

Effects of Oestrogenic Silage on Some Clinical and Endocrinological Parameters in Ovariectomized Heifers

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Nwannenna A.I., A. Madej, T.J.-O. Lundh and G. Fredriksson: Effects of oestrogenic silage on some clinical and endocrinological parameters in ovariectomized heifers. Acta vet scand. 1994, 35, 173-183. – The influence of phytoestrogens was studied in 3 ovariectomized Swedish Friesian heifers fed 20 kg of 100% red clover silage per heifer/day for 14 days. Behaviour, reproductive organs and pituitary response to exogenous gonadotropin-releasing hormone (GnRH) injections were monitored. Clinical effects like oedema and mucous discharge in the vulva, presence of milky fluid in the mammae and increases in teat size and the cross-sectional distance of the uterus were observed in heifers fed red clover silage. Fluid accumulation in the uterus, visualized by means of ultrasonography, had still not disappeared 30 days after the red clover silage had been completely withdrawn. Red clover silage appeared to reduce the magnitude and duration of the pituitary response to GnRH injections.

reproductive organs; LH; GnRH; phytoestrogens; red clover.

Introduction

Phytoestrogens are oestrogen-mimics produced in high amounts by leguminous plants such as clover, lucerne and soyabeans. Red clover is quantitatively the most important source of phytoestrogens in Sweden. The concentration of phytoestrogens in red clover is normally about 0.5-2.5% of dry matter (Pettersson *et al.* 1984). The amount of phytoestrogens in red clover varies very little between different varieties of red clover. A greater variation depends on other factors such as climate (Pettersson *et al.* 1984), phosphorus sup-

ply and light (Cocks *et al.* 1980). The concentration of phytoestrogens in animal feed also depends on the time of harvest and methods for preservation. Field cured hay reduces the oestrogenic activity by about 70% (Nilsson 1960). On the other hand, ensiling not only preserves the oestrogenic activity (Kallela 1975, Kallela 1980) but even increases it (Jorgensen & Freymiller 1974, Kallela 1980). Oestrogenic compounds from plants are classified as isoflavones and coumestans and their structure are similar to non-steroidal synthetic oestrogens (Price & Fenwick 1985). It has long been known that phytoestrogens cause both temporary and permanent infertility in sheep (Bennetts *et al.* 1946, Adams 1989). Reproductive disturbances have also been reported in cattle in some countries (Thain 1966, Kallela

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1968, Kallela *et al.* 1984, Pettersson *et al.* 1984, Shemesh & Shore 1987). However, it has been suggested that the ingestion of phytoestrogens by cattle might result in temporary but not in permanent infertility (Adams 1989). Although the reason(s) for this difference in response has been the subject of controversy (Braden *et al.* 1971, Lundh *et al.* 1990), it seems probable that dissimilarities in phytoestrogen metabolism are involved. The manner in which phytoestrogens disturb the oestrogen and gonadotropin balance in animals has mainly been studied in sheep (Findlay *et al.* 1973, Hearnshaw *et al.* 1977, Rodgers *et al.* 1980, Bindon *et al.* 1982, Adams & Martin 1983, Montgomery *et al.* 1985). To our knowledge, no hormonal studies have been carried out in cattle exposed to phytoestrogens. The aim of this experiment was therefore to monitor changes in mammary glands and reproductive organs and the LH response to administration of exogenous GnRH in ovariectomized heifers fed red clover silage.

Materials and Methods

Animals and feeding

Three clinically healthy, normally cycling heifers of the Swedish Friesian Breed, about 2 years old and weighing 550-600 kg, were used. In November, the heifers were bilaterally ovariectomized through a flank incision as was described by Åström (1972) but using other premedication. Briefly, sedation was performed with Rompun® *vet.* (Bayer) (0.1 mg/kg of xylazine *i.m.*) including local nerve block with Xylocaine® adrenaline (Astra) (10 mg/ml of lidocaine and 5 µg/ml of adrenaline). The mesovarium was slowly crushed with the ecraseur and the ovary retained in the hand. Then, the peritoneum, musculature and skin were sutured. The general condition of the animals was scarcely affected by the operation.

The heifers were kept indoors and normally received about 15 kg of nonestrogenic hay (mainly Timothy grass) and 750 g concentrates (39% barley, 39% oats, 11% soya, 7% rapeseed and 4% other additions) per heifer per day except during experimental feeding periods. However, they had free access to mineral licks and good drinking water at all times. To provide phytoestrogens the animals were fed red clover silage during the experimental period. The high performance liquid chromatographic method described by Pettersson & Kiessling (1984) was used to determine the oestrogen contents of homogenous samples taken from hay, concentrates, and red clover silage fed to the heifers in this experiment. The hay and concentrates contained no oestrogens. The red clover silage contained a total of 1.74 g oestrogens (1.01 g formononetin, 0.03 g daidzein, 0.61 g biochanin-A, and 0.08 g genistein) per kg wet weight. The fodder for the heifers was gradually changed by decreasing the proportions of hay and increasing the proportions (20%, 30%, 50%, 50%, 75%, 75%) of red clover silage over a period of 6 days to allow the rumen microbial populations in these heifers to adapt. From day 7 to day 20 the fodder consisted entirely of red clover silage. At 0730 and 1430 h each heifer received a maximum of 10 kg silage per feeding, corresponding to about 35 g phytoestrogens per day. Beginning on day 21, silage was gradually replaced with hay over another period of 6 days.

Blood sampling

Two months after ovariectomy, intravenous jugular catheters were introduced into the heifers under local anaesthesia. Prior to feeding with red clover silage and on the last day of maximum red clover feeding, 10 ml blood samples were collected into heparinized tubes from each heifer at 10 min intervals for 6 h

(0830 - 1430 h). A single intramuscular injection of 100 µg synthetic GnRH (gonadorelin; Nialutin[®], Boeringer Ingelherm Agrovvet/BI-vet) was given to each heifer 60 min after the start of sampling. All blood samples were centrifuged immediately after collection. Plasma samples were stored at -20°C until analysis.

Clinical examination

The condition of the reproductive organs and behaviour of all heifers were clinically evaluated prior to the beginning of the feeding experiment. Subsequently, these examinations were repeated twice a week until slaughter. Observations on udder growth, its consistency and signs of milk secretion were done accordingly. Teat length was measured according to the method of *Gardnar & Adams (1986)* except that permanent markers were used instead of tattoos. To measure teat circumference, we used thin, long nylon strings, calibrated (mm) in the middle along a distance of 10 cm. Each cm interval was given a different colour. The string was circled round the base of the teat and the circumference was recorded. Contact between teat and string was ascertained without pressure on teat or space between teat and string.

The colour of the vulva was recorded, and it was examined for signs of mucous discharge and oedema. Arbitrary scores were assigned to changes in each parameter, ranging from 1 (no change) to 5 (severe change). The sum of the scores for changes in the 3 parameters was recorded for each period of vulva examination.

Uterine response was assessed by rectal palpation and transrectal ultrasonography. The uteri were palpated and scanned 3 days before ovariectomy, 4 days before the feeding of red clover silage started and, thereafter, twice weekly. Palpation was used to check for the presence of tone. Uterine tone was scored

from 1 (flabby or atonic) to 5 (contracted and firm).

Ultrasound examinations were performed using an Aloka SSD-210DX (Aloka Co. Ltd., Tokyo, Japan) portable real-time B-mode linear scanner with a 5 MHz transducer inserted into the rectum. The uterus was located by rectal palpation and then scanned around the bifurcation. Screen images of the uterine horns were then frozen for cross-sectional distance measurements, examination of the uterine lumen and Polaroid[®] photography.

Heifers were slaughtered 30 days after the last red clover silage feeding. The uteri were then visually examined to check phytoestrogen effects.

Changes in teat length and uterine diameter were expressed in percent of individual pre-treatment values.

Hormone assay and analysis of LH data

Luteinizing hormone concentrations were measured in all blood samples using a heterologous radioimmunoassay validated for bovine plasma according to *Forsberg et al. (1993)*. Assay sensitivity for 100 µl plasma, calculated from the precision profiles, averaged 0.7 µg/l. The intra-assay coefficients of variation were < 8% for concentrations exceeding 1 µg/l. Inter-assay coefficients of variation were < 10.6% for concentrations of 3.2, 6.6, and 11.7 µg/l

LH values in µg/l were plotted against collection time (6 h) for each heifer to visualize the magnitude and duration of response to GnRH injection in each case. Area under the curve (AUC), in mm², for each profile was determined using an electronic integrator (Digiplan, Kontron Messgeräte, FRG). The area due to pituitary response was calculated by subtracting the area above basal levels that follow injection of GnRH from the total area. The basal LH level for GnRH treatment pro-

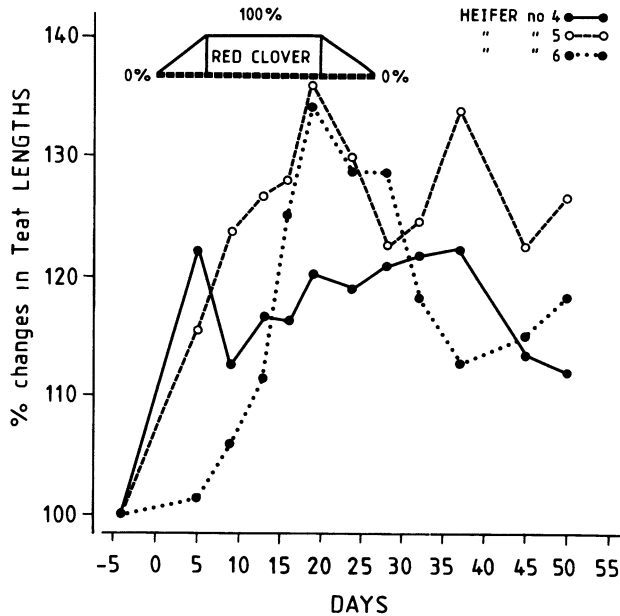


Figure 1. Percent change in teat length in relation to the initial values for individual ovariectomized heifers fed red clover silage. The timing of the introduction and withdrawal of silage is shown within the box entitled RED CLOVER.

files was calculated as the mean of all values before GnRH injection plus their 2 standard deviations. The duration of LH response to GnRH was calculated as the time interval between the point of GnRH injection and the point at which the response curve dropped back to basal levels. Peak time was defined as the time interval between injection of GnRH and the highest LH concentration within the response interval.

Results

Clinical examination

The percent change in average teat lengths, uterine diameters, and the scores for vulva as well as uterine tone, are presented for each heifer in Fig. 1-4.

Teat length increased by 22% within 5 days in heifer no. 4. In heifers nos. 5 and 6 teat length

peaked at about 35% on day 18 (Fig. 1). Changes in teat circumference were negligible. Milky fluid secretion from mammae was observed in heifers nos. 4 and 5.

In all 3 heifers the vulva responded to red clover by changing colour (from pale to pink), swelling, and discharging mucus. The sum of the response scores was almost identical in all heifers. The responses became apparent 13 (no. 4) and 16 (nos. 5 and 6) days after introducing red clover silage and continued to increase until a peak was reached 6 days after complete withdrawal (Fig. 2). Just before slaughter vulva scores were still at level 3 in 2 heifers, whereas in the third heifer it had dropped to zero.

Prior to ovariectomy palpation indicated that the uteri had a very strong tonus (score = 5) in heifers 4 and 5 and moderate tonus (score = 3) in heifer no. 6. Corresponding ultrasonogra-

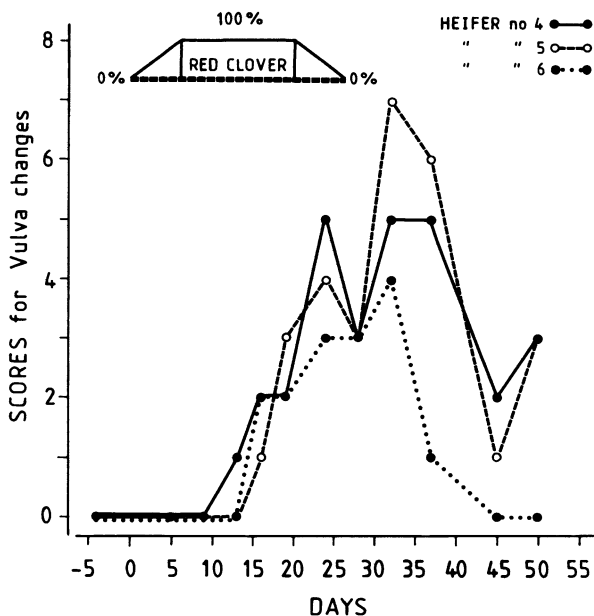


Figure 2. Vulva scores for individual ovariectomized heifers fed red clover silage. The timing of the introduction and withdrawal of silage is shown within the box entitled RED CLOVER.

phy revealed clear echogenic endometrial folds with a nonechogenic, stellate lumen for heifers 4 and 5. A more clearly distinguishable myometrium and homogenous grey lumen without evidence of interstitial oedema was seen in heifer 6. Heifers nos. 4 and 5 were confirmed to be in oestrus based on the presence of the characteristic oestrous discharge from the vulva a few hours before ovariectomy.

Two months after ovariectomy, and prior to the feeding of red clover silage, all uteri were hardly palpable and lacked tone (Fig. 3). The ultrasonographs showed cornua that were very much reduced in size and lacked demarcation between myometrium and endometrium. In response to the ingestion of red clover silage, uteri gradually acquired tonus and became palpable. Uterine tone reached a maximum between days 19 and 24 and declined thereafter (Fig. 3). Ultrasonography re-

vealed cross sections of uterine horns that were increased in size in a fluctuating manner, sometimes with mixed patches of echogenic and nonechogenic areas in the lumen. The percent increase in the average diameter of the uterine horns reached a maximum on day 13 in heifer 4 (30%), day 20 in heifer 5 (50%), and day 28 in heifer 6 (40%) (Fig. 4).

On 1 of the red clover silage exposure days, 1 heifer seemed restless and mounted other heifers.

After slaughtering the heifers, gross examination of the reproductive genitalia revealed accumulations of greyish watery fluid in the anterior vagina (15-23 ml) of all 3 animals and in the uterus (8 and 15 ml) of 2. The anterior third of the left ovarian tube with a diameter of 4 mm in heifer no. 4 was blocked and contained fluid. Cervices were relaxed and the external os felt patent on palpation.

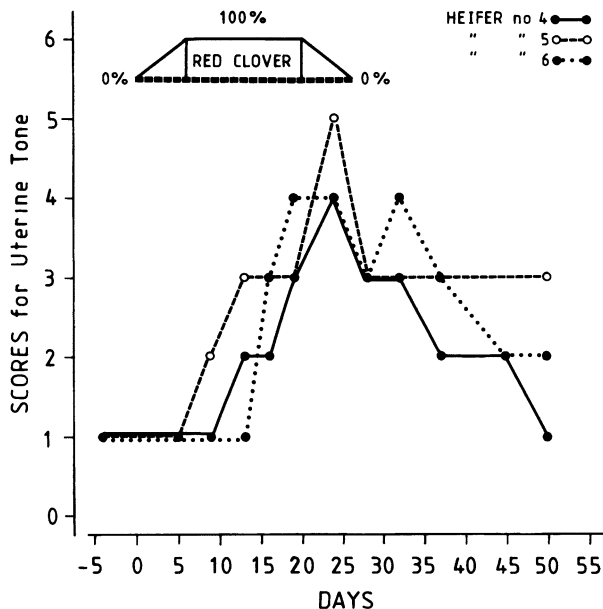


Figure 3. Uterine tone scores for individual ovariectomized heifers fed red clover silage. The timing of the introduction and withdrawal of silage is shown within the box entitled RED CLOVER.

LH values

Plasma LH concentrations increased greatly within 5-10 min of injecting GnRH, in every case where grass hay or red clover silage was being ingested (Fig. 5). The duration of this response was longer during hay feeding than during exposure to red clover silage in 2 heifers (293 min vs 226 min in heifer 4 and 264 min vs 247 min in heifer 5). The time that elapsed before this response reached maximum was also 10 min longer during hay feeding in both these heifers. Additionally, it appeared that both the total and response AUC were greater during the control period in the same heifers (i.e., total AUC – 4823 mm² vs 4338 mm² in heifer 4 and 4377 mm² vs 2894 mm² in heifer 5; response AUC – 3286 mm² vs 2486 mm² in heifer 4 and 3017 mm² vs 2099 mm² in heifer 5). Heifer no. 6 responded oppositely.

Discussion

This paper reports on the effects that oestrogenic red clover has on the mammary glands and the reproductive organs of ovariectomized heifers. Teat length reached a maximum after about 12 days of maximum exposure to red clover and declined about 2-3 weeks following withdrawal. Similar observations were made by us (Nwannenna 1993) in a similar experiment with ovariectomized ewes and by Adams (1977) in wethers grazing on oestrogenic subterranean clover over a period of 10 days. The change observed in the teat circumference of ewes was not seen in these heifers.

The increase in vulva scores during red clover feeding is in agreement with clinical field observations made by Adler & Trainin (1960) who reported swollen vulvae with hyper-

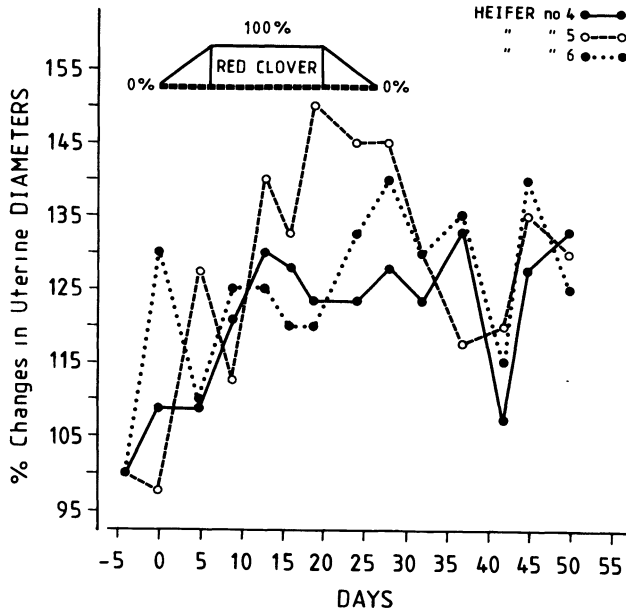


Figure 4. Percent change in the uterine diameter, in relation to the initial values, for individual ovariectomized heifers fed red clover silage. The timing of the introduction and withdrawal of silage is shown within the box entitled RED CLOVER.

aemic, glistening mucous membrane in alfalfa-fed heifers in various stages of their oestrous cycle.

The uterine response to ovariectomy included a reduction in the cross-sectional distance of the uterine horns by an average of about 20% and decrease in the tone score from 3-5 to 1. These responses were gradually reversed when heifers were exposed to red clover silage. *Pierson and Ginther* (1987) found that the ultrasonographic appearance and mean distances from the dorsal surface of the uterine body to the ventral surface were influenced by the stage of the oestrous cycle. The mean distances were highest around ovulation. Similarly, clinical examination of anestrous cows will show a flabby and atonic uterus which reverses and becomes erect and turgid during oestrus (*Settergren* 1980, *Ro-*

berts 1986). Endometrial folds and the myometrium were visible in the ultrasonographic pictures taken 3 days before oestrus and ovariectomy in 2 of our heifers. *Fissore et al.* (1986) reported that it is usually possible to distinguish the endometrial folds and myometrium as well as small accumulations of fluid in the uterine lumen with ultrasonography in oestrous cows. They suggested these findings to be due to the high oestrogen concentrations typical of the perioestrous period. In our study, this endometrial fold architecture seen before ovariectomy became distorted, with disorganized locations of fluid pockets, when heifers were exposed to phytoestrogens. We presumed that this distortion represented some kind of pathology which could be analogous to the doughy and pendulous uteri found in cows grazing on oestrogenic subterranean

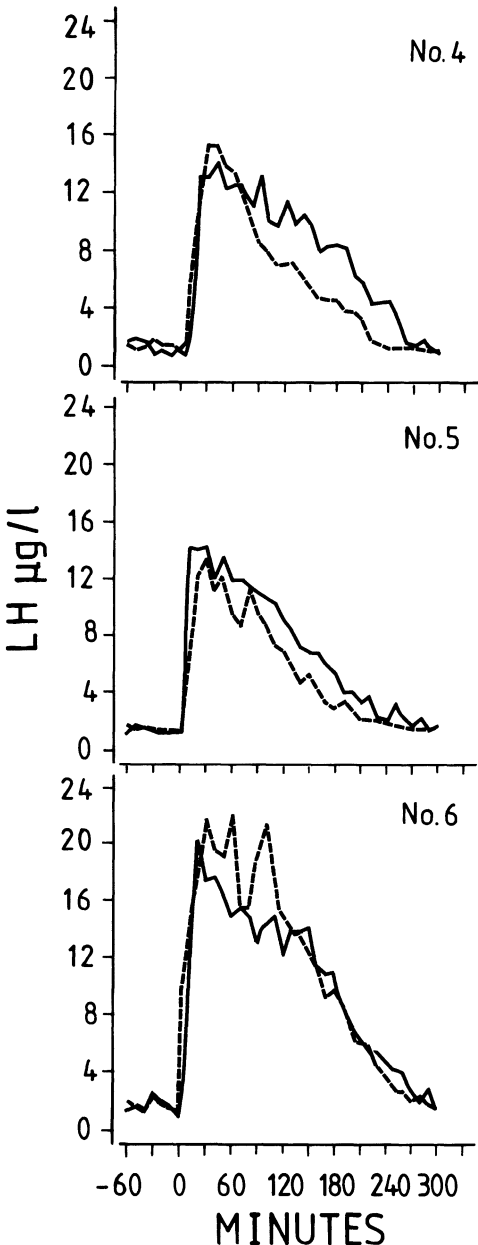


Figure 5. Plasma LH concentrations in individual ovariectomized heifers injected with GnRH before (—) and during (-----) exposure to phytoestrogens.

clover by *Thain* (1966). Further more, this is probably a primary stage (in these heifers) of the cystic endometrial condition reported by *Bennetts et al.* (1946) in ewes grazing subterranean clover pasture.

The mounting behaviour seen in one of our heifers is in agreement with previous reports of mounting in heifers on red clover pasture (*Kallela* 1968) and heat periods in cattle grazing on alfalfa (*Adler & Trainin* 1960) as well as the pattern of sexual behaviour in ovariectomized heifers treated with oestradiol (*Katz et al.* 1980).

The synthetic decapeptide known as GnRH acts on the anterior pituitary to initiate a release of LH in intact (*Schams et al.* 1974, *Jensen et al.* 1983) or ovariectomized heifers (*Hausler & Malven* 1976). Our findings of a lower total as well as a reduced response LH area under the curve after GnRH injection in 2 heifers fed red clover silage compared to when they were fed hay are in agreement with the report by *Hinshelwood et al.* (1986). These authors found that the release of LH in response to GnRH in ovariectomized cows differed, depending on when GnRH was administered in relation to oestradiol-17 β pre-treatment. The area under the LH curve was smaller for cows receiving the GnRH injection 2.5 h after oestradiol-17 β than for those receiving the placebo. No such difference was found when GnRH was administered 5 h after oestradiol-17 β injection. In ovariectomized cows the concentration of LH can be inhibited by oestradiol-17 β for up to 12 h (*Rhodes et al.* 1980, *Madej et al.* 1981, *Kesner et al.* 1981). These authors concluded that the first phase of this inhibitory period, i.e. 2-4 h after oestradiol administration may occur owing to a relatively low responsiveness to GnRH. Plasma concentrations of formononetin and its metabolite daidzein in cows fed red clover silage increase significantly within 1 h after

feeding (Lundh *et al.* 1990). Furthermore, Markiewicz *et al.* (1993) found that daidzein had oestrogenic activity as a result of its interaction with oestrogen receptors. The data on low LH response to GnRH during phytoestrogen exposure, found here, require corroboration in future experiments using several more animals.

In conclusion, a daily intake of 35 g phytoestrogens from Swedish red clover silage induce changes in the behaviour, reproductive organs and may affect the pituitary response to GnRH in ovariectomized heifers.

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Sammanfattning

Kliniska och endokrinologiska förändringar hos ovariectomerade kvigor som utfodrats med ostrogen silage.

Inverkan av vaxtostrogener studerades på 3 ovarie-

ektomerade kvigor, svensk låglandsboskap (SLB), som vardera utfodrades med 20 kg 100 procentigt rodtkloverensilage per dag i totalt 14 dagar. Förändring i beteende, reproduktionsorgan och hypofysens respons på exogent gondadotropinfrisättande hormon (GnRH) undersöktes. Kliniska effekter som ödem och förändring av mucus i vulva, mjölksekretion samt ökning i spenstorlek och uterusstorlek

observerades under rodtkloverensilageutfodring. Ackumulering av vatska i uterus påvisades med ultraljudsteknik. Denna vatskeansamling var bestående även 30 dagar efter avslutad rodtkloverutfodring. Rodtkloverensilage tycks reducera magnituden och varaktigheten på hypofys-responsen av GnRH.

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