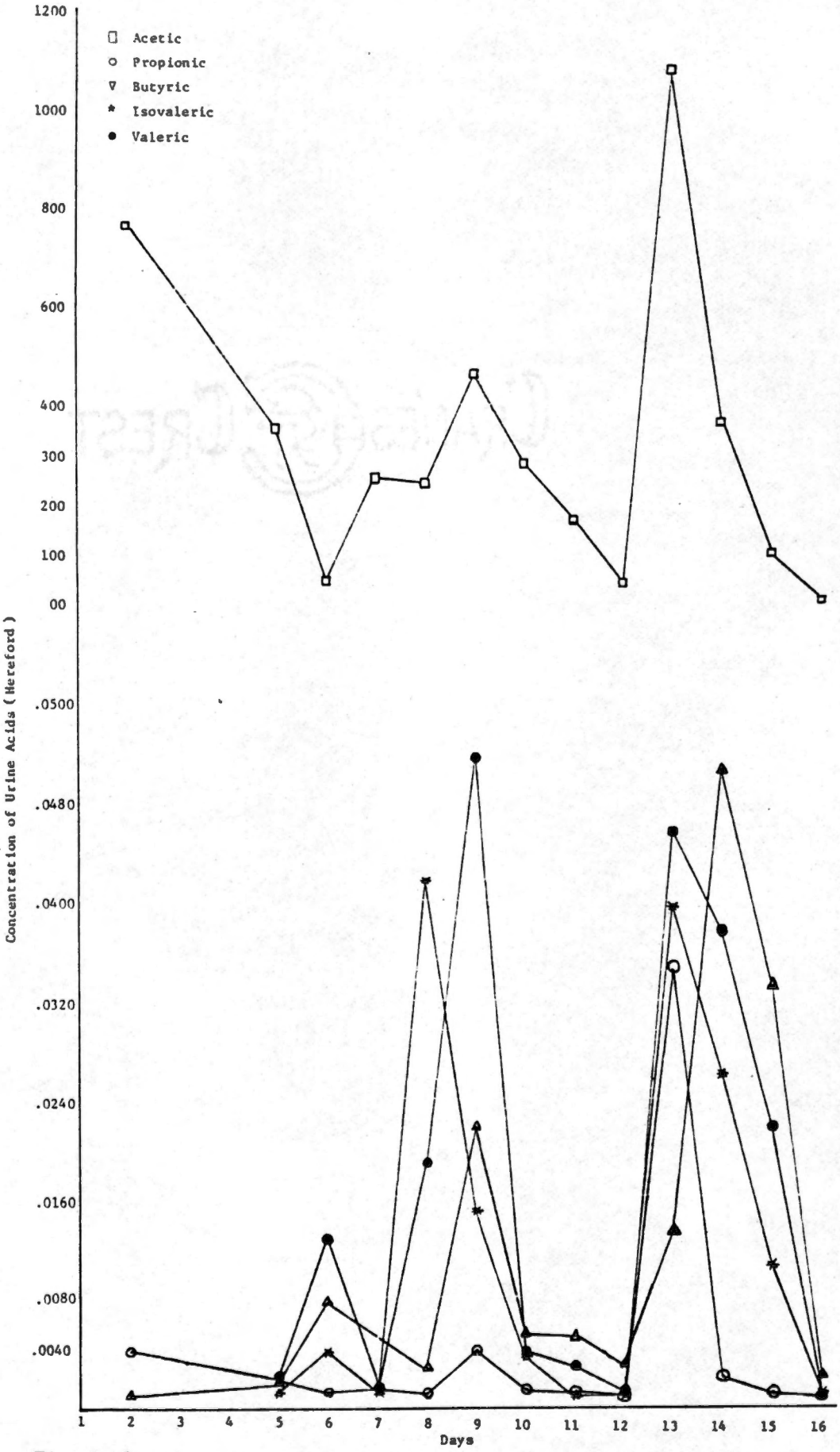

Figure 1. Concentration of Urine Acids, Cow 1 (Hereford).

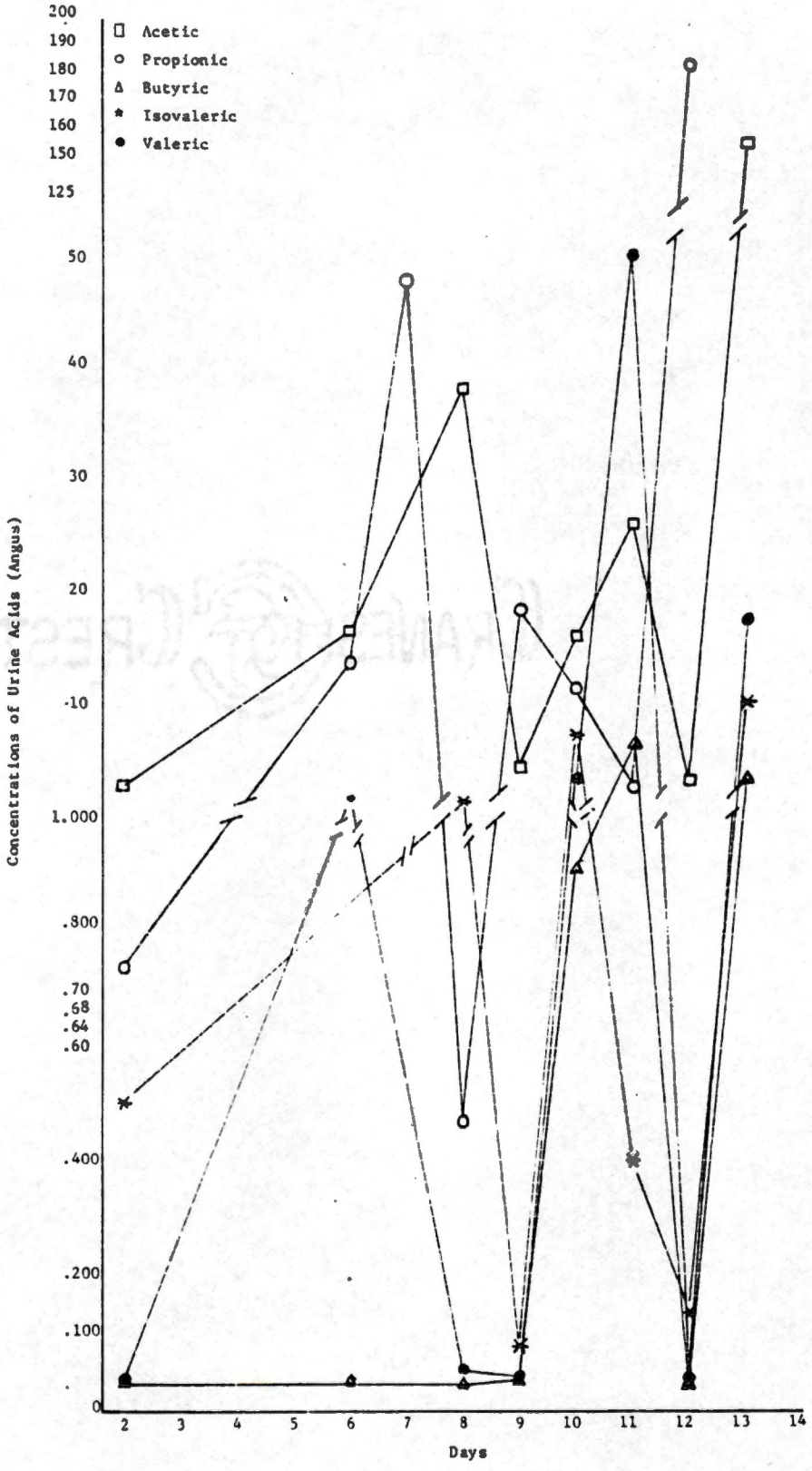

Figure 2. Concentrations of Urine Acids, Cow 1 (Angus).

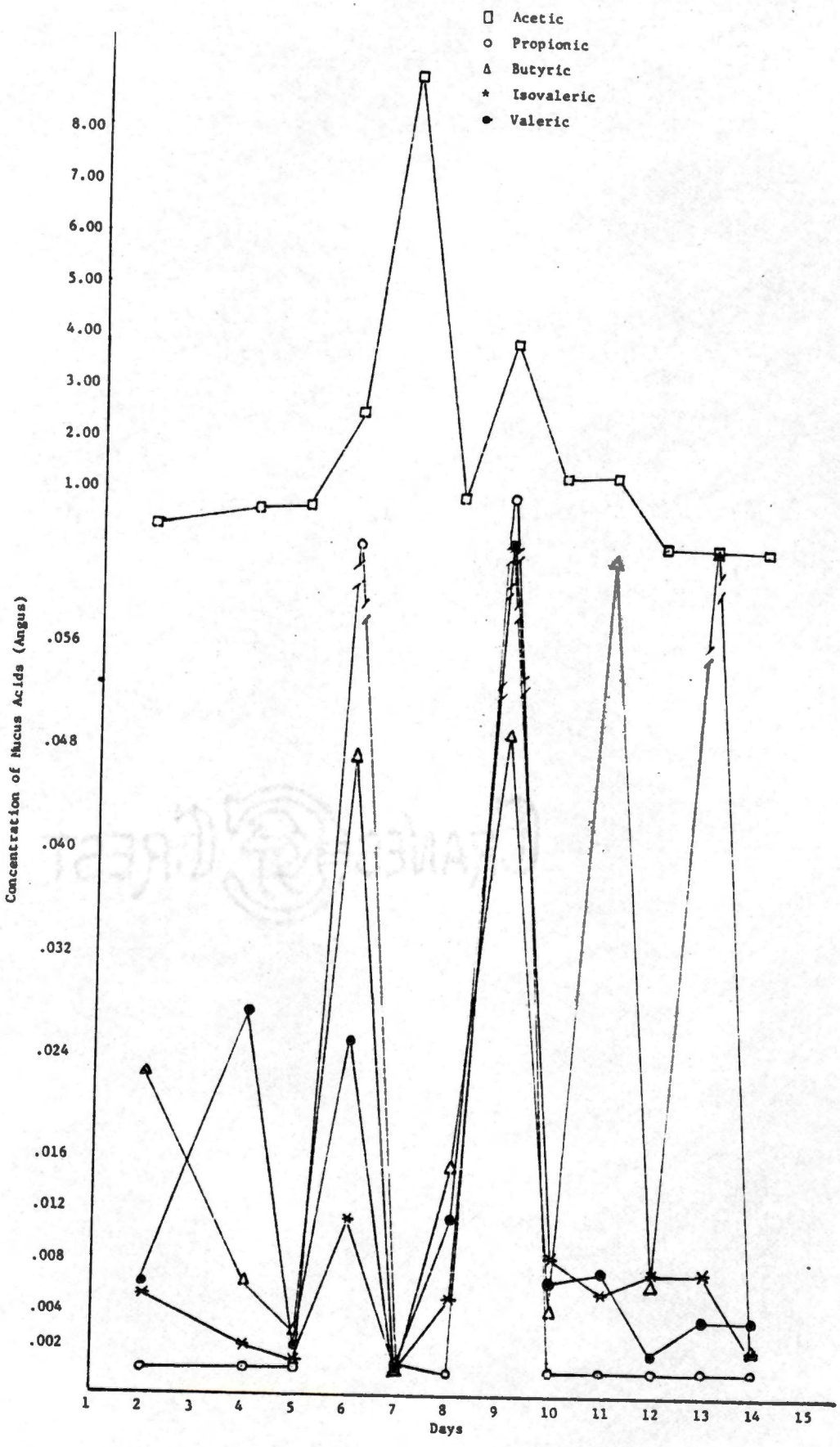

Figure 3. Concentration of Mucus Acids, Cow 2 (Angus).

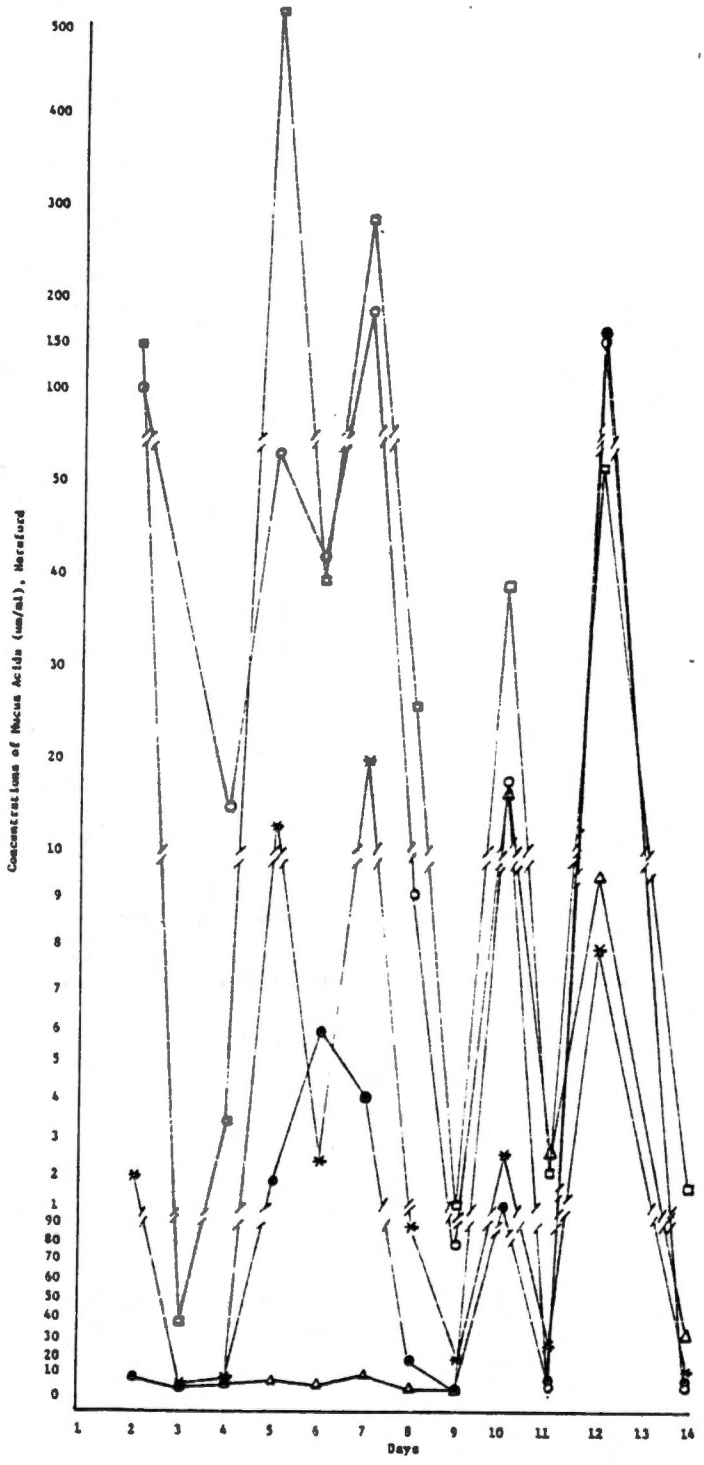

Figure 4. Concentration of Mucus Acids, Cow 2 (Hereford).

TABLE VI

CORRELATIONS AMONG VOLATILE FATTY ACIDS IN URINE AND MUCUS FROM HEREFORD HEIFER.

		ACETIC ACID		PROPIONIC ACID		BUTYRIC ACID		ISOVALERIC ACID		VALERIC ACID	
		URINARY	MUCUS	URINARY	MUCUS	URINARY	MUCUS	URINARY	MUCUS	URINARY	MUCUS
ACETIC ACID	URINARY	1.00									
	MUCUS	.27	1.00								
PROPIONIC	URINARY	.75**	.31	1.00							
	MUCUS	-.06	.77**	.06	1.00						
BUTYRIC	URINARY	.51*	-.12	.31	-.24	1.00					
	MUCUS	-.10	.08	.58*	.10	-.11	1.00				
ISOVALERIC	URINARY	.88**	.17	.71**	-.12	.37	.03	1.00			
	MUCUS	-.09	.37**	.59*	.47**	-.11	.41**	.04	1.00		
VALERIC	URINARY	.48*	-.11	.28	-.22	.99**	-.12	.30	-.12	1.00	
	MUCUS	-.09	.89**	.59*	.88**	-.11	.79**	.04	.52**	-.12	1.00

* $< .01$ $P < .05$ ** $P < .01$

TABLE VII

CORRELATIONS AMONG VOLATILE FATTY ACIDS IN URINE AND MUCUS FROM ANGUS HEIFER.

		ACETIC ACID		PROPIONIC ACID		BUTYRIC ACID		ISOVALERIC ACID		VALERIC ACID	
		URINARY	MUCUS	URINARY	MUCUS	URINARY	MUCUS	URINARY	MUCUS	URINARY	MUCUS
ACETIC ACID	URINARY	1.00									
	MUCUS	.17	1.00								
PROPIONIC	URINARY	.58**	.22	1.00							
	MUCUS	-.11	.60**	-.11	1.00						
BUTYRIC	URINARY	-.02	-.22	.30	-.08	1.00					
	MUCUS	-.12	.55**	-.11	.91**	-.08	1.00				
ISOVALERIC	URINARY	.50*	.39*	.23	-.11	-.10	-.12	1.00			
	MUCUS	-.10	.60**	-.10	.99**	-.06	.94**	-.10	1.00		
VALERIC	URINARY	.05	-.12	.55**	-.05	.64**	-.05	.42*	-.12	1.00	
	MUCUS	-.11	.59**	-.11	.99**	-.08	.91**	-.11	.98**	-.05	1.00

* .01< P< .05 **P < .01

Considerable difficulty was encountered in establishing an extraction process that would provide consistent results. Attempts to reproduce Fitz's (1975) extraction process were unsuccessful. A thick, "wax-like" precipitate was formed when the alkaline samples were heated in a water bath. Since the tampons were washed with ether, water and isopropyl alcohol, it was assumed that they were carrying no residue. Likewise, the glassware and all utensils were washed similarly to insure no residue. However, R. T. Jacobs, Johnson and Johnson Laboratories (personal communication), indicated that the lubricant being used (K-Y Jelly) contained a protein extracted from sea weed. Phosphoric acid added to the samples precipitated this protein. A "wax-like" layer was still present but in a much smaller quantity than before. On the basis of a suggestion by Dr. Sharon Melton (personal communication) a water extraction process was used which removed the "wax-like" material. Protein removal was enhanced by freezing the samples prior to gas chromatography. Length of time the samples were held in the frozen state was not critical.

In the current study, the animals were used on several occasions during the collection period for classroom demonstrations resulting in failure to obtain urine samples on those days. At other times people not associated with the study may have disturbed the cows and caused them to urinate at other than the designated collection intervals. Other than loss of urine samples, there is no evidence that this additional handling interfered with the outcome of the study.

The initial objective of this study was to characterize the temporal pattern of production of the fatty acids during the estrous

cycle. With this information one should be able to prepare an appropriate mixture of the acids in sufficient quantities to permit behavorial studies with both bulls and cows. This was not accomplished.

In retrospect more attention should have been devoted to behavorial studies. The reaction of bulls to mucus as well as urine should be compared. It may be that despite the male stimulatory action of the vaginal contents in monkeys, as demonstrated by Michael and associates, the similar activity in cattle may reside in the urine. Further studies should include behavioral responses of both cows and bulls along with chemical studies.

CHAPTER V

SUMMARY

A procedure for extraction of short chain fatty acids from vaginal mucus and purification of the extract to permit fatty acid analyses by gas chromatography was developed. Physiological estrus was induced in two ovariectomized heifers, an Angus and a Hereford, by sequential injections of progesterone and estradiol. Concentrations of fatty acids (acetic, propionic, butyric, isovaleric and valeric) were determined. Concentrations of these acids were higher in urine than in mucus samples from both females. They were higher in both urine and mucus samples from the Angus female than in the Hereford female.

Bulls exposed to urine samples collected from the Angus heifer 24 hours after estrogen injections exhibited sexual excitement. Urine collected from the Hereford heifer failed to stimulate bulls.

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VITA

Thomas Eugene Bianconi was born in Nashville, Tennessee on July 21, 1950. He was raised on a small farm in rural middle Tennessee and attended local schools before his completion of high school in Nashville, Tennessee in June, 1968. He entered The University of Tennessee, Knoxville, in the fall of 1968 but stopped his education in the winter of 1969 to enter the United States Air Force. He graduated with a Bachelor of Science degree in Animal Science in the spring of 1975. He entered graduate school at The University of Tennessee, Knoxville, in the summer of 1975. He is married to the former Barbara Lee Murphree of Nashville, Tennessee.