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Changes in the Vulvular and Vestibular Tissue of the Bovine During the Estrous Cycle as Determined by the use of Near Infrared Interactance

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CHANGES IN THE VULVULAR AND VESTIBULAR TISSUE OF THE BOVINE DURING THE ESTROUS CYCLE AS DETERMINED BY THE USE OF NEAR INFRARED INTERACTANCE

by

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Robert Alan Kunzler

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ABSTRACT

CHANGES IN THE VULVULAR AND VESTIBULAR TISSUE OF THE BOVINE DURING THE ESTROUS CYCLE AS DETERMINED BY THE USE OF NEAR INFRARED INTERACTANCE

by

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Near infrared reflectance spectroscopy is routinely used for the analysis of quality components of feedstuffs. Near infrared spectrophotometers, coupled with a fiber optic probe, could enable direct measurements of the live animal. This study was conducted to characterize changes in the vulva and vestibule during the bovine estrous cycle using near infrared (NI) spectroscopy. Sixteen cycling Holstein cows were observed for estrus twice daily from 40 days postpartum for three estrous cycles or until conception was verified. In addition, weekly rectal palpations, cowside milk progesterone tests, and tailhead chalk were used to aid in estrous detection. Near infrared spectra of both the vulva and the vestibule were collected daily (Model 6500 with 1.83 m fiber optic probe, NIR Systems Inc., 1100 to 2500 nm). Twenty-four estrous periods were confirmed. Vulvular and vestibular

spectra of estrous and nonestrous cows were compared. Spectral differences occurred in three regions at 1700 nm, 1790 nm, and 1880 nm ($P < .05$). These regions are possibly associated with changes in carbohydrate, protein, and water content. Results indicate that direct NI analysis of the live cow is possible and changes in the vulvular and vestibular tissue during the estrous cycle can be detected. However, accurate estrous detection using NI interactance is not practical at the present time because of individual cow variability.

(93 pages)

STATEMENT OF THE PROBLEM

Introduction

Milk production and reproductive performance play major roles in determining the profitability of a dairy herd. Milk production is dependent on reproduction because lactation is induced by pregnancy and calving. Milk production also depends on the cow's genetics and the environment she is exposed to. Therefore, a knowledge of the relationship between milk production and reproduction is important for profitability of the dairy. Shanks et al. (61) associated 40% of the dairyman's direct health costs with reproduction. These costs were divided between reproductive and insemination costs. It has been estimated that 20% to 25% of all dairy cows are culled because of poor reproductive performance (11). Expenses associated with reproduction increase dramatically when the cost of replacing these culled cows is considered. In 1981 it was estimated that United States dairymen lost $960 million because of poor reproductive performance (31).

Artificial insemination (AI) is a valuable tool for the genetic improvement of animals. In Utah, 80% of the dairy cattle are artificially inseminated (43). AI allows the dairyman to improve his herd through the genetic improvement of the cows, to control venereal diseases, and to increase safety by eliminating the use of dangerous bulls on the dairy. The use of artificial insemination has made accurate estrous detection a key to the reproductive performance of a dairy

herd. Field studies have shown that only 52% of estrous periods are detected (40, 64). The trend toward larger herds, and more efficient feeding arrangements minimizes contact between the cow and the herdsman. Less contact makes it more difficult to identify cows in estrus. Other problems dairymen encounter in the detection of estrus include extremes in temperature, high production, confinement housing, and nutritional limitations, causing an energy imbalance, all of which reduce the expression of estrus (63, 66, 68). Visual aids have been developed to help the dairyman detect more estrous periods. These include the use of video tape, heat mount detectors, chin ball markers, and wax crayon on the tailhead.

Estrous detection by visual means consists of identifying the behavioral changes in the cow at the time of estrus. A wide variation exists in the behavioral changes of cows associated with estrus. To help overcome the behavioral variation that exists in cows, researchers are investigating the physiological changes that occur during the estrous cycle, as a means of finding more objective indicators of estrus. These changes include increased activity, changes in uterine contractions, changes in the vaginal cytology, shifts in the cellular water content of the vulvular tissue, shifts in the protein content of the edema fluid, blood flow increases to the uterus, and chemical changes in the cervical mucus.

During the estrous period, activity of the cow increases several hundred percent as measured by pedometers (40). Contractions of the uterus increase in number and move toward the oviducts during early estrus and toward the cervix after the end of estrus (30). Vaginal epithelial cells undergo cyclic changes during the estrous cycle. Estrus is characterized by an extension of the superficial layer of the vagina due to distension of the cells with mucus (57). Ezov et al. (21) found that during estrus, the cellular water content of vulvular tissue shifted from the inside of the cell to the outside. The rise in estrogen, associated with estrus causes an increase in the protein content of edematous fluids of the reproductive tract, and an increase in blood flow to the uterus (2, 54). Alliston et al. (3) found that crystallization patterns formed from cervical mucus collected during estrus were more fernlike and more extensive than during the luteal phase.

Merilan (45) reported that nuclear magnetic resonance (NMR) could be used to detect changes in vaginal mucus associated with estrus. The main drawbacks to this method are the price of the equipment, collection of mucus from cows not in estrus, and purification of the sample before analysis.

Near infrared reflectance (NIR) spectroscopy is a method for rapidly and reproducibly measuring the chemical composition of samples with little or no sample preparation.

Near infrared reflectance spectroscopy has been used to measure quality components in grains for many years. Recently its use has been expanded to forages and industrial applications (71).

Near infrared spectroscopy has four advantages over chemical analysis: speed, simplicity of sample preparation, multiple chemical analysis with one operation, and nonconsumption of the sample. Near infrared reflectance spectroscopic measurements can be made in less than two minutes. Forage sample preparation is simple, consisting of grinding the sample to ensure sample homogeneity, and placing it into the sample cup for analysis.

There are some disadvantages in using the NIR spectroscopic method of analysis. Disadvantages include instrumentation requirements; dependance upon calibration procedures; and complexity in the choice of data treatment. The NIR spectroscopic method requires high-precision spectroscopic instrumentation, because a small change in the reflectance at specific wavelengths must be measured. Calibration is required for each constituent and is valid for only the same type of sample. Once the data has been collected there are several treatments for the data, and an optimum treatment has not been agreed upon.

All substances of plant and animal origin are composed of constituents possessing functional groups of atoms that absorb

in the NIR region. These groupings include -OH, -CH, and -NH. The NIR spectrophotometer is responsive to hydrogen stretching and bending. The O-H bonding, related to water, occurs many times in the NIR region. However, the 1950 nm area is the primary region for water determinations (74). For this reason, the NIR spectrophotometer is very sensitive to changes in the water content of the sample.

With this knowledge, preliminary research was conducted at Utah State University which studied the use of NIR to analyze vaginal mucus and determine if the NIR spectroscopic method of analysis could be used as an estrous detection aid (12). Spectra in second derivative showed a shift in the water region to the left and a depression in peak height as estrus approached. This was probably due to an influx of ions as vaginal secretions increased in preparation for mating. The main drawbacks to this method were obtaining mucus from nonestrous heifers and the inability of the NIR spectrophotometer to measure the changes in situ.

The recent development of a fiber optic probe may allow the researcher to analyze the changes which occur in situ.

Problem Statement

Near infrared reflectance spectroscopy has been used by researchers to analyze the chemical constituents in many substances including forages, meat products, dairy products,

and fruits. The development of fiber optic probes enables direct measurements of the live animal. Therefore, it may be possible to characterize the changes in vestibular tissue throughout the estrous cycle and use it as an aid in estrous detection.

Purpose and Objectives

The objectives of this study follow:

1. To evaluate the efficiency of a NI spectrophotometer coupled with an infrared fiber optic probe for in situ spectral analysis of vulvular and vestibular tissue in dairy cattle.
2. To correlate changes in NI spectra of vulvular and vestibular tissue with changes in the estrous cycle of the cow.
3. To determine if changes in the spectra of the cow's vulva and vestibule could be used to predict and identify the day of estrus.

LITERATURE REVIEW

The Estrous Cycle

The dairy cow is a polyestrous animal. That is, estrus occurs regularly throughout the year. The estrous cycle is the time between estrous periods. In the bovine, the cycle ranges from 18 to 24 days in length with an average length of 21 days. Longer and shorter estrous periods sometimes occur, but are often the result of cystic ovarian disease or early embryonic death (14).

Estrus is the time when the female will accept the male for breeding. The length of the estrous period varies from six to 30 hours with an average of 18 hours. Studies have found that 65% of cows exhibit behavioral signs of estrus lasting less than 16 hours, and 25% of cows exhibit these signs for less than 8 hours (20, 38, 50).

The estrous cycle consists of two ovarian phases, the follicular and the luteal phases, which are separated by ovulation. The follicular phase refers to the period from regression of the corpus luteum to the following ovulation. The length of the follicular phase in the bovine is 4 to 5 days (30). Follicle growth involves hormonally induced proliferation and differentiation of both theca and granulosa cells. This leads to the increased ability of follicles to produce estradiol and to respond to the pituitary

gonadotropins. Estradiol has a positive feedback on the hypothalamus; that is it increases the release of gonadotropin-releasing hormone (GnRH). Gonadotropin-releasing hormone is transported to the pars distalis via the portal vessels of the pituitary stalk where it attaches to basophil cell receptors and stimulates the synthesis or release of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) (18). During the follicular phase, tonic levels of FSH and LH stimulate estrogen secretion from theca cells of the maturing follicle (23, 55). Follicle-stimulating hormone induces granulosa cell sensitivity to LH, which prepares the granulosa cells for luteinization. Luteinizing hormone stimulates the theca cells to produce testosterone, which is converted by the granulosa cells into estradiol 17_{β}. Production of estradiol determines which follicle will gain the LH receptors necessary for growth, maturation, ovulation and luteinization (15). The preovulatory LH surge is initiated by the increase in circulating estrogen and decreased progesterone levels, which cause the hypothalamus to release an acute amount of GnRH (18). Increases in serum LH correspond to the maturation of the ovum, ovulation, and formation of the corpus luteum (23). Ovulation occurs approximately 30 hours after the preovulatory surge (9).

Prostaglandins ($PGF_{2\alpha}$ and PGE_2) play important roles in ovulation. Prostaglandin $PGF_{2\alpha}$ induces the follicular wall to

break down allowing ovulation to occur. Following ovulation, LH and PGE_2 transform the follicular layers into the corpus luteum (67).

The period of corpus luteum activity is known as the luteal phase. The luteal phase is 16 to 17 days in length in the bovine (30). The functional life span of the corpus luteum is totally dependent upon the fate of the fertilized oocyte. If implantation occurs following ovulation, the corpus luteum undergoes extensive enlargement and continues to synthesize and secrete progesterone. By contrast, if the oocyte fails to be fertilized or implantation does not occur, the corpus luteum undergoes atrophy and eventual degeneration (10). Progesterone produced in the corpus luteum has several functions, including preparing the endometrium for implantation, secretion of uterine milk to nourish the embryo, and maintaining pregnancy (32). Estrogen is produced in small quantities during the luteal phase with a minor peak between 7 and 11 days (28, 35). The estrogen peak is associated with the development and subsequent regression of the mid-cycle follicular waves. In the presence of a functional corpus luteum, this peak is unable to initiate the necessary follicular growth and the acute release of LH for ovulation (36). Corpus luteum regression is not caused by a decreased secretion of pituitary luteotrophic hormones (LH and prolactin), but by the action of a luteolytic factor, $PGF_{2\alpha}$

which is released by the uterus (33). The changes which occur during regression include a thickening of the walls of the arteries, a decrease in cytoplasmic granulation, a rounding of the cell outline, and peripheral vacuolation of the large luteal cells (10). These changes are apparent by the 18th day of the cycle which corresponds to decreased levels of progesterone. Following these changes in the corpus luteum, the level of estradiol-17_{β} increases and a new follicular phase begins (17).

Female Reproductive Organs

The female reproductive organs are composed of the ovaries, oviducts, uterus, cervix, vagina and the external genitalia. The internal organs, consisting of the ovaries, oviducts, uterus and cervix, are supported by the broad ligament. The ligament consists of the mesovarium, the mesosalpinx, and the mesometrium. In cattle, the attachment of the broad ligament is beneath the ileum. The uterine horns are arranged like a ram's horns, with the convexity dorsal and the ovaries located near the pelvis.

The ovary is composed of the medulla and the cortex. The ovarian medulla consists of irregularly arranged fibroelastic connective tissue and extensive nervous and vascular systems that reach the ovary through the hilus (attachment between the ovary and the mesovarium). The ovarian cortex consists of

connective tissue with fibroblasts, and collagen, and some reticular fibers. The ovarian cortex contains the ovarian follicles or corpora lutea depending on the stage of the estrous cycle. The connective tissue of the cortex contains many fibroblasts, some collagen and reticular fibers, blood vessels, lymphatic vessels, nerves and smooth muscle fibers (46).

The oviduct is divided into four sections, fimbria, infundibulum, ampulla, and isthmus. The fimbria transports ovulated ova from the ovarian surface to the infundibulum. The fimbria consists of ciliated cells which extend from the infundibulum and guide the ovum into the infundibulum. The ostium abdominal is the funnel-shaped opening in the infundibulum which allows the ovum to enter the oviduct. The ampulla connects the infundibulum to the constricted section of the oviduct, called the isthmus. The infundibulum, ampulla and isthmus are composed of a mucosal lining, the muscularis and serosa. The mucosal lining consists of one layer of columnar epithelial cells. The muscularis consists of smooth muscle fiber and connective tissue (29). The serosa is a thin muscle layer connecting the muscularis to the mesosalpinx.

The uterus consists of two uterine horns, a body and the internal os of the cervix. The uterine horns and the body of the uterus consist of a mucous membrane lining (endometrium), an intermediate smooth muscle layer (myometrium) and an outer

serous layer (perimetrium). The internal os of the cervix is a sphincter which helps keep foreign objects out of the uterus.

The cervix is a fibrous organ that separates the uterus and the vagina. The cervix is composed mainly of connective tissue, mucus secreting cells, ciliated cells, and small amounts of smooth muscle. The connective tissue of the cervix is made of ground substance, fibrous constituents, and cellular elements (29).

The bovine vagina is a fibromuscular tube that is attached to the neck of the cervix and connects directly with the external genitalia. The fibromuscular tube consists of three layers the surface epithelium, the muscular coat and serosa. The surface epithelium is composed mainly of glandless stratified squamous epithelial cells, except in the cranial section near the cervix where some mucous cells are present. The muscular coat consists of a thick inner circular layer and a thin outer longitudinal layer. The latter continues for some distance into the uterus. The serosa is composed of loose and dense connective tissue, nerve bundles and groups of nerve cells.

The external genitalia consist of the vestibule, clitoris, labia minor, and labia major. The vestibule extends inward approximately 10 cm where the external urethral orifice opens into the ventral surface of the vagina (29). Posterior

to the orifice lies the suburethral diverticulum. Gartner's tubes (remnants of the wolffian ducts) open into the vestibule posteriorly and laterally to the Gartner's ducts. The glands of Bartholin secrete a viscid fluid during estrus and have a tuboalveolar structure. The vestibule consists of a structure similar to that of the vagina. The clitoris is concealed in the mucosa of the vestibule. It is composed of erectile tissue covered by a stratified squamous epithelium and it is well supplied with sensory nerve endings. The labia minor is within the labia major and is composed of a core of spongy connective tissue with large sebaceous glands on the surface. The labia major is the fold of skin on either side of the entrance of the vulva. The integument of the labia is richly endowed with sebaceous and tubular glands. These glands contain fat deposits, elastic tissue, and a thin layer of smooth muscle (29).

Histological Changes in the Vagina during the Estrous Cycle

Histological changes in the vagina are mostly confined to a period of rapid growth, two days prior to estrus, followed by regression during the remainder of the cycle. The growth period is the result of the action of estrogen, produced at estrus in increasing quantities. The regression occurring during the luteal phase is probably the result of lowered levels of estrogen (57).

During estrus, the surface epithelial cells of the vagina are at their maximal height due to distention of the cells with mucus. Surface epithelial cells also vary in the number of cell layers, depending on the area of the vagina. Some areas consist of a single layer while others consist of two to five layers of polyhedral or squamous type cells in small clusters adjacent to the membrane propria. One day postestrus, the surface epithelium resembles that of estrus. The columnar cells are reduced in height, and there are slight increases in the number of layers of squamous cells. Two days after estrus, the surface epithelium reduces further in height and changes to a cuboidal form. The luminal borders of the cell begin to become irregular. The layers of squamous cells reach their maximum at this stage of the cycle. Three days postestrus the superficial epithelial cells vary from columnar to cuboidal and remain so during the rest of diestrus. Squamous cells decrease gradually from this point and become minimal at estrus. During proestrus the surface epithelium begins to increase in cell depth with the cells changing from cuboidal to columnar in form (57).

Changes in the Vulvular Mesenchyme During the Estrous Cycle

During estrus the bovine vulva becomes edematous as determined by clinical and histological observations (57, 60). The edema of vulvular mesenchyme during estrus is caused by a

shift in cellular water from the inside of the cells to the outside (21). Figure 1 shows graphically the absolute grams (g) and relative percent dry matter (DM), extracellular water (ECW), and intracellular water (ICW) in 100 g of estrus and diestrus vulvular tissue. Absolute weights are expressed as the areas of each sector forming a half-circle, whereas relative weights are expressed by the size of the central angle of each sector. During estrus DM, ECW, and ICW of vulvular mesenchyme tissue increased by weight 18.4 g, 38.0 g, and 17.6 g, respectively, resulting in a total increase of 74.0 g per 100 g of tissue during diestrus (21). These absolute weight increases resulted in no change in percent DM (24.2 vs. 24.5), a decrease in percent ICW (56.0 vs. 42.3) and an increase in percent ECW (19.8 vs. 33.2). The additional weight of bovine vulvular mesenchyme during estrus is 75% water and 25% DM, as evidenced by the absence of change in the percentage of these components in estrus compared with diestrus (Figure 1).

The changes in cellular water content from inside to outside as the cow approaches estrus are gradual as circulating estrogens in the blood increase. Postestrus the reverse is true; circulating estrogens decline and progesterone levels increase causing the cellular water to diffuse back into the cell. The increase in ECW is primarily responsible for the increase in total tissue water during

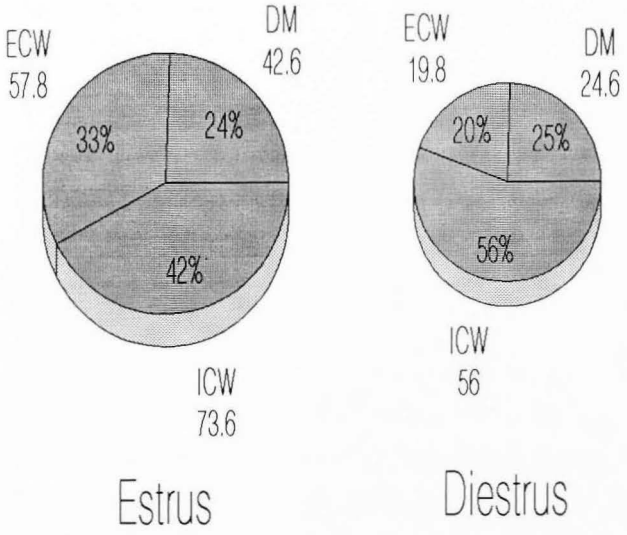

Figure 1. Changes in the cellular water content.
DM = Dry matter.
ECW = Extracellular water.
ICW = Intracellular water.

estrus (21). The increase in ECW is consistent with data on bovine myometrial tissue. Hawk et al. (34) found that the ECW of myometrial tissue changed from 37.0% during the luteal phase to 45.8% during estrus.

During estrus, cellular density of the vulvular mesenchyme is lowest, due to the distension of the cells with mucus, and should reduce the percent dry matter (DM). However, Ezov et al. (21) found no decrease in percent DM, because of a concomitant increase in the dry matter content per cell. Peterson et al. (54) found that natural and experimental estrogen stimulation of uterine tissue increased the protein content of edema fluids.

The concentrations of potassium, sodium, and chloride ions in the extracellular compartment have also been shown to vary throughout the estrous cycle (25, 34). Hawk et al. (34) found that potassium levels were lowest in the endometrium during estrus. As ECW increases, tissue sodium and chloride should also increase. Studies are unclear as to whether or not a change occurs in the level of sodium during the estrous cycle. Hawk et al. (34) found that sodium increased, while Ezov et al. (21) found that sodium and chloride do not increase. They explained that their seeing an increase in sodium and chloride was due to an equal decrease in the concentration of extracellular sodium and chloride (21). The decrease in sodium and chloride in the extracellular

compartment is consistent with that of plasma (25). Plasma and extracellular ion concentrations showed similar characteristics.

Vaginal Mucus

Mucus production increases at the time of estrus to help carry sperm to the sight of fertilization. The increase in mucus production is due to an increase in circulating estrogen and a decrease in progesterone. Estrogen stimulates the secreting cells of the oviduct, uterus, cervix, and vagina to increase mucous production. These secretions accumulate in the vagina and mix with leukocytes and cellular debris from the oviduct, uterus, cervix, and vaginal epithelia (29). Roark et al. (57) found that the largest volume of mucus was secreted during the first three hours of estrus and that the amount of mucus secreted gradually decreased as the interval from the beginning of estrus increased.

Mucus is a hydrogel consisting of water, carbohydrates, trace elements, enzymes, and a mucin component. The mucin is composed of three or more of the following substances: glucose, maltose, mannose, amino acids, sialic acid, and trace elements, which form a three-dimensional network. Mucous secretions are heterogenous in composition due to the presence of low and high viscosity components. The low viscosity component, a yellowish fluid readily aspirated into a

capillary tube, is composed of nonmucin proteins (characteristic of serum proteins), salts, lipids, and carbohydrates. The high viscosity component is a clear substance, containing mucins that are the glycoproteins, or glycopeptides, responsible for the gel formation (29). The macromolecules of mucins are arranged in long, thread-like glycoprotein molecules with oligosaccharide and sialic acid sidechains (39). Analysis of the mucins from the preovulatory and postovulatory period show a significant difference with respect to sialic acid and hydroxy-amino acid content (39).

Vaginal mucus has many unique characteristics which cause it to exhibit several rheologic properties, such as flow elasticity, viscosity, and ferning. Flow elasticity of vaginal mucus follows a cyclic pattern. The flow elasticity begins to rise 24 hours preceding estrus and does not return to normal values until about 60 hours after estrus. Flow elasticity is measured by an oestroscope and readings of 5 mm or more correspond with estrus. The oestroscope is accurate in predicting the onset and end of estrus (60).

Viscosity of vaginal mucus also follows a cyclic pattern. Scott et al. (60), using an emptying tube viscosimeter, found that the viscosity of mucus varied from 1 poise during estrus to 1,000 poises during midcycle. Cyclic variations in the viscosity of the mucus cause periodic changes in the permeability of spermatozoa in the cervical canal (57).

Ferning patterns of mucus are known to be related to the ovarian activity of the female. Alliston et al. (3) found that cervical mucus shows a ferning pattern of crystallization when dried on a glass slide, becoming evident 3.5 days before estrus. These patterns reached a maximum in extent at the time of estrus and began to decline before ovulation occurred. Vaginal mucus was found to be less reliable than cervical mucus as an indicator of approaching estrus. The ferning pattern is associated with the high chloride content of the mucus and does not occur with mucus obtained at stages of the cycle when progesterone levels are high or during pregnancy (30).

These changes in the mucus that occur during estrus are necessary for the penetration of the sperm and fertilization of the ovum (24).

Estrous Detection Methods

Artificial insemination (AI) is an important tool used by dairymen for increasing the genetic potential of their cows. AI requires the job of detecting cows in estrus. There are two types of estrous detection errors which a dairyman can make. The first is failure to observe the behavioral signs of estrus. Heat detection efficiency is a measure of a person's ability to identify cows in estrus. Peter and Bosu (53) found that in the first ovulation postpartum, only 19% of cows

exhibit behavioral signs of estrus. This study also found that 64% of total ovulations were not associated with behavioral changes at estrus. A study in Ohio found that dairymen were observing 1.7 estrous periods per cow per year and missing 1.9 periods, resulting in a 47% estrous detection efficiency rate (4). Other studies indicated that only 52% of estrous periods are observed by dairymen (40, 64). Oltenacu et al. (52) found that increasing detection rates from poor (35%) to good (75%) resulted in a $60 increase in returns per cow per year. The second error a dairyman can make in heat detection is to inseminate cows that are not in estrus. This measure is called heat detection accuracy. Peter and Bosu (53) found the pedometer to be 93% accurate in predicting the third ovulation postpartum as determined by palpation per rectum and milk progesterone. However, only 79% of these ovulations were associated with behavioral signs of estrus.

Estrous detection involves observing the behavioral changes in the cow brought about by increased levels of estrogen. Some of the reasons that estrous detection is a problem include exhibitation of limited behavioral signs of estrus, environmental factors, housing, feeding arrangements, and inadequate time spent by the dairyman observing the cows. Foote (22) listed the following behavioral signs used to detect estrus:

Primary Sign:

Standing when mounted by the male or other females.

Secondary Signs:

Nudges, sniffs, and mounts other cows.

Chin rests.

Swollen vulva with clear mucus discharge.

Decreased appetite with lower milk production.

Manure on the cow's flanks.

Congregating in certain areas and walking or running along fences.

Ruffled hair on the cow's tail head.

Increased activity.

Signs of being nervous, bawling, etc.

Raising tail when contacted by others.

Up when other cows are lying down.

A number of factors need to be considered by the dairyman in observing these behavioral changes. It has been shown that 70% of mounting activity occurs between 6:00 p.m. and 6:00 a.m. (20, 50). Olds et al. (51) found that nighttime activity is more prevalent in hot weather. Hurnik et al. (38) estimated that mounts last 10 seconds or less, making it even more difficult to verify the primary sign of estrus. Cows which come into estrus as a group increase mounting activity three to five times as they tend to seek each other out.

Many aids have been developed to help dairymen detect the behavioral changes associated with estrus. One of the most popular is a pressure-sensitive device that is placed on the backs of animals and which changes colors when an animal stands for mounting (most common type: KaMAR detectors). Larger herds apply wax crayon or paint to the cow's tailhead. The absence or smudging of the chalk is an indication that the cow has been mounted. Teaser animals with chin ball marking devices are also used in estrous detection. The chin ball marker marks the female when she stands to be mounted. Physical activity increases with the onset of estrus; pedometers have been used to measure this increase.

The KaMAR detector is the most efficient of the detection aids. With proper placement on the cow and visual observation, a detection rate as high as 95% has been observed (22). Williams et al. (62) found the use of KaMAR detectors and visual observation produced a detection rate of 84%. One difficulty is placement of the detectors too far back on the cow. This increases the rate at which the detectors are lost or rubbed off. In one study, 25% of the detectors were lost due to rubbing and licking (22).

Another disadvantage of the KaMAR is that it can be rubbed against fences, trees, stalls and change colors even though the cow is not in estrus. Stevenson and Britt (64) found that 56% of color changes seen were associated with an

estrus, while 44% were false positives.

Wax crayon or paint applied in a strip on the tail head is increasing in use on many large commercial dairy operations. With this method and visual observations by the herdsman, a 79% estrous detection rate was observed (19). The drawbacks to this method include the time requirement to paint or chalk the cows daily, the need of head locking stanchions for restraint, and cows acquiring a taste for the paint and licking it off. Ducker et al. (19) found that only 51.1% of the cows with partial tail paint removal were actually in estrus as determined by milk progesterone and palpation per rectum.

The use of teaser bulls, steers, and testosterone-treated cows with marking devices has yielded a detection rate as high as 87% (42). The drawbacks to having a teaser animal are as follows: the animal tends to become aggressive with the herdsmen so they must be handled with caution; feeding an animal which could otherwise be sold for beef is not necessarily cost-efficient; and the stall that the teaser animal uses could be used for an additional milking cow.

Pedometers are used by humans to measure distance travelled. Pedometers can be attached to the leg of the cow to measure the increased activity associated with estrus. Physical activity during estrus increases from 293% to 393% in 90% of cows at estrus (40, 70, 73). Kiddy (40) noted that the

use of pedometers and visual observation produced a detection rate of 68%. Yet another study found that pedometers combined with visual observation increased estrous detection rate to 93% (73). Pedometers must be monitored on a daily basis and need to be coupled with visual observation. Moreover, pedometers for humans are not rugged enough to withstand the rigors of being on a cow's leg (40).

In addition to the behavioral changes associated with estrus, there are many nonvisual changes that have been investigated for detecting estrus. Nonvisual signs are very important because they are less subjective and it is possible to use remote sensing and automation. Some of the nonvisual changes include changes in the progesterone level in the milk, electrical resistance of the vagina, chemical composition of vaginal mucus, body temperature, and pH of vaginal mucus.

Milk varies in the concentration of progesterone depending on the stage of the cycle. Progesterone in the milk can be determined with the use of a cowside enzyme linked immunosorbent assay (ELISA) to verify visual observations of estrus. Williams et al. (73) found heat detection accuracy based on milk progesterone to vary from 29% to 95%. Gartland et al. (26), using milk progesterone tests coupled with veterinary palpations, found that estrous detection rates of 72% could be obtained. The main drawback to using this method solely for the detection of estrus is the variability among

cows. Progesterone declines rapidly three to five days before estrus and can remain low until six or eight days postestrus. Milk progesterone tests can be used most effectively to verify whether or not the cow is in estrus and estimate the level of estrous detection errors in a herd.

The vagina exhibits changes in electrical properties that have been investigated as possible aids in the detection of estrus. Electrical conductivity of the vagina and mucus are known to increase at estrus, as determined by an electrical probe inserted into the submucosa of the vagina. Smith et al. (62) found that bipolar electrical probes inserted into the vaginal submucosa were 100% accurate in discriminating between a cow in estrus (± 1 day) and ones that were not. However, the imprecision in determination of the onset of estrus and time of ovulation in this trial leaves a question as to whether electrical conductivity was due to estrus or ovulation (62). The disadvantages of this method include the cleaning of the probe, disease transmission, and the variability and instability of the measurement data. Depth of insertion, instability of contact between the vagina mucosa and the probe, posture of the cow, and unequal pressure on the probe all influence the measurement data and hence the reliability of the probe for detecting estrus.

Bovine cervical mucus exhibits cyclic changes during the estrous cycle. Merilan (45) used nuclear magnetic resonance

(NMR) to examine the cyclic changes in bovine cervical mucus and to correlate these changes to estrus. Nuclear magnetic resonance spectra showed an abrupt change between early and mid-estrus. Qualitatively, the mucous spectra from the early to mid-estrous period closely resembled the water control spectrum. This was expected because of the higher moisture content of the mucus at estrus (57). A second transition in NMR spectra occurs at six to 15 days postestrus (45). This transition appeared to be the consequence of decreased mucous hydration in concert with molecular changes in the structure of mucous polymers. Although NMR evaluation of cervical mucus samples may be used to detect changes associated with estrus in cattle, the price of the equipment, time consumed in collection of the mucous sample, and purification of the sample before analysis preclude it from practical use.

The effect of estrogen on uterine blood flow in several animals is well documented (1). Indirect evidence for this effect derives mainly from the old observation that the color of uterine blood becomes bright red following estrogen administration (13, 56). Abrams et al. (1) found in sheep that 1 to 2 mg of estrone raised the uterine blood flow and lowered the change in temperature (uterine cavity-aorta) from .2 to .4 to less than .05°C after 1 1/2 to 2 hours. The metabolic activity of the uterus and uterine blood flow lead to a difference in temperature during estrus. Abrams et al.

(1) found temperature to increase slightly at the onset of estrus and declined more precipitously before ovulation. The changes in temperature are not substantial enough to be used in the detection of estrus.

The pH of the vagina shows a marked decrease one day before the onset of estrus (59). With the beginning of estrus, there is a further decrease in the pH, and the minimal value is observed immediately before ovulation (59). Roark and Herman (57) likewise observed a decrease in the pH during estrus. They found a minimum pH four to six hours after the onset of estrus. The drawbacks to this method are the variability due to location of the pH probe and the variation between individual cows.

Near Infrared Spectra

The NI region of the electromagnetic spectrum lies between the visible and mid-infrared regions. It is generally accepted that the NI region is between wavelengths of 600 and 2500 nm. Near infrared spectra arise from the light-induced asymmetric stretching vibrations of hydrogen bonds in the functional groups of molecules (71). NI spectra consists of harmonic overtones of mid-infrared fundamental bands and combination bands generally associated with hydrogen-atom stretching-bond deformations (58). Harmonic overtones occur from 750 nm to 2100 nm, and are one-half and one-fourth of the

frequency of the substance in the 2000 nm to 2500 nm region. Figure 2 shows the NIR spectra (log 1/R) of water. Note that the first overtone for water occurs at 1500 nm, about one-half the frequency of that found at 2000 nm. Once the first overtone has been identified, equations have been developed to predict the frequencies of the higher overtones (44). Combination bands occur from 2000 nm to 2500 nm and are the difference between the frequencies of two or more fundamental bands or harmonic vibrations (5).

The assignment of overtones and other bands occurring in the NI region involves identifying atomic associations likely to occur in the molecules of the substance of interest (i.e. -CH, -NH, -OH, etc.). The next step is to search the literature for information concerning the assignment of fundamental absorbers in the NI region, and then to calculate the position where overtones and combinations of fundamentals are likely to occur (44).

Spectroscopy method of analysis offers some unique advantages to scientists. First, the signal-to-noise (S/N) ratio is quite good. (The S/N ratio is that of the signal reaching the detectors from the sample at specific wavelengths to that of all unrelated background signals reaching the detectors.) The intensities of the overtone and combination bands are orders of magnitude less than those of their corresponding fundamental bands. This disparity is not a

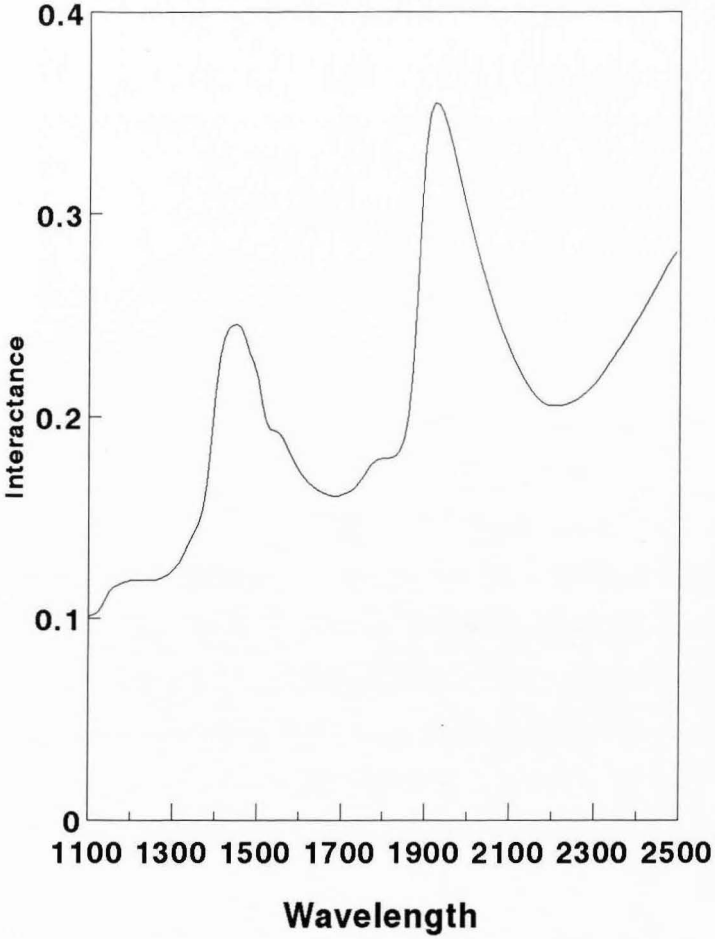

Figure 2. Near Infrared Spectra of water (log 1/R).

disadvantage because the light sources used are intense and the lead sulfide detector is quiet (5). This results in a low S/N ratio. Another advantage is that the low molar absorptivities yield uniform signal intensities, so dynamic range can easily be handled. Each of the peaks and curves present in Figure 2 represent several unresolved vibrations. In almost all instances the individual vibrations are related, and this relationship is repeated several times across the spectra. This intercorrelation that exists in the NI region allows multi-term calibration equations to be developed for relating the chemical composition to the spectrum.

Once the NI spectra has been collected, it is necessary to run the spectra through a math treatment to deal with overlapping peaks and large baseline variations. Derivatives are the method used to enhance absorption bands in the NI region. The math treatment most commonly used is second derivative. The second derivative is able to separate overlapping absorption bands; larger concentrations are found in the more negative peaks. The second effect of the second derivative is to remove baseline shifts. The first derivative also has these two effects, but to a lesser extent, and has a geometric interpretation as the slope of the spectrum at each wavelength. Higher-order derivatives also exhibit these effects, but they are more sensitive to noise and generate more artifacts than the lower-order derivatives. The three

most common methods of calculating derivatives are the finite-difference, the Fourier transform, and the Savitzky-Golay methods (37).

Near Infrared Spectrophotometer Instrumentation

The theory of infrared spectroscopy has been around for more than 160 years, but it was not until the 1930's that the first custom-made instruments appeared in industrial laboratories. In the 1950's Karl Norris began to investigate optical properties of dense, light scattering materials. By 1969, his work led to the development of a computerized, near-infrared spectrophotometer that was used to collect spectra of meat samples (56). In 1975, at a workshop sponsored by USDA-ARS and the American Forage and Grassland Council, it was suggested that NIR spectrophotometer could be used to estimate forage quality. This initiated the cooperative research that provided the first evidence that NIR spectrophotometer could measure forage quality. In 1979, a network was funded and established to use the NIR spectroscopy method of analysis to evaluate forage quality.

Major components of the NIR spectrophotometer include: lenses and mirrors, a radiation source, detectors, and computers (44). The lenses and mirrors used in the spectrophotometer are for the collection and focusing of radiation and are made of aluminum with a silver or gold

coating. The radiation source for the spectrophotometer is a tungsten-halogen lamp. Gratings are the dispersion elements generally used and serve to disperse the radiation according to wavelengths. There are three types of spectrophotometers, fixed, variable, and tilting. Infrared detectors can be classified into two broad categories according to their principle of operation: thermal detectors and photon detectors. Computers serve to collect and store the data during collection (44).

Research with NIR spectrophotometer has been extensive over the last 15 years on different agricultural products (72). Spectrophotometers have been used to analyze freeze-dried and powdered blue cheese, which resulted in correlations of .93, .91, and .81, between the spectrophotometer and laboratory analysis of protein, ash, and ph, respectively (27, 69). Studies analyzing meat products (including bacon, all-meat frankfurters, and bologna) found correlations of .974 and .974 for water and fat, respectively (8). Kruggel (41) analyzed meat products and found that changes in meat temperature cause considerable variation in the analysis of fat and moisture content in meat samples. The sodium chloride content of meat has also been analyzed and a correlation of .96 has been noted (7).

Recent research at the United States Department of Agriculture, Beltsville Research Center, in the area of NIR

NI transmittance has led to the development of the infrared interactance method (42, 48, 49). Interactance differs from reflectance in that interactance has unknown depth penetration and less space for light to bounce around the molecules. The interactance method also allows direct analysis in situ. The range used to develop interactance is between 700 nm and 1100 nm. The NI spectrophotometer uses a single beam rapid scanning monochromator and a fiber optic probe. The fiber optic probe conducts the radiation from the monochromator to selected sites on the specimen, collects the reflective and interactive radiation, and conducts it back to the detector (16). Reflective radiation is the radiation from the lamp which is reflected from the surface of the sample. Interactive radiation is the radiation which actually penetrates the sample and interacts with the molecules of the sample. The spectrophotometer is standardized by measuring a signal from a reference teflon block (1 cm thick) before scanning the substance of interest. The spectrophotometer computes interactance (I) at each wavelength by dividing the signal obtained at each site by the signal from the reflectance standard (I = Es/Er; where Es = energy received from the subject, and Er = energy received from the reference). All data are processed to log (1/I) to be similar to absorption spectra plotted as log (1/T) (16). Earlier work in analysis of agricultural foodstuffs has shown that log

(1/T) varies linearly with concentration of a specific absorber in a mixture with other materials (47).

Conway et al. (16) used NI interactance for the in situ analysis of the body fat composition in humans. Near infrared interactance successfully predicted percent body fat in one of the subgroups used in the project (r = .91). However, analysis of the female subjects by the NI interactance slightly overestimated percent body fat. There were no apparent adverse affects in using the NI interactance spectroscopy to determine body fat composition. Results from this study indicate that NI interactance may provide a rapid, safe way for the in situ analysis of percent body fat in humans. In particular, NI interactance appears to be observer-independent (once anatomical location of the site is identified) and useful in very lean and obese subjects.

MATERIALS AND METHODS

Introduction

Dairy cattle from the Utah State University herd were used in this research project. The herd consisted of 245 registered and grade Holsteins housed at the Caine Dairy Research and Teaching Center, Wellsville, Utah. The cattle were fed a total mixed ration consisting of haylage, corn silage, whole cotton-seed, distiller's dried grain, beet pulp, mineral and vitamin supplements, and long-stemmed alfalfa hay. The cows were milked twice daily. Housing included both tie stalls and enclosed freestalls.

Thirty-two lactating dairy cows (one primiparous, 31 multiparous) were selected from the herd at 40 days postpartum. The cows in this experiment calved between March 16, 1990 and May 20, 1990. Cows having a recent history (present lactation) of reproductive problems, including dystocia, retained placentas, severe metritis, or cystic ovaries were not used. Cows were palpated per rectum weekly to determine ovarian structures, cyclicity, uterine tone, stage of cycle, and pregnancy. This aided in detection of estrous and identification of problem cows. Problem cows were identified as those cows exhibiting anestrus or a cystic condition after beginning the experiment. Cows with prolonged or severe problems were removed from the study. Remaining

cows were left on the project until three verified estrous periods were collected, or until pregnancy resulted. The average length of the collection period was 65 days. Spectral collection of pregnant cows continued until they were verified to be pregnant by rectal palpation 36 days postbreeding, at which time they were removed from the experiment.

Visual observation for estrus consisted of moving the cows onto a small pasture adjacent to the housing facility so that they could be observed for behavioral changes associated with estrus. The cows were turned onto the pasture at 6:00 a.m. for 2 1/2 hours. Visual observation in the afternoon consisted of the farm personnel watching for estrus for one hour beginning at 2:30 p.m. immediately prior to the p.m. milking. In the evening between 8:00 and 9:00 p.m., the cows in the freestalls were again placed on pasture for observation. As an aid to the visual observation of estrus, wax crayon was applied in a 15.24 cm long 5.08 cm wide strip on the tailhead.

In addition to these visual aids, milk samples were taken to monitor milk progesterone levels. Milk samples were collected at two-day intervals throughout the study, beginning on day 40 postpartum. Milk samples were analyzed using a cowside milk progesterone test (Accufirm Rapid Progesterone Test, Immucell, Portland, Maine). The milk progesterone tests

were used to help verify the stage of the estrous cycle and anticipate future estrous periods.

Blood samples were collected via venous puncture of the coccygeal artery or vein on days during which the cow exhibited primary or secondary signs of estrus. All blood samples were placed into 10 ml tubes containing .10 ml of 15% EDTA solution (Vacutainer, Becton Dickinson, Rutherford, New Jersey). Blood samples were then centrifuged to separate the red blood cells from the plasma. The plasma was decanted and frozen in five ml polypropylene vials (Baxter Scientific Products, Irvine, California). The samples were stored in a freezer at the Caine Dairy Research and Teaching Center at (-1.11°C) until progesterone analysis could be performed.

Plasma progesterone concentrations were determined by radioimmunoassay (RIA) at the Utah State University Reproductive Physiology Laboratory. Plasma progesterone concentrations were determined using the Coat-A-Count Progesterone RIA kit (Diagnostic Products Corporation, Los Angeles, California) (see Appendix A).

Spectra were collected daily using a computerized near infrared spectrophotometer, model 6500, built by NIRS Systems Inc,(Silver Spring, MD.) coupled with a 1.83 m fiber optic probe. The head of the probe was 3.65 cm squared and 4.13 cm long. The end of the probe had seven openings, three emitters (.16 cm wide) and four detectors (.08 cm wide). A 386SX

laptop computer, Dell Model 316 LT was attached by a 9.14 m long serial cable to the spectrophotometer. The spectrophotometer is a single beam rapid scanning monochromator. Spectra were collected from 1100 nm to 2500 nm at 2 nm intervals. A piece of polyethylene (Reynolds Saran wrap) was placed over the head of the fiber optic probe to keep the probe clean and prevent the spread of vaginal infection. The polyethylene was replaced between cows.

The spectrophotometer required a warmup period of not less than 15 minutes for the proper collection of spectra. If the collection of spectra began before the spectrophotometer was warm, the repeatability and accuracy of the collected spectra was very low. During this experiment, the spectrophotometer was allowed to warm up for 30 minutes before the collection of the spectra. After the warmup, the NI spectrophotometer was standardized by measuring a signal reference from a polyethylene covered Teflon block (1 cm thick) before collection of the spectra from the first cow. The computer divided the sample spectra from the standard to compute interactance. Spectra collection throughout the barn required moving the computer several times, and reestablishing the power. Each time the power was reconnected the machine was allowed a brief warm-up period and a new standard was taken.

Collection of the Spectra

Collection of the spectra required a minimum of two people, one to clean the vulva and position the probe during the collection, and the other to collect the milk samples and run the computer. Cows housed in the freestalls were restrained using a head-locking chute for the collection of spectra. An additional person usually helped maneuver the cows into the chute. Cows housed in the tie stalls did not require any additional restraint other than the collar and neck chain. The spectrophotometer was carried to the animals on a backpack frame. The 9.14 m long serial cable connecting the computer and spectrophotometer allowed ample room to work around the animal. The computer was positioned on a cart away from the cow to avoid possible mishaps. The actual collection of the spectra took 28 seconds, with the average time to process a cow of approximately 3 minutes. Processing included wiping the vulva, applying wax crayon to the tailhead, collecting a milk sample on the appropriate day, and actual collecting of the spectra.

Two anatomical sites on the bovine were selected for the collection of spectra in this experiment. The first was the external portion of the vulva, midway between the top and bottom of the external opening and on the midline of the animal. The second site was 3.81 cm inside the vestibule on the left wall. After the spectra were collected, the person

operating the computer observed the spectra to see if any abnormal patterns existed. Possible causes of abnormal spectra included the cow moving during the collection, excess stray light from the barn, and excessive force in the placement of the probe. Spectra which showed abnormal patterns were repeated. The cows showed little behavioral signs of stress while the scanning was taking place.

After spectral collection was completed, the computer calculated the interactance (I) at each wavelength by dividing the signal obtained at each site by the signal from the reflectance standard (I = Es/Er; where Es = energy received from subject, and Er = energy received from the reference). All data were processed to log (1/I) to be similar to absorption spectra plotted as log (1/I). The computer then stored the spectra for later analysis.

During this experiment it was very important to make sure that the estrous periods that were observed were positive so that they could be used in a prediction equation. The details for a positive estrous period are discussed later. Because not all estrous periods met the requirements for a positive estrus, some had to be eliminated before the final analysis.

Editing of Observations

The first step in editing the number of observations was to eliminate cows which continued to be anestrus one or more

months after being placed in the study as determined by visual observation and palpation per rectum. Three cows were in this category. The next group that was edited from the data set included cows in which only secondary signs were observed. This further reduced the number of cows on the experiment by an eight. Culling is a necessary part of sound management practices and an additional four cows were culled during the project. Two cows were culled because of leg fractures which were the result of slipping and falling on the concrete floor. Two others were culled because daily milk production dropped below 35 pounds. Spectra data on one additional cow were excluded because she was inseminated prior to the project and was determined to be pregnant from this breeding. None of the cows excluded from the data set completed an entire estrous cycle. A summary of all eliminations is shown in Table 1.

The verification of a positive estrus was crucial to the experiment so that the observations would be used to develop an equation to predict estrus using NI spectra. Therefore, the next major step in preparing the data set was the elimination of those estrous periods which did not meet the criteria set for estrus. For a positive estrus, the following criteria had to be met:

1. The cow stood to be mounted.
2. The blood progesterone was low.
3. The milk progesterone was low.

TABLE 1. Reasons for exclusion of cows from the final data set.

Group of cow	Number of cows
Total cows in initial sample	32
1. Cows culled from herd[1]	4
2. Cows noncycling[2]	3
3. Exhibiting only secondary signs of estrus	8
4. Bred 37 days postpartum	1
Total cows used on the project	16

[1]These cow did not exhibit any estrous periods before they were culled from the herd.

[2]These cows were put on the experiment and were still anestrus (as determined by visual observation and palpation per rectum) 30 or more days after being included in the experiment.

4. The wax crayon on the tailhead was gone.

Or

5. The cow became pregnant from breeding at that estrus.

A total of 48 estrous periods were monitored by changes in the ovarian structures as determined by palpation per rectum and changes in the milk progesterone levels in the remaining 16 cows. However, all estrous periods did not meet the criteria
for a verified estrus.

The first step in editing estrous periods for the final data set was to eliminate those estrous periods missed as determined by rectal palpation of ovarian structures and changes in the milk progesterone levels. These estrous periods were eliminated because of a lack of visual signs of estrus. Eight estrous periods were determined to have been missed during the project. Seven additional estrous periods were eliminated because the cows exhibited only secondary signs of estrus. Embryo transfers were performed on three of the cows in the experiment, consequently another six estrous periods were lost. These estrous periods were eliminated because the natural changes in the vulva and vestibule may have been altered by the hormone preparations used to induce superovulation. Two cows were thought to be pregnant because no changes were noted in their ovarian structure for 32 days, but upon palpation per rectum at 36 days, these cows were

determined to be open. Prostaglandins were used to treat these cows so that they could be inseminated again. The administration of prostaglandins induced two estrous periods, which were eliminated because of possible alterations of the vulva and vestibule. One cow in the study was determined to be cystic, by palpation per rectum. Upon diagnoses, prostaglandins were administered to her and the corresponding observations were eliminated. A summary of the reasons for estrous period removal is shown in Table 2.

Treatment of the Spectra

Spectra were sorted into files by cow and site location (in and out); then the files were separated into categories. The categories included the following:

1. Those cows exhibiting positive signs of estrus, meeting all criteria.
2. Questionable estrous periods meeting most, but not all criteria for a positive estrus.

Criteria for classifying the files into questionable estrus included a combination of the following: no mounting behavior was observed; blood progesterone was high; milk progesterone was high; and the wax crayon was missing.

To compensate for the variable length of each estrous cycle, only days -10 to +10 (day 0 = estrus) were used in the statistical analysis.

TABLE 2. Reasons for excluding of estrous periods from the final data set.

Reason for removal	48
1. Missed estrous periods[1]	8
2. Secondary signs only	7
3. Estrous periods lost due to embryo transfer[2]	6
4. Prostaglandins administered[3]	2
5. Estrous period lost due to cow being cystic[4]	1
Total estrous periods used in the analysis	24

[1]These estrous periods were determined to have been missed by the changes in the milk progesterone tests and changes in the ovarian structure as determined by palpation per rectum.

[2]During the experiment 3 cows were used from the project to perform embryo transfer on, the corresponding estrous periods which were observed were not used in the final analysis.

[3]During the project it became necessary to administer prostaglandins to two cows and the corresponding estrous periods were discarded.

[4]One cow was determined to be cystic by palpation per rectum, the treatment of which resulted in an estrous period which was discarded.

Upon observation and consultation with Dr. D. Clark it was determined that the region from 1900 to 2500 nm should be excluded from the analysis because of noise (see Figure 3). Mean interactance at each wavelength was calculated for the day of estrus and all nonestrous days (no estrus). The day of estrus was coded 1 and nonestrus was coded 2. Students t-test was used to compare the mean reflectance of estrus and nonestrus for each wavelength. The significant wavelengths as determined by the t-test were then used in a discriminant analysis to predict estrus and nonestrus cows based on the spectra collected.

RESULTS AND DISCUSSION

Sample Identification

Milk production and reproduction are closely associated but highly variable. Therefore, it is necessary to compare the production and reproduction of the 16 cows in the final analysis versus the herd in which the cows came from.

Twenty-four estrous periods from 16 cows were used in the final analysis. These cows had an average production of 10,013 kg kilogram (kg) of milk for their current lactation (Table 3). This was slightly above the rolling herd average (9,612 kg) at the Caine Dairy Teaching and Research Center at the time of the trial. Sample and herd milk production was substantially higher than Dairy Herd Improvement Association (DHIA) herds in Utah which averaged 8,728 kg in 1990 (65).

Mature equivalent correction factors are a means of adjusting milk production records for differences in age. The mature equivalent for milk production of the group was 11,153 kg as compared with 10,208 kg for the herd and 9,677 kg for the Utah DHIA average. High production has been associated with a greater incidence of retained placentas and associated reproductive problems (70). Wagner et al. (68) found that high milk production and infertility during the early postpartum period (45 to 90 days) are closely associated. The negative effect which high production exerts on early postpartum fertility might be directly on the endocrine system

TABLE 3. Summary of production and reproduction of cows. (N=16)

Group	Sample Mean	Sample Range	Herd Mean	State Ave[1]
Age in years	4.5	3-8	3.78	3.95
Lactation number	3.3	2-8		
Actual Production	10,013	6,495-12,918	9,612	8,728
Mature equivalent	11,153	6,473-15,427	10,208	9,677
Reproduction				
Serv. Conc.[2]	2.29	1-7	2.00	
Calving interval	13.54	11-17.5	13.30	13.5
Percent detection	83.30[3]		51.00[4]	47.0[5]

[1]Elimination of the cow which had seven services before being confirmed pregnant would lower the services per conception for the trial group by .7.

[2]This is actual detection rate determined from the number of estrous periods observed compared to the number of estrous periods which should have been observed (as determined by palpation per rectum and changes in milk progesterone levels).

[3]This percentage is an estimate from the herds DHIA records.

[4]This percentage is an estimate from the state summary of DHIA records.

or indirectly through a reduction in energy intake. The direct effect on the endocrine system may be an increase in adrenal corticosteriods and suppression of the hormones involved in reproduction. High milk production has been associated with postpartum body weight losses. Studies have associated many of the fertility problems of high milk-producing cows with inadequate nutrient intake (68).

Sample cows averaged 4.5 years in age with a range from three to eight years. The average age at last calving was 3.78 and 3.95 years for the herd and DHIA herds in the state, respectively.

The reproductive performance of the sample cows was similar to the remainder of the herd. Services per conception averaged 2.29 as compared with 2.00 for the herd. However, one sample cow was bred seven times after embryo transfer, before conception occurred. This increased the average services per conception of the sample by .7. Spalding et al. (63) found that cows in the highest quartile of 305-day milk yield had a 20.5% lower conception rate on first service than cows in the lowest yield quartile. They also found that cows in the third and later lactation generally have lower conception rates as well as longer intervals to first service than younger cows. Stress and health problems associated with advancing age are possible reasons for this decrease in reproductive performance. This relationship was also apparent

in this study as the older cows in the sample averaged three services per conception compared with younger cows which averaged 2.17. The calving interval of the sample was also similar to herd and state DHIA averages. The calving interval for the sample averaged 13.54 mos. compared to 13.30 mos. for the total herd and 13.50 mos. for the state.

The estrous detection efficiency was high (83.3%) for the sample cows. This was determined by comparing the number of estrous periods in which primary or secondary signs were observed with the number of estrous cycles as determined by milk progesterone levels and changes in ovarian structures. The estimated DHIA estrous detection efficiency for the herd was 51%. The DHIA detection efficiency is estimated based on the average interval between breedings. The state average for the detection of estrus is 47% from state DHIA records (65).

The estrous detection regimen for this project was quite intensive. The cows were observed for 2.5 hours in the morning between 6 a.m. and 8:30 a.m., again in the afternoon for one hour before the 2:30 p.m. milking, and an hour in the evening starting at 8:00 p.m. In addition to visual observations, a wax crayon was applied to the tailhead and checked daily for removal. Milk progesterone samples were taken at two-day intervals and palpations per rectum on a weekly basis increased the detection rate. The rate obtained in this study is similar to the detection rates found by

Otenacu et al. (52) when three visual observation periods were used for the detection of estrus. They noted a detection of 75% when three 45-minute periods were used at eight-hour intervals.

With visual observation, it is extremely difficult to achieve 100% efficiency. Peter and Bosu (53) found that 89% of the cows in their study ovulated within 21 days of calving, but that only 35% of the total ovulations were associated with behavioral changes at estrus. It has been shown that 70% of mounting activity occurs between 6:00 p.m. and 6:00 a.m. (20, 50). Olds et al. (51) found that night-time activity is more prevalent in hot weather. Hurnik et al. (50) estimated that mounts last 10 seconds or less, making it difficult to verify the primary sign of estrus.

Estrous detection at the Caine Dairy consists of visual observation in the morning and again in the afternoon before milking. Stevenson and Britt (64) found detection rates similar to those at Caine dairy when visual observations alone were used to detect estrus. They found in a control group of 155 expected estrous periods that only 51% were identified using visual observations.

From reviewing production and reproductive data, it appears that there are no adverse effects from NI exposure and the collection of spectra on the reproductive organs, milk production, and reproductive performance of the cows in this

study. Visual observation of the sites of collection showed no apparent adverse reactions from the collection of NI spectra. This is consistent with Conway et al. (16) found no adverse affects in scanning humans with the NIR spectrophotometer to determine body fat composition.

Interactance Spectral Modification

Interactance spectra were collected from 1100 nm to 2500 nm during the project. Figure 3 is an example of a spectra taken on day 0 (estrus). Upon examination of the spectra, it was determined to exclude data for the wavelengths above 1900 nm because of the noise associated with these wavelengths. The major sources of noise in spectral measurements are the glass fibers in the 1.83 m probe.

Probably one of the biggest disadvantages to NI analysis is that, except for perhaps water, we have very little idea as to what the spectrophotometer is actually measuring at a specific wavelength. We can only infer, based on past research, the types of chemicals and bonds seen at specific wavelengths.

There are two regions in which strong absorbers are found as shown in Figure 3. The first region is between 1150 nm and 1250 nm. This region is the bond vibrations associated with C-H stretch (second overtone) with the main structures including CH, HC=CH, CH_2, and CH_3 (37). The second region is

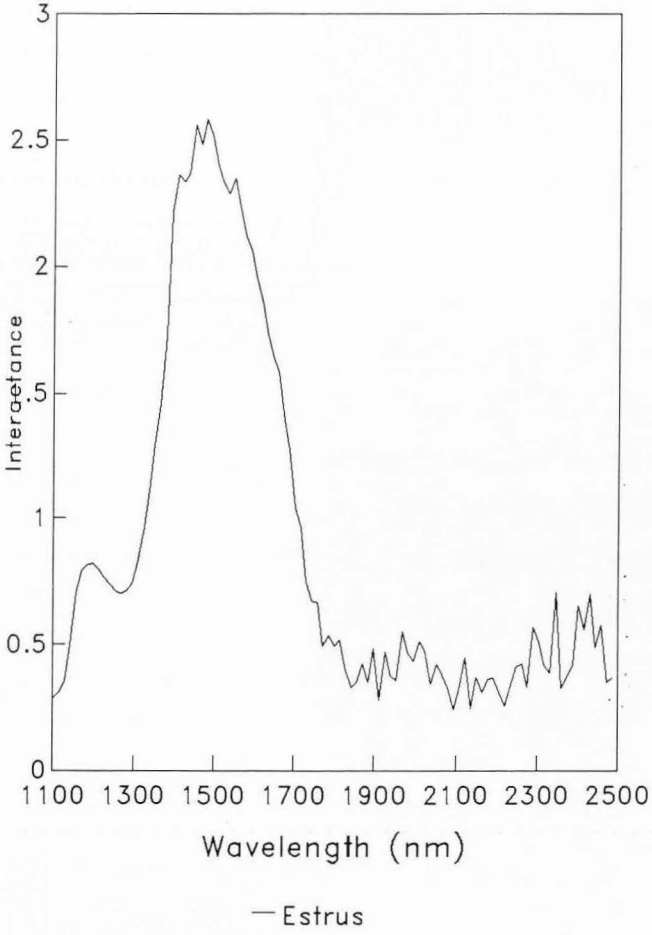

Figure 3. Spectra collected from 1100 to 2500.

from 1350 nm to 1600 nm. This region is probably associated with the bond vibrations, O-H stretch (first overtone) and the N-H stretch (first overtone). The structures associated with this area include ROH, CH_2, aromatic, $CONH_2$, sucrose, starch, CONHR, protein, RNH_2, C=H, and glucose (37).

Regression Analysis

Linear regression is the most common method used to equate spectra with chemistry (37). Multiterm linear regression uses the information at a number of wavelengths to isolate the effect of a single absorber and to normalize the baseline. There are a variety of ways to choose the wavelengths used in multiterm linear regression, the most common being a forward stepwise procedure (step-up). The step-up procedure selects the wavelength giving the best single-term calibration as the first independent variable; then it finds the best wavelength to add as a second variable in a two-term regression, and finally it continues adding variables until some stopping criteria are met. This method is used when there is minimal environmental or chemical noise in the spectra.

Partial least-squares (PLS) regression is a form of linear regression. This method is especially suitable for spectra with both experimental (spectral, environmental, etc.) and chemical noise (72). Partial least-squares differs from

simple linear regression in that it searches and uses all of the information in the spectrum to determine the concentration of the unknown. PLS attempts to explain the largest and most consistent changes in the spectrum avoiding the noise. The main limitations to PLS are that only inconsistent noise can be eliminated and that there is an intercorrelation between the wavelengths.

In the original data set, the dependent variable, day of the estrous cycle, was coded. Day -10 (10 days prior to estrus) was given a code of 1; the remaining days were assigned a positive integer from 2 to 21, estrus being 11 and day +10 (10 days postestrus) being 21. Results of the PLS are found in Table 4. The high number of nonestrus days made it difficult to predict the day of estrus as seen by the R^2 of.03 for the vestibule and an R^2 of .02 for outside the vulva. The standard error of calibration (SEC) was 5.80 for the vestibule and 5.83 for outside on the vulva. The standard error of cross validation (SEVC), which gives an estimate of how a similar data set would behave given this treatment, was 5.86 for the vestibule and 5.88 for the vulva.

The second variable was called heat (estrus vs. nonestrus), the estrus days were coded as 1 and all of the nonestrus days were coded as 2. Table 5 shows the results of this coding. The variable heat had the same problem of unequal frequency as the day of the cycle. The number of

TABLE 4. Results of the partial least squares linear regression of the vestibule and the vulva (N=486). Variable day.

Site	Variable	Mean[1]	SEC[2]	RSQ[3]	SECV[4]	1-RV[5]
Vestibule	Day	11.00	5.80	.02	5.86	.01
Vulva	Day	10.93	5.83	.02	5.88	.00

[1]The coding used for this variable was from 1 to 21 starting on day -10 (10 days prior to estrus) to +10 (10 days postestrus) with estrus being coded as day 11.
[2]Standard Error of calibration.
[3]R^2.
[4]Standard Error of Cross Validation.
[5]r^2.

TABLE 5. Results of the partial least squares linear regression of the vestibule and the vulva (N=486). Variable heat.

Site	Variable	Mean[1]	SEC[2]	RSQ[3]	SECV[4]	1-RV[5]
Vestibule	Heat	1.95	.21	.02	.22	.00
Vulva	Heat	1.95	.21	.02	.22	.00

[1]The coding used for this variable was either 1 for estrus or 2 for nonestrus.
[2]Standard Error of Cross Validation.
[3]R^2.
[4]Standard Error of Cross Validation.
[5]r^2.

estrus days (N=24) was very small compared to the nonestrus days (N=462); this influenced the regression in favor of the nonestrus observations. The R^2 of the vestibule was .02 and the vulva was .02. The SEC for the vestibule was .22 and the SEC for the vulva was .22. The SECV for the vestibule using the variable heat was .22 for the vestibule and .22 for the vulva.

The tremendous amount of variation in the spectra which could not be explained using regression analysis could be due to a number of factors. These include unequal frequencies of estrus and nonestrus observations; pressure differences applied to the probe during collection; individual cow variability; cyclic hormone patterns; and the short intervals at which cellular changes being monitored probably take place.

The problem of unequal frequencies of estrus and nonestrus observations is inherent in the bovine estrous cycle, because for only one day in 21 is a cow in estrus. Winsgry (75, unpublished data) found, in using the NI for body condition scoring in dairy cattle, that the amount of pressure applied during collection can significantly change the spectra. He also found that most of this variation could be eliminated by one person collecting the spectra and being careful to apply equal pressure. The cellular changes which occur at estrus start on day 18 or 19 of the cycle and follow a pattern of increasing cell size and a shift in the cellular

water content from inside to the outside, which reaches its peak at estrus. Because these changes do not occur during the remainder of the cycle, it was difficult to plot a linear function to the cellular changes as they occur only around estrus. Estrogen and progesterone are the main hormones responsible for the changes seen in the vestibule and vulva. These follow a cyclic pattern throughout the cycle. For these reasons other forms of analysis such as Student's t-test and discriminate analysis were used to examine spectral differences throughout the estrous cycle.

Changes in the Spectra: Estrus vs. Nonestrus

The estrus and nonestrus spectra were compared at the same two sites. The spectra from the vulva showed some variation due to the color of the vulva and the inability to collect the spectra without some stray light (Figure 4). The spectra in Figure 4 shows more noise above 1800 nm. This excess noise could have been caused by stray light coming in through the opening of the vulva. The spectra of the vestibular wall did not show this variation (Figure 5).

The spectral math treatments applied to the spectra (log 1/I) include the first and second derivative.

The mean absorbance at each of the different wavelengths was averaged for all the estrus and nonestrus days. The data were then analyzed using a Student's t-test. Results of the

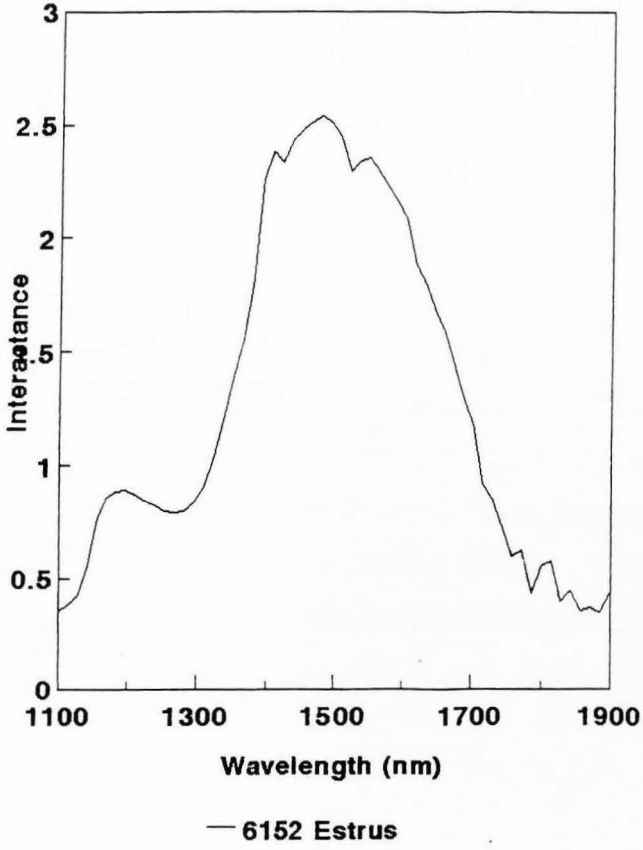

Figure 4. Outside spectra Log (1/I).

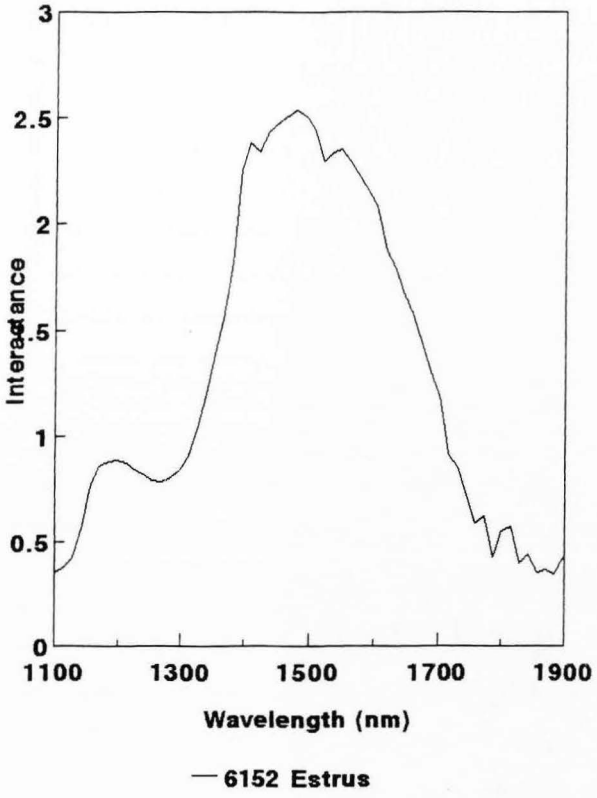

Figure 5. Inside spectra (log 1/I).

t-test showed a number of regions with three or more consecutive means that were significantly different ($P < .05$) (Estrus vs. Nonestrus). Three regions of the spectra which showed this difference were at 1695 nm to 1705 nm, 1790 nm to 1800 nm, 1880 nm to 1900 nm ($P < .05$).

Figure 6 shows the average spectra of the vestibule (First derivative). Spectra changes in the first derivative are difficult to interpret because the changes occur on the sides of peaks and there are peaks which are inverted. The first significant change is found in the region between 1695 nm and 1705 nm. The spectra shows a shift to the right which is probably associated with the change in carbohydrates. This is probably due to a decrease in carbohydrates in the mucus and a decrease in the carbohydrate concentration in the cells of the vulva and vestibule. Differences in carbohydrates in bovine mucus were found by Zaaijer and Van DerHorst (76). Results from their study indicated that differences existed in the carbohydrate composition around estrus, during mid-cycle and late-cycle (76). They found a decrease in the carbohydrate content two days before the onset of estrus. Iacobelli et al. (39) analyzed cervical mucins from the preovulatory and postovulatory period and found a difference with respect to fucose, sialic acid and hydroxy-amino acid content.

The next region, from 1790 nm to 1800 nm, also shows a

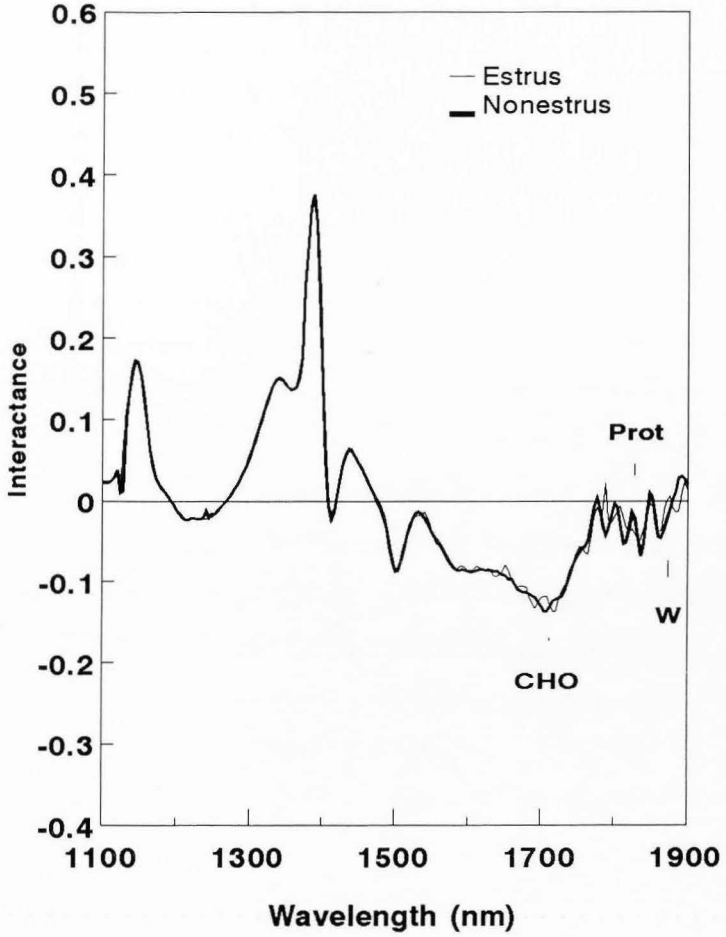

Figure 6. Vestibular spectra 1st derivative
CHO = carbohydrate region.
Prot = protein region.
W = water region.

shift to the right. This region is probably associated with a change in the protein content (37). The literature is unclear as to whether there is a change in the protein content associated with estrus. Evoz et al. (21) found that the cell size during estrus increased 74%, which caused the cell membrane to become thinner due to the distension of the cell with mucus.

The last region in Figure 6 to show a change is the region between 1880 nm and 1900 nm. The spectra in this region show a shift to the right and a depression in peak height. This region is probably associated with the changes in water (37). The change in the water region could be a combination of the changes in the water content of the vaginal cells and an increase in the water content of the vaginal mucus. Ezov et al. (21) noted that the vulvular mesenchyme is 74% heavier during estrus than during diestrus. The additional weight is about 75% water and 25% dry matter, as evidenced by the absence of change in the percentage of these components at estrus compared to diestrus (21). The additional weight per unit of tissue resulted from an increase in the mean weight per cell. Hawk et al. (34), working with the uterus, found that the endometrial water content and water distribution varied between estrus and the luteal phase. Cows in estrus had the highest water content and highest percentage of water in the extracellular phase. The changes in the

bovine uterus result from the effects of rising levels of estrogen.

The changes in all three regions could also be explained as an increase in water content simply by the water dilution of both the carbohydrate and protein fractions of the vestibular cells and mucus.

The second derivative is the most commonly used math treatment for the spectra. Figure 7 shows the average spectra (estrus vs. nonestrus) of the vestibule. When Figures 6 and 7 are compared, the changes in the three regions are more easily seen in the second derivative; however, the significance at each wavelength was lower. The changes in the spectra follow the same pattern as the first derivative, showing a decrease in the carbohydrate and protein content and an increase in the water content when estrus and nonestrus were compared.

The vulvular spectra showed the fewest significant wavelengths. This could be due to excess noise since the opening of the vulva is part of the site for spectral collection. Another possible reason could be excess manure on the vulva, although care was taken to remove it. Figure 8 shows the average spectra (estrus vs. nonestrus) of the vulva (first derivative). These changes are similar to the changes found inside the vestibule, mainly a decrease in carbohydrate and protein concentration and an increase in the water in

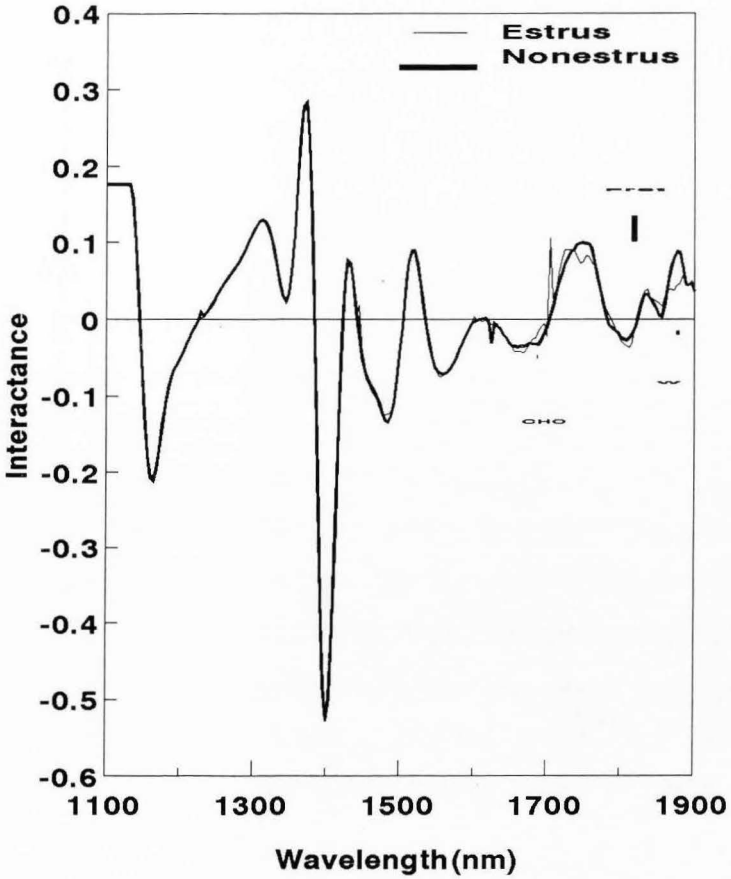

Figure 7. Vestibular spectra second derivative.
CHO = carbohydrate region.
Prot = protein region.
W = water region.

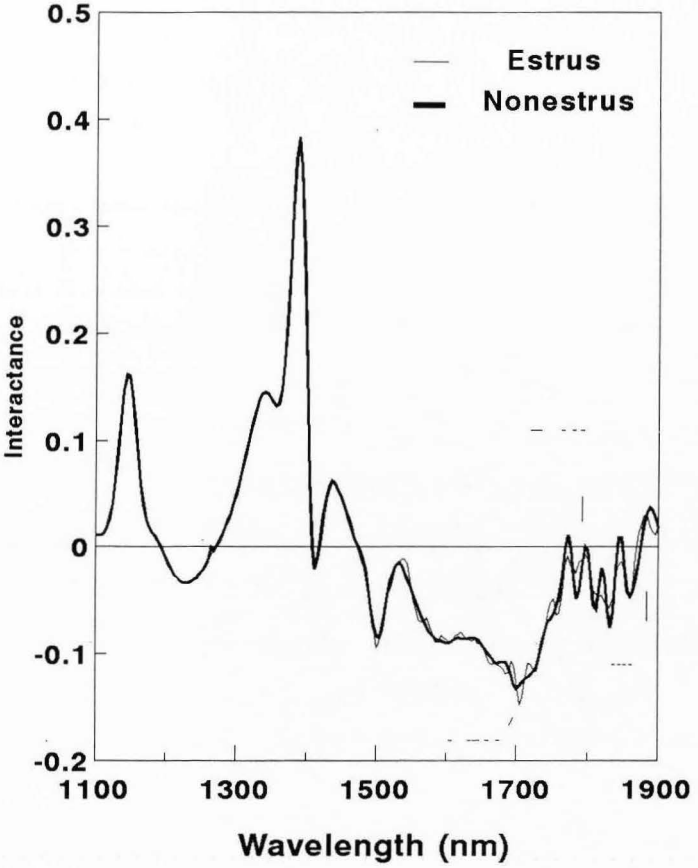

Figure 8. Vulvular spectra first derivative.
CHO = carbohydrate region.
Prot = protein region.
W = water region.

preparation for mating. Figure 9 shows the average spectra (estrus vs. nonestrus) of the vulva (second derivative). The vulva spectra, both first and second derivative, show a pattern similar to those found inside the vestibule, but not as pronounced.

Discriminate Analysis

Wavelengths from the three regions identified by the t-test were used in a discriminate analysis (inside and outside, first and second derivative). The discriminate analysis was done to see if these wavelengths could be used to discriminate between the estrus and nonestrus spectra. The results using discriminant analysis of the vestibular spectra in the first derivative are shown in Table 6. The table shows that of the 24 estrous periods, 21 (87.5%) of the estrous periods were correctly identified. Although the discriminate analysis identified 87.5% of the estrous periods correctly, it also identified 116 (25.11%) of the 486 days which were nonestrus as estrus. The average error rate of prediction for Table 6 is 26%.

It was important to determine at which stage of the estrous cycle the classification errors occurred. Table 7 is a frequency distribution of the nonestrus days misclassified as estrus by the discriminate analysis. The results show that 31% of the misclassified days were two days either side of

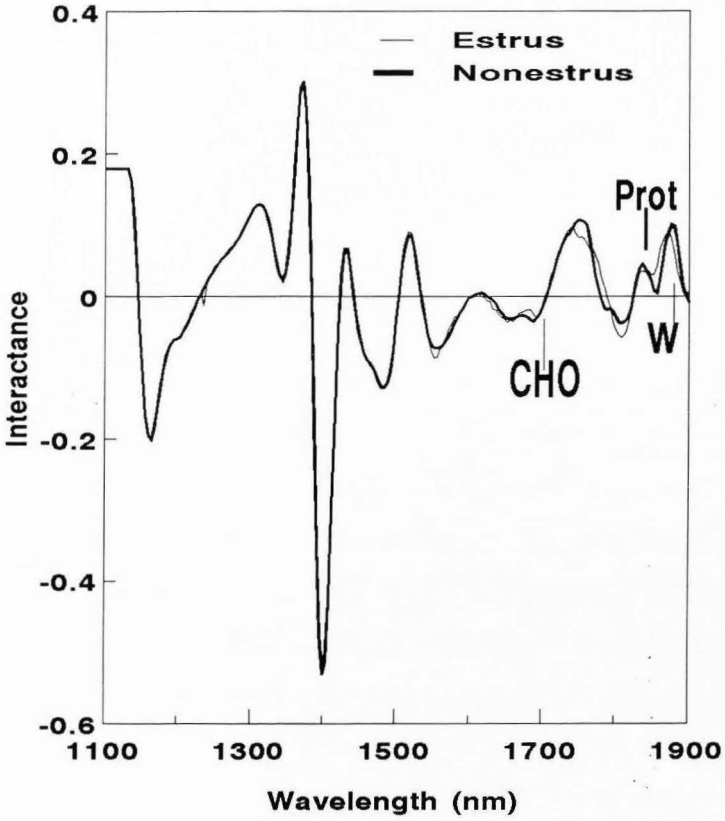

Figure 9. Vulvular spectra second derivative.
CHO = carbohydrate region.
PROT = protein region.
W = water region.

TABLE 6. Results of the discriminate analysis of the Vestibule in first derivative (Error rate of 26%).

	Estrus	Nonestrus	Total
Estrus	21[1]	3	24
Nonestrus	116[2]	346	462
Total			
Percent	137	349	486

[1]The number of estrous periods that were correctly classified as being in estrus (87.5%).

[2]This number represents days which were not in estrus and were misclassified as being in estrus (25.11%) the first derivative and inside the vestibule was 26%.

TABLE 7. Days that were misclassified as estrus.

Day of cycle	Frequency
-9	1
-8	5
-7	7
-6	5
-5	6
-4	6
-3	5
-2	8
-1	13
1	8
2	7
3	6
4	4
5	8
6	5
7	7
8	3
9	5
10	7
Total	116

estrus with the remaining misclassified days scattered randomly throughout the cycle. This seems to indicate that there is a greater likelihood to misclassify a nonestrus day, as estrus approaches. This is to be expected, since most of the histological and chemical changes associated with estrus occur in a narrow window three to four days before and after estrus.

Results of the t-test showed fewer significant wavelengths for the vestibule in the second derivative than in the first derivative; therefore, fewer wavelengths were used in the prediction (Table 8). Table 8 shows that of the 24 estrous periods, only 16 (66.67%) were correctly identified. The discriminate analysis also identified 177 (38.71%) out of 486 nonestrus days as estrus. The average error rate of prediction for the second derivative was 37%.

Table 9 shows the results of the discriminate analysis for the vulva in the first derivative. This shows that of the 24 estrous periods, 17 (70.83%) were correctly identified. The discriminate analysis also identified 153 (33.12%) out of 486 nonestrus days as estrus. The average error rate of prediction was 32%.

The fewest significant wavelengths were found using the vulva in the second derivative (Table 10). Table 10 shows that of the 24 estrous periods, 17 (70.83%) were correctly identified. The discriminate analysis also identified 162

TABLE 8. Results of the discriminate analysis of the vestibule in second derivative (Error rate 37%).

	Estrus	Nonestrus	Total
Estrus	16[1]	8	24
Nonestrus	177[2]	285	462
Total			
Percentage	193	293	486

[1]The number of estrous periods that were correctly classified as being in estrus (66.67%).

[2]This number represents days which were not in estrus and were misclassified as being in estrus (38.31%).

TABLE 9. Results of the discriminate analysis of the vulva in first derivative (Error rate 34%).

	Estrus	Nonestrus	Total
Estrus	17[1]	7	24
Nonestrus	153[2]	309	462
Total			
Estrus	170	316	486

[1]The number of estrous periods that were correctly classified as being in estrus (70.83).

[2]This number represents the number of nonestrous periods that were misclassified as being in estrus (33.12).

TABLE 10. Results of the discriminate analysis of the vulva in second derivative (Error rate of 34%).

	Estrus	Nonestrus	Total
Estrus	17[1]	7	24
Nonestrus	162[2]	300	462
Total			
Percentage	179	307	486

[1]The number of estrous periods that were correctly classified as being in estrus.

[2]This number represents the number of nonestrous periods that were misclassified as being in estrus.

(35.06%) out of 486 nonestrus days as estrus. The average error detection rate was 34%.

In reviewing the results in tables 6 to 9, it becomes apparent that the best results of the discriminate analysis were found using vestibular spectra in the first derivative (Table 6). The best results were probably found inside the vestibule because of a cleaner collection site and the changes in the vaginal mucus would combine with the cellular changes to give a more pronounced change. However, even the best results using discriminate analysis show that substantial variability has yet to be accounted for. Although changes exist in the vestibular spectra between estrus and nonestrus, detection of estrus using these data would result in a large portion of nonestrus cows being identified as being in estrus.

CONCLUSIONS

The results of this study indicate that the NIR spectrophotometer coupled with a fiber optic probe can be used to detect changes in the vulva and the vestibule of the bovine during the estrous cycle. The regions most sensitive to discriminatory analysis include the 1700 nm, 1790 nm, and 1880 nm regions and were similar between the two sights of collection. These regions are probably associated with the decreased carbohydrates and protein concentration and an increase in water. Changes are most likely brought about by increasing levels of circulating estrogen during the time of estrus. The best discriminatory results from the study were noted using the vestibular spectra in the first derivative. There are several reasons for this. The spectra of the vestibule had less noise because the collection probe could be positioned so there was less stray light entering the vestibule. The vulva possesses some external color differences that affected the spectra, which were not found in the vestibular tissue. Although care was taken during the experiment to make sure the sight of collection was clean, the vulva is still more likely to be contaminated with manure and other foreign material that could have affected the spectra.

The reason the first derivative math treatment was better than the second was probably that many unknown constituents

changed during the estrous cycle. This made it even more difficult to identify the actual chemical or cellular changes occurring at the time of estrus. The first derivative measured the slope of the various constituents, and the changes in the spectra occurred on the side of the peaks. The first derivative inside also had some inverted peaks in the three regions of change.

The results of this experiment were consistent with other experiments with the chemical and cellular changes that occur at estrus. Estrus is associated with decreases in carbohydrate and protein concentrations and an increase in the water content. The increase in the water content of the vaginal mucus and the shift in the cellular water from the inside to the outside of the cell may have had a dilution effect on the other constituents.

During the experiment, many problems were encountered which could have affected the results. One of the biggest difficulties was in restraint of the cows during the collection of the spectra. Although the cows did not seem to show any signs of pain, they seemed to be nervous when their vulvas were manipulated to collect the spectra of the vestibule. This improved as the cows became accustomed to the daily regimen. Another difficulty faced during this experiment was the loss of cow numbers. This was mainly due to the strict requirements we had for a positive estrus. The

way to overcome this would be to increase the number of cows in the beginning sample. Upon completion of the project, it was observed in another study using the NI spectrophotometer that the amount of pressure applied at the sight of collection changed the spectra. Although care was taken during the experiment to apply equal pressure, three different people were involved in the collection of the spectra. If the trial were to be repeated, it would be necessary to train one person to collect the spectra in order to eliminate this variation.

The results of this study indicated that the NI spectrophotometer can detect compositional changes in situ. Other studies are now underway to determine the body fat composition in sheep and the use of the NIR spectrophotometer in condition scoring of dairy cattle.

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APPENDIX

Appendix A
Progesterone Assay Procedure[a]

All components must be at room temperature before use.

1. Plain Tubes: Label four plain (uncoated) 12x75 mm polypropylene tubes total counts (T) and nonspecific binding (NSB) in duplicate.

2. Coated Tubes: Label fourteen Progesterone Ab-Coated tubes A (maximum binding) and B through G in duplicate. Label additional antibody-coated tubes, also in duplicate, for controls and patient samples.

3. Pipet 100ul of the zero calibrator A into the NSB and A tubes, and 100 ul of each of the calibrators B through G into correspondingly labeled tubes. Pipet 100 ul of each control and patient sample into the tubes prepared.

4. Add 1.0 ml of buffered (^{125}I) progesterone to each tube. Vortex.

5. Incubate for 3 hours at room temperature.

6. Decant thoroughly.

7. Count each tube for one minute in a gamma counter.

[a]Coat-A-Count^{R} Progesterone Assay Kit
Diagnostic Products Corporation
Los Angeles, CA. 90045