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I am submitting herewith a thesis written by Samuel G. Duncan entitled "Reproduction and Lactation in the Rat: A Bioassay for Fescue Toxicity." I recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Animal Science.

Gerald R. Bratton, Major Professor

We have read this thesis
and recommend its acceptance:

Accepted for the Council:

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Graduate Studies and Research

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REPRODUCTION AND LACTATION IN THE RAT:

A BIOASSAY FOR FESCUE TOXICITY

A Thesis

Presented for the

Master of Science

Degree

The University of Tennessee, Knoxville

Samuel G. Duncan

December 1980

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DEDICATION

This thesis is dedicated to my wife, Deborah, who made it possible, my daughter, Ann, who spent many fatherless nights while I studied, and often was the object of misdirected frustrations. To my mother, Betty, who somehow provided impetus in her statement "They can't take what you know" but still leaves her major question "So now what can you do?" unanswered and to my dad, E. L., whom I don't think has ever been surprised by the latest announcement, whatever it may be.

Sam Duncan

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ABSTRACT

The objective of this research was to develop a quick, easy to interpret, inexpensive bioassay of potentially dangerous fescue pastures. A further aim was to identify the mechanisms of action of the toxin(s) of fescue in aglactia and abortion reported to occur in mares.

The feeding of ground fescue hay to laboratory rats may be an effective way to assay for fescue toxicity, thus circumventing the tedious and costly process of extraction in developing a dependable bioassay of toxic fescue. Although the results of using extracts of fescue and the ground hay are similar, neither adequately distinguishes between toxic or non-toxic forages when reproductive efficiency and lactation of rat dams are assessed. However, the rat pups seem to be able to distinguish the toxic from the non-toxic forages as a result of in utero changes. The gestation ration fed to dams influenced pup survival in this study ($P<.01$) and pup hemoglobin ($P<.05$). Conducting the bioassay at low (1.1 C) environmental temperatures enhanced the toxic effects of the fescue on pup survival. Pups nursing fescue fed dams had a lower (40%) survival rate than did the controls (90%).

TABLE OF CONTENTS

CHAPTER		PAGE
INTRODUCTION		1
	A. Assumption and Limitations	3
	B. Historical Considerations	4
	C. Survey of Recent Research	5
I.	MATERIALS AND METHODS	9
II.	RESULTS	19
III.	DISCUSSION	29
IV.	CONCLUSION	48
LITERATURE CITED		50
VITA		56

LIST OF TABLES

TABLE		PAGE
1.	Summary of the parameters for trials 1, 2 and 3	18
2.	The effects of aqueous and alcoholic extracts on reproductive performance of rats, trial 1	20
3.	The effects of aqueous and alcoholic extracts of fescue on the hemoglobin values of male and non-pregnant female rats, trial 1	21
4.	The effects of temperature and ration on the reproductive performance of rats, trial 2	22
5.	The effects of lactation ration of dams maintained at 28.9 C, on pup survival, average 5-day weight gain, and stomach milk content of crossfostered pups, trial 2	23
6.	The effects of lactation ration of dams maintained at 1.1 C on pup survival, average 5-day weight gain, and stomach milk content of crossfostered pups, trial 2	24
7.	The effects of ration on the reproductive performance of rats, trial 3	25
8.	The effects of ration on milk volume, total solids, and percent fat of the rat, trial 3	27
9.	The effects of gestation ration of dams on pup survival, average 5-day weight gain, stomach pH, stomach milk content, and hemoglobin of rat pups, trial 3	28
10.	Composition of rations fed during trials 2 and 3	30
11.	Chemical composition of fescue forages fed to rats	31

INTRODUCTION

Fescue grass is readily adapted to all soils and environmental variations prevalent in the southeastern United States and for this reason it has been suggested as the grass of choice for most classes of livestock. However, it does on occasion, cause economic losses in horses reported to range in excess of $500,000 per year (Hutton, 1980). In the last 2 decades there has been considerable effort to elucidate the causative agent or agents that produce fescue toxicity, yet the answers have not been forthcoming. Cattle, sheep and perhaps horses continue to present with fescue foot, hard fat lesions, reproductive losses, weight loss, or decreased milk production.

Some method of evaluating potentially dangerous pastures would be a valuable asset at the present time. Several models have been evaluated and most have used extracts of fescue. Cattle have been used as an experimental animal, (Cunningham, 1949; Bowman, et al., 1973; Lorey, et al., 1973; Williams, et al., 1973; Yates, et al., 1973) but they require expensive housing and costly feed. Crickets have also been used as a bioassay model (Assay et al., 1975). A high degree of mortality in the crickets was associated with the cation fraction of extracts of fescue. However, this entity is probably different from that associated with "fescue foot" since the toxic compound in this syndrome appears to be associated with the anionic

fraction. Cats, dogs, sheep, and guinea pigs have been used but have failed to produce a consistent response (Yates, et al., 1962). Rats have also been used and abortion and stillbirth in this species has been associated with both aqueous and alcoholic extracts of fescue (Daniels, et al., 1973; Jacobson, et al., 1973; Williams, et al., 1973), as well as skin and tail temperature changes.

Tennessee, Georgia, and Missouri veterinarians have described thickened placentas, weak foals, stillbirth, abortion, and aglactia as commonly associated signs of fescue toxicity in mares. A recent survey conducted by the Tennessee State extension veterinary personnel (personal communication) revealed that fescue abortion and aglactia have been reported for 50 counties in Tennessee.

The reputedly high incidence of fescue-related abortion and aglactia in mares needs to be confirmed. Furthermore the continued diagnosis of this problem in mares grazing fescue pastures suggests the need for a model to evaluate potentially dangerous pastures. However, the cost of maintaining adequate numbers of mares mitigates against the use of this species to test the toxic effects of tall fescue. The rat, on the other hand, is less expensive to feed and house, reproduction is quicker and dosing is simplified in the rat allowing the use of smaller volumes of hay or extracts (Daniels, et al., 1973).

The purpose of this study was to develop a bioassay that was inexpensive, easily performed and easily

Our aim in developing the bioassay was to use ground hay instead of extracts in order to achieve a less expensive, quicker assay of pasture fields. In addition the research was aimed at identifying the mechanisms underlying aglactia in the mare, as well as the abortion phenomenon. We also wanted to assess the survival of the neonate if birth was successful.

A. Assumptions and Limitations

A major assumption was that agalactia in the rat would mimic agalactia seen in the horse. However, hormonal control of lactogenesis and galactopoieses in the rat is different than in the horse. In the rat, prolactin is luteotropic rather than lactogenic as in the horse, thus possibly seriously limiting the extrapolation of rat lactation data to the horse.

The rat can yield young at a much faster rate than can the horse or cow. This then leads to the assumption that the laboratory rat would make a valuable model to assess the effects of fescue on reproduction. Using the rat allows removal of individual animal variations as litter mates can be crossfostered across treatments. The serious limitation in this assumption is that the mare has a diffuse placenta and the rat has a discoid type placenta. It is not known if there is a difference in the rate of transfer of nutrients across these types of placentas or if the rat will

respond to the same stimulus in utero as will the horse. The length of time the rat fetuses are exposed to the toxicants of fescue may also be so short that they do not yield a similar response, e.g., the thickened placenta of the mare.

B. Historical Considerations

Fescue grass (festuca arundincea schreb) is variously known as Randall grass, Williams' grass, Reed fescue, Evergreen grass, Giant fescue, "Wonder grass," Ditch bank fescue, King fescue and Tall fescue. Cultivars also exist, such as Kentucky 31 (KY-31) or G1-306, G1-307, and Kenwell to further complicate the identification of toxic pastures.

Various crossbreds such as fescue x rye or fescue x bluegrass hybrids also show signs of toxicity (Yates, et al., 1973; Bruchner, et al., 1973).

The common environmental conditions involved in fescue toxicity was summed up by Klussendorf (1955), when he characterized the situation by saying that toxic fescue usually grows on wet, poorly drained land or on banks of ditches where water is abundant. He reported that the grass was coarse and unpalatable, being eaten only when other pasture grasses were not available. He concluded that because fescue is a tall coarse grass, it often sticks up above the snow that covers the weaker pasture plants, thus

accounting for the more predominate number of fescue toxicity cases reported during the winter.

Merriman (1955) reported that cattle in Tennessee presented additional symptoms to those that had previously been described. He found that affected cattle had an increased respiration and a decreased heat tolerance, as well as an increase in sensitivity to sunlight. He also reported that affected cattle tended to stay in ponds while unaffected cattle continued to graze. Merriman also reported that affected cattle were often emaciated, stood with an arched back, and appeared anesthetized. Additionally, a bioassay involving rats fed fescue seed had a decreased feed intake.

C. Survey of Recent Research

Fescue toxicity has been associated with levels of nitrogen fertilization (Mott, et al., 1971; Garner, et al., 1973; Rumsey, et al., 1979; Studeman, et al., 1973; Jacobson, et al., 1973). Increased incidence of fescue toxicity cases parallels increased nitrogen levels. Aspergillus terres, and Fusarium sp. fungal invasions (Yates, et al., 1969; Molina, et al., 1978; Grove, et al., 1973; Farnell, et al., 1973; Ellis, et al., 1968) have also been shown to decrease feed intake and cause lesions of fescue foot. Perloline and perlolidine alkaloid levels in fescue have also been cited as possible causitive agents (Boling et al., 1975; Robbins,

et al., 1973; Yates, et al., 1962; Gentry, et al., 1969; Bruchner, et al., 1973; Hemken, et al., 1979). Environmental temperatures have been incriminated in the fescue toxicity syndrome. Cold weather (Pulsford, 1950; Garner, et al., 1973; Jacobson, et al., 1973) and hot weather (Merriman, 1957, Studeman, et al., 1973, Hemken, et al., 1979) have been shown to decrease animal performance.

Fescue contains a vasoactive compound (Cunningham, 1949; Walls, et al., 1970; Garner, et al., 1973; Yates, et al., 1973; Williams, et al., 1973; Jacobson, et al., 1963, 1973; Bowman, et al., 1973; Corley, et al., 1973) which has been linked to decreased blood flow in cattle. Corley, et al., (1973) has characterized a 50% decrease in flow to the limbs while blood vessels have swollen walls and decreased lumen size in areas adjacent to necrotic tissue. Regional blood flow has been determined in the rat fed both alcohol and water extracts of fescue. Blood flow is decreased to many major organs (Bruckner, et al., 1973). However, effects on blood flow to the reproductive organs or mammary organs have not been assessed.

The interruption of blood flow in the extremities can be a result of thrombosis initiated by <u>in vivo</u> platelet aggregation or by erythrocyte aggregation (Corley, et al., 1973). The exact cause has not been established nor is there total agreement on the change in blood vessels, perivascular edema, hair follicle hemorrhage, or congestion.

Williams, et al., (1973) has documented these changes while Garner, et al., (1973) only rarely observed vascular edema and in general reported negative histological findings in fescue toxicity. Unremarkable histopathological findings have also been reported by Daniels, et al., (1973), Corley et al., (1973), Jacobson et al., (1973) and Bruckner, et al., (1973). These authors have not, however, reported on the reproductive system.

Internal temperatures are increased and tail temperatures are decreased in cattle and rats (Jacobson, et al., 1963, 1973; Garner, et al., 1973; Yates, et al., 1973; Corley, et al., 1973), further suggesting changes in the vasculature of the extremities. Extensive studies of histological changes have not been reported although dry gangrene and ischemia have been described in both species of animals.

The preceding suggestions of vasoactivity are further substantiated by reports of agalactia not only in cattle and swine (Bowman, et al., 1973), but it also may be a problem in horses grazing fescue pastures (Daniels, et al., 1973; Garret, 1980; Gibbons, 1968). However, mammary histology has not been reported for animals grazing fescue or fed extracts. Reproductive problems associated with fescue pastures have been reported in cattle (Studeman, et al, 1973), sheep (Yates, et al., 1973), swine and horses (Daniels, et al., 1973; Garret, 1980; Gibbons, 1968).

Daniels, et al., (1973) has further shown that stillbirths and resorption of fetuses occur in the rat fed fescue extracts. He also reported lower litter weight gains for pups from fescue fed dams and a decrease in hemoglobin of these pups.

CHAPTER I

MATERIALS AND METHODS

Three feeding trials were conducted in an attempt to develop a bioassay for fescue toxicity. The question of possible abortants in fescue was evaluated by these reproductive critera:

(1) number of pups born alive
(2) number of pups stillborn
(3) number of decidua (implantation sites in the uterus)
(4) histology and histopathology of the uterus.

Aglactia reported to be produced by fescue was studied by:

(1) number of pups surviving to 5 days after birth
(2) five day weight gain of surviving pups
(3) quantity of milk obtainable at the end of five days of lactation
(4) milk total solids and fat content
(5) histology and histopathology of the mammary glands

The role of the neonate in its own survival was assessed by:

(1) stomach milk content of the pups at the end of 5 days of nursing
(2) stomach pH, if the pup survived 5 days, to determine the ability of the pups to utilize milk obtained from the dam.

An additional parameter was measured in the search for a consistent change easily detected. This parameter was the hemoglobin of the dams at parturition (trial 1), and at 5 days post-partum (trial 3). Hemoglobin was also measured in the pups (trial 3). These values were determined since

hemoglobin values have been reported to be lowered in rats given fescue extracts (Daniels, et al., 1973).

A total of 126 nulliparous Sprague-Dawley rats were used in these trials (trial 1 also utilized 19 males of the same strain and colony). Feed and water were given ad libitum, and 14 hours of light were provided. The basal ration for all trials was a commercial feed (Purina Lab Chow). When this ration was to be mixed with a fescue or orchargrass hay (trials 2 and 3) both the commercial feed and the hay were ground separately in a Wiley Mill (20 mm screen) and mixed after grinding.

Matings were confirmed by vaginal smears and the day that sperm were found in the smear was designated day 1 of gestation. Females were housed individually in plastic boxes 51 x 23 x 16.5 cm for the duration of gestation, except for trial 1, where males remained with the females until day 20 of gestation.

In all trials animals were observed daily for signs of toxicity. At parturition females were observed for signs of dystocia (increased squealing, prolonged parturition, and uterine hemorrhage) or abnormal behavior.

Trial 1

This trial was conducted with 39 rats housed at an environmental temperature of 84 F (28.9 C). The fescue hay used in trial 1 was obtained from Blount County, Tennessee,

and had been blamed for the abortion of three Arabian mares. Two fescue extracts were made after the procedure described by Williams, et al., (1975). The grass was chopped and steeped for 7 days in either 95% ethanol, or distilled water. After 7 days the extract was filtered through several layers of cheesecloth, steeped again for 4 days and strained again through cheesecloth. The hay was then allowed to steep for 1 additional day and strained as before. The extracts obtained were then combined, e.g., water with water, alcohol with alcohol fractions. These extracts were then dried with charcoal filtered air until they were almost dry. Following sufficient drying the extracts were reconstituted with distilled water so that 1 ml contained the equivalent of either 1 mg or 2 gm of fescue based on the original dry weight of the hay that was extracted. A control to evaluate any effects from an ethanol residue was prepared by filling a beaker with ethanol which was allowed to sit for the same amount of time that the extracts were steeped. The ethanol was then dried in the same manner as the extracts and rehydrated with distilled water.

These extracts were administered by stomach tube daily, at doses of 1 ml. The extracts were given from day 12 of gestation to the day of parturition.

Immediately after parturition the dams were sacrificed by cervical dislocation and blood was collected for hemoglobin determinations (American Optical Hemoglobinometer, Beckman DU-2 spectrophotometer). An abdominal

incision was made and the uterine decidua counted. The uteri were removed and fixed in neutral buffered formalin for later histological examination. Males were sacrificed at the same time and in the same manner as the bred dams. Blood was collected and hemoglobin determinations were made.

Trial 2

The alkaloid content of fescue has been incriminated by others in fescue toxicity studies (Hemken, et al., 1979, Yates, et al., 1973). The fescue hays used during trial 2 were obtained from Dr. L. P. Bush, University of Kentucky, Lexington, Ky. The Kentucky-31 (Ky-31) variety of fescue contained approximately twice the amount of perloline alkaloids as the G1-307. These hays had been shown to decrease milk production in dairy cattle (Hemken, et al., 1979).

In trial 2 four rations were fed. The rations were all compounded with the basal commercial feed to contain 16% of: (1) orchardgrass (CO),(2) fescue G1-307 (LF) a low perloline alkaloid variety, (3) Kentucky-31 (HF) a high perloline cultivar, and (4) the control ration (CC) which was the basal commercial feed without added grass. Orchardgrass was selected as the control forage in this trial because it is the second most abundant forage grass in Tennessee (J. Burns personal communication).

The rations were fed to 24 females maintained at an environmental temperature of 84 F (28.9 C) and to 24

females maintained at an environmental temperature of 34 F (1.1 C), to test the role of ambient temperature in the fescue toxicity syndrome. Females were divided into 4 groups of 6, in both environments.

Pups born in trial 2 were crossfostered such that each dam kept two of her own pups and received 2 pups from each of the other three treatment groups. This gave the dams standarized litters of 8 pups. It has previously been shown in our laboratory that 8 pups are optimal for litter weight gains (Whitworth, 1967).

Pups were allowed to nurse for 5 days, and were weighed daily. On day 5 of nursing, pups were decapitated and the abdomens opened. The stomach was removed and emptied of its contents. The stomach milk contents were then weighed to assess lactation of the dams.

Two dams of each ration group were selected for vascular permeability studies to determine if vascular abnormalities were evident in the adults. The dams were anesthetized using .05 mg/kg Nembutal interperitonealy. A 1% solution of Evans' Blue dye was injected into the left femoral vein and allowed to circulate for 1 hour. The dams were then sacrificed by cervical dislocation and examined for evidence of vascular permeability.

The other dams were sacrificed on day 5 of lactation. Uteri and mammary glands were collected for histological study. The tissues were fixed in 10% neutral

buffered formalin. Two animals from each treatment were perfused with 2.5% glutaraldehyde, after heparin administration, to provide histological fixation controls. Mammary tissues were embedded such that the four treatments were represented in each paraffin block to provide processing controls. Routine hemotoxylin and eosin stains were used for both the uterus and mammary glands.

Trial 3

Trial 3 utilized 58 females and was conducted similar to trial 2. The exceptions were: (1) only the 1.1 C environmental temperature was used, since it was found that this temperature had more effect than did the 28.9 C temperature of trial 2. (2) an additional ration containing 16% corncobs (CD) was used to test the effects of added fiber that might have been responsible for the results of trial 2. (3) the hay used for extracts in trial 1 was fed at 2 levels (16% and 50%) to determine if the ground hay would yield the same results as extracts of stockpiled hay (SP).

Some additional parameters were also measured during trial 3. The weights of the paired adrenal glands of the dams were obtained. Milk volume and quality were assessed in 2 dams from each treatment. Quality was assessed by the total solids and fat of the milk. Total solids were determined by drying 60 ul samples in aluminum

drying pans for 16 hours at 60 C. After weighing, the dried sample was transferred to a filter crucible containing 10 gms of sand. The crucible and sand had been dried for 4 hours at 500 C. The crucible, with sample, was then placed in an ether extraction apparatus and allowed to extract for 4 hours in order to determine the fat content of the milk. Milk was obtained by the use of a milking machine designed after Cox (1937)(Figure 1). Oxytocin (Pitocin) was given to the dams intramuscularly (5 I.U.) following general anesthesia with .05 mg./kg Nembutal. Hand milking was employed when mammary glands were not to be collected for histological sections.

Pups were color coded with marking pens to assess the effects of gestation ration, lactation ration, and the survival of pups in each treatment group. Stomach pH of rat pups was measured. In measuring the stomach pH the fundic region of the stomach was measured rather than the pyloric area to eliminate the possibility of increased HCl flow as a result of expressing the curd from the stomach.

Selenium and vitamin E injections were given to 6 rats from each group on day 18 and on the day of parturition. The dose given was based on the recommended dosage for mares and was calculated to be .4 ml/rat (Biotec).

Proximate and mineral analysis of the feedstuffs were done by routine procedures described in the AOAC (1975).

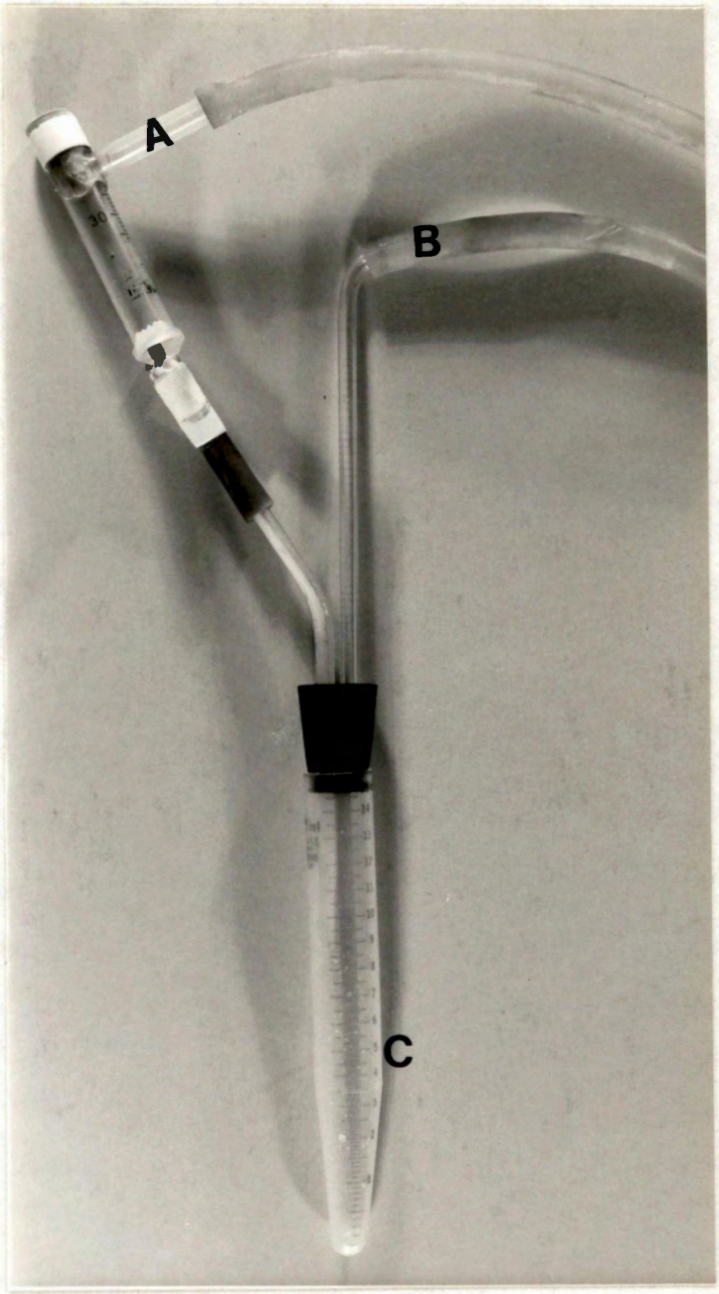


Figure 1. Teat cup - A, A 3cc syringe barrel, with a needle guard welded to the side. A pulsating vacuum is applied here (7 mmHg) to massage the teat, (20-25 pulses per minute). The vacuum may be broken by finger, (two man operation), or by increasing the hose length, by foot, (one man operation) or by a conventional dairy pulsing unit.

B, is connected to a constant vacuum of 7 mmHg, and milk is collected in the graduated cylinder, C.

The hays were further tested for the presence of parasitic, toxin producing, saprophytic species as described by Tuite (1969). The hay was cut to 5 mm lengths and 3 to 4 pieces placed on a sterile agar plate and incubated for 48 hours at room temperature.

The results of these trials were analyzed by analysis of variance procedures, functional categorical analysis (Chi-square analysis) was performed on the pup data, and means were compared by orthagonal contrasts. These procedures have been described by Goodknight and Barr (1979).

Table 1 summarizes the parameters for trials 1, 2, and 3.

TABLE 1

SUMMARY OF THE PARAMETERS
FOR TRIALS 1, 2 and 3

	Trial 1	Trial 2	Trial 3
No. of pups born alive	X	X	X
No. of pups stillborn	X	X	X
No. of decidua	X	X	X
Litter birth weight	X	X	X
Dam hemoglobin	X		X
Male hemoglobin	X		
Pup hemoglobin			X
5-day weight gain (pups)		X	X
Stomach milk content		X	X
Stomach pH (pups)			X
Milk volume, total solids, and fats (dams)			X
Pup survival for 5 days		X	X
Adrenal gland weights (dam)			X
Uterine histology	X	X	X
Mammary histology		X	
Proximate analysis (ration)		X	X
Selenium levels of hays used			X
Fungal cultures of hay used	X	X	X
Feed intake			X

CHAPTER II

RESULTS

The results of trial 1 indicated that there were no differences between the control dams and the treated dams for the number of pups born alive, the number of pups stillborn, the number of decidua, embryonic mortality or dam hemoglobin. These results are summarized in Table 2. However during this trial the males receiving the 1 mg water extract had lower hemoglobin value than the control males or non-pregnant females ($P<.01$, Table 3).

The feeding of ground toxic fescue hay and orchardgrass in trial 2 gave no indication as to which hays might be toxic. The number of pups born alive, the number of pups stillborn, the number decidua and embryonic mortality were not effected by ration or ambient temperature. These results are summarized in Table 4.

Pup survival was decreased in the litters nursing fescue fed dams at 28.9 C, but the 5-day weight gain of survivors showed no difference between forages. Stomach milk content of pups was lower in the fescue fed groups than in the controls (.31 gms vs. .15 gm, Table 5).

At 1.1 C stomach milk content and pup survival were again higher in the control groups, and 5-day weight gain was not different (Table 6).

The results of trial 3 have been summarized in Table 7. The number of pups born alive, the number of pups

TABLE 2

THE EFFECTS OF AQUEOUS AND ALCOHOLIC EXTRACTS ON REPRODUCTIVE PERFORMANCE OF RATS, TRIAL 1

	control	water		alcoholic	
		1 mg	2 gm	1 mg	2 gm
No. of litters	3	4	5	3	5
Pups born alive (P<.1)	5.3* ±3.9	6.75 ±2.2	9.2 ±2.7	5.0 ±3.2	12.2 ±1.2
Pups stillborn (P<.6)	.6 ± .3	1.0 ± .7	1.2 ± .5	3.3 ±2.8	.2 ± .2
No. of decidua (P<.2)	8.3 ±4.2	11.4 ±4.1	12.4 ±1.2	9.5 ±3.2	13.0 ±1.1
Embryonic mortality (%)	28.9	32.0	16.1	12.6	4.6
Dam hemoglobin gm/100 ml (P<1.0)	10.0 ±2.0	8.0 ±.8	8.6 ±.36	9.0 ±.5	8.6 ±.36

* Means ± standard error, except embryonic mortality which is means only.

TABLE 3

THE EFFECTS OF AQUEOUS AND ALCOHOLIC EXTRACTS OF FESCUE ON THE HEMOGLOBIN VALUES OF MALE AND NON-PREGNANT FEMALES, TRIAL 1

	Control	H_2O ext.	ETOH ext.	Non-pregnant Females
No. Animals	3	9	7	5
Hemoglobin G/100 ml	10.8 ±.4	7.5 ±.6	9.9 ±.6	10.7 ±.4

* Means ± standard error.

($P < .01$)

TABLE 4

THE EFFECTS OF TEMPERATURE AND RATION ON THE REPRODUCTIVE PERFORMANCE OF RATS, TRIAL 2

	Ration							
	CC		CO		LF		HF	
	84	34	84	34	84	34	84	34
No. of litters	6	6	3	4	6	5	4	4
Pups born alive (P<.6)	9.6 ±1.1	11.0 ±1.5	12.0 ±1.5	12.2 ±2.0	9.6 ±1.9	10.4 ±2.8	11.5 ±1.9	11.7 ±2.0
Pups stillborn (P<.2)	.16 ± .16	.5 ± .3	1.0 ± .5	.25 ± .25	.6 ± .3	4.2 ±2.5	2.0 ±1.6	.25 ± .25
No. of decidua (P<.2)	11.0 ±1.4	15.4 ± .8	13.0 ±1.7	15.5 ± .86	13.5 ±1.3	16.0 ± .5	13.5 ±1.4	13.7 ±1.4
Embryonic mortality (%)	11.3	15.4	0.0	19.6	24.4	8.8	0.0	12.7

* Means ± standard error, except embryonic mortality which is means only.

CC, control; CO, orchardgrass; LF, low perloline fescue, (G1-307)
HF, high perloline fescue, (Kentucky-31)

TABLE 5

THE EFFECTS OF LACTATION RATION OF DAMS MAINTAINED AT 28.9 C, ON PUP SURVIVAL, AVERAGE 5-DAY WEIGHT GAIN, AND STOMACH MILK CONTENT OF CROSSFOSTERED PUPS*, TRIAL 2

	CC	CO	LF	HF
No. of pups born	44	24	53	32
Pup survival (%)	90 ±.04	87 ±.06	77 ±.05	78 ±.07
5-day weight gain (gms)	3.2^{a} ±.4	1.9^{b} ±.2	2.8^{a} ±.28	1.0^{b} ±.3
Stomach milk content (gms)	$.31^{a}$ ±.02	$.35^{a}$ ±.04	$.28^{b}$ ±.02	$.15^{b}$ ±.02

* Means standard error. ($P < .001$)
Means with different superscripts are different.

CC, control; CO, orchardgrass; LF, low perloline fescue, (G1-307); HF, high perloline fescue (Kentucky-31)

TABLE 6

THE EFFECTS OF LACTATION RATION OF DAMS MAINTAINED AT 1.1 C ON PUP SURVIVAL, AVERAGE 5-DAY WEIGHT GAIN, AND STOMACH MILK CONTENT OF CROSSFOSTERED PUPS*, TRIAL 2

	CC	CO	LF	HF
No. of pups born	51	32	32	32
Pup survival (%)	90^{a} $\pm.02$	65^{b} $\pm.08$	40^{c} $\pm.08$	71^{b} $\pm.08$
5-day weight gain (gms)	6.5^{a} $\pm.3$	7.9^{b} $\pm.9$	3.1^{c} $\pm.5$	6.1^{a} $\pm.7$
Stomach milk content (gms)	$.23^{a}$ $\pm.01$	$.1^{b}$ $\pm.01$	$.08^{c}$ $\pm.02$	$.15^{a}$ $\pm.03$

* Means standard error. ($P<.001$)
Means with different superscripts are different.

CC, control; CO, orchardgrass; LF, low perloline fescue, (G1-307); HF, high perloline fescue (Kentucky-31)

TABLE 7

THE EFFECTS OF RATION ON THE REPRODUCTIVE PERFORMANCE OF RATS, TRIAL 3

	CC	CD	CO	LF	HF
No. of litters	10	10	8	6	7
Pups born alive (P<.1)	8.9* ±1.1	10 ±.5	6.75 ±1.4	8.8 ±.79	6.0 ±1.9
Pups stillborn (P<.5)	1.8 ± .4	1.7 ±.5	3.5 ±2.1	.8 ±.4	2.4 ± .5
No. of decidua (P<.3)	12.2 ± .8	14.1 ±.7	17.6 ±5.2	11.5 ±1.1	10.7 ±1.7
Embryonic mortality (%)	12.29	17.02	41.82	16.52	21.49
Adrenal weight (mg) (P<.04)	63.18[a] ±4.4	62.7[a] ±4.4	57.0[a] ±5.0	70.1[b] ±7.0	77.4[b] ±7.6
Hemoglobin gm/100 ml (P<.4)	11.8 ± .4 (3)	11.3 ± .5 (8)	12.8 ±1.0 (3)	11.8 ±.7 (5)	10.9 ±.4 (4)

* Means ± standard error, except embryonic mortality which is means only. Mean with different superscripts are different.

CC, control; CD, corncob ration; CO, orchardgrass; LF, low perloline fescue, (G1-307); HF, high perloline fescue (Kentucky-31)

stillborn, the number of decidua, embryonic mortality and hemoglobin of dams did not differ. However, during this study the adrenal glands were found to be heavier in the fescue fed dams than the controls (63.18 mg vs. 70-77.4 mg). Milk volume, total solids, and percent fat were not different (Table 8).

Pup data for trial 3 is summarized in Table 9. Pup survival was lower in all groups of trial 3 than in trial 2. The orchardgrass and Kentucky 31 groups in trial 3 had higher survival rates than did the control or the corncob fed group. The group receiving the low perloline fescue G1-307 had the lowest survivial rate.

Five day weight gain did not indicate the toxic forage. The controls did not differ from the Kentucky-31 group, nor did the corncob gestated group differ from the G1-307 fescue. The orchardgrass group made the greatest gains in this trial.

Hemoglobin values were lower in the G1-307 fescue gestated group than the control, corncob or orchardgrass group, but the Kentucky-31 group pups had the highest hemoglobin values.

Stomach milk content of pups in trial 3 were the same in all groups. However, stomach pH was less acid in the G1-307 fescue group and the Kentucky-31 than the controls, corncob or orchardgrass group.

TABLE 8

THE EFFECTS OF RATION ON MILK VOLUME, TOTAL SOLIDS, AND PERCENT FAT OF THE RAT, TRIAL 3

	CC	CD	CO	LF	HF
No. of animals (dams)	6	6	4	5	6
Volume (ml) ($P<.2$)	.23* ±.06	.24 ±.07	.35 ±.10	.30 ±.03	.14 ±.07
Total solids (%) ($P<.2$)	28.9 ±1.1	27.4 ±2.1	28.4 ±2.0	24.1 ±2.2	28.7 ±1.2
Fat (%) ($P<.9$)	8.9 ±1.4	9.6 ±1.6	9.7 ±1.9	8.5 ±1.6	10.3 ±1.7

* Means ± standard error.

CC, control; CD, corncob ration; CO, orchardgrass; LF, fescue (G1-307), HF, fescue (Kentucky-31).

TABLE 9

THE EFFECTS OF GESTATION RATION OF DAMS ON PUP SURVIVAL, AVERAGE 5-DAY WEIGHT GAIN, STOMACH pH, STOMACH MILK CONTENT, AND HEMOGLOBIN OF RAT PUPS*, TRIAL 3

	CC	CD	CO	LF	HF
Pup survival (%) ($P<.01$)	46^{a} (56) $\pm.06$	46^{a} (97) $\pm.05$	67^{b} (70) $\pm.05$	32^{c} (53) $\pm.06$	65^{b} (45) $\pm.07$
5-day gain (gms) ($P<.07$)	1.42^{a} (26) $\pm.27$	2.5^{b} (45) $\pm.31$	3.0^{c} (47) $\pm.27$	2.1^{b} (17) $\pm.5$	1.8^{a} (30) $\pm.4$
Stomach milk content ($P<.5$) (gms)	.1 (23) $\pm.02$	.1 (34) $\pm.03$	.1 (33) $\pm.02$	.1 (17) $\pm.02$	.1 (29) $\pm.03$
Stomach pH ($P<.005$)	4.1^{a} (20) $\pm.26$	3.9^{a} (15) $\pm.23$	3.6^{a} (21) $\pm.25$	5.8^{b} (4) $\pm.7$	4.7^{b} (23) $\pm.3$
Hemoglobin (gms/100 ml) ($P<.001$)	11.3^{a} (2) $\pm.3$	10.5^{a} (21) $\pm.4$	10.8^{a} (18) $\pm.5$	9.2^{b} (13) $\pm.4$	14.8^{c} (6) $\pm.4$

* Means $\pm$ standard error.
Means with different superscripts in rows are different.

( ) number of pups

CC, control; CD, corncob ration, CO, orchardgrass; LF, fescue (G1-307); HF, fescue (Kentucky-31)

CHAPTER III

DISCUSSION

Management factors

Management factors such as nitrogen fertilization levels of pastures (Garner, et al., 1973; Studeman, et al., 1973), the presence of fungi on hay or pastures (Ellis, et al., 1969; Yates, et al., 1969) selenium levels of the feed (hay) (Garret, 1980) and the alkaloid level (Gentry, et al., 1969; Buckner, et al., 1973; Robbins, et al., 1973; Boling, et al., 1975) of fescue have all been cited as causative agents in the fescue toxicosis syndrome. Management factors in these trials were held constant, e.g., the levels of nitrogen fertilization of the pastures that the forages received were essentially the same, as were the selenium levels of the feed, (Table 10). The fungal populations were negative for saprophytic species. Only the alkaloid levels of the forages differed. The G1-307 (LF) alkaloid level was only 1/2 that of the Kentucky-31 (HF), (Table 11).

Others have reported that cold environmental temperatures enhance the symptoms of "fescue foot" (Garner, et al., 1973; Goodman, 1952; Stern, 1973; Merriman, 1955), and warm temperatures seem to be related to "summer slump" in cattle (Hemken, et al., 1979). Lowered environmental temperatures in these trials decreased pup survival across

TABLE 10

COMPOSITION OF RATIONS FED
DURING TRIALS 2 and 3

Components	Rations				
	CC	CD	CO	LF	HF
% DM	85.89	85.87	84.44	87.21	90.12
% Crude prot.	24.86	22.77	25.30	24.66	24.66
% Crude fiber	3.99	9.04	7.73	7.13	7.44
% Ash	6.90	5.90	6.76	7.50	7.28
% Ether ext.	3.72	5.58	5.47	5.87	6.62
% ADF	5.56	12.54	9.86	8.73	9.29
% Lignin	.903	1.90	1.39	1.11	1.27
% cwc	20.23	30.41	19.79	72.31*	22.62
Ca mg/g	10.75	5.50	8.29	7.56	7.50
P mg/g	6.96	5.06	7.49	7.81	6.74
Mg mg/g	2.00	1.11	1.63	2.13	1.79
K mg/g	8.31	6.79	9.23	13.02	10.74
Se ppm	.218	.147	.179	.174	.162

% cwc for the ration containing the G1-307 fescue is probably the result of incomplete mixing and not a true value.

* CC, control; CD, corncob; CO, orchardgrass, LF, fescue (G1-307); HF, fescue (Ky-31)

TABLE 11

CHEMICAL COMPOSITION OF FORAGES FED TO RATS*

Variety and year	Crude protein	Acid detergent fiber	Ether extract	Ash	Perloline	Perlolidine
	(%)	(%)	(%)	(%)	(mg/g)	(mg/g)
Ky 31-1975	14.7	36.2	4.3	8.2	417	138
G1-307-1975	16.5	33.9	5.1	8.4	281	92

* Henken, et al., 1979, with permission.

all treatments and therefore is probably not related to the feed as much as it was to the housing itself.

Abnormal behavior

Abnormal behavior changes were seen in trial 1. These changes were not evident until the day of parturition. Two dams receiving the 2 gm equivalent alcoholic extracts began biting their own tails. Two other dams receiving 2 gm equivalent alcoholic extracts appeared to have no maternal instincts such as pup retrieval or nursing. Both of these dams ate their live litters of 12 pups each, within 24 hours of birth. The abnormal behavior changes that occurred in this study may suggest that the dams were stressed by the feeding of fescue extracts.

The increase in adrenal gland weights (trial 3) (Table 7, page 25) of the fescue fed dams and the deaths of some dams before and during parturition also suggest that feeding fescue hay may cause stress. Furthermore it was observed that during trial 3 those dams that received the stockpiled fescue had very rough hair coats. This too is probably an indication of stress since the rations fed were essentially the same nutritionally and rough hair coats were not seen in the controls or the orchardgrass fed groups. Stress in cattle as a result of grazing fescue pastures has been reported by others (Merriman, 1955; Jacobson, et al., 1973; Garner, et al., 1973).

Pups born alive

Litter size (the number of pups born alive) is a direct consequence of the number of ova released, the efficiency of fertilization, and pre-and post implantation embryonic mortality. These results show that the feeding of fescue hay or extracts of fescue did not decrease the number of pups born alive, nor did it affect the number of decidua (implantation sites in the uterus), (Tables 2, 4, 7, pages 20, 22, 25).

Embryonic mortality however, was increased 20% in the orchardgrass group and 29% in the G1-307 fescue group over the controls. Nevertheless this parameter did not differ between the non-toxic and the toxic forages, (Tables 2, 4, and 7, pages 20, 22, 25).

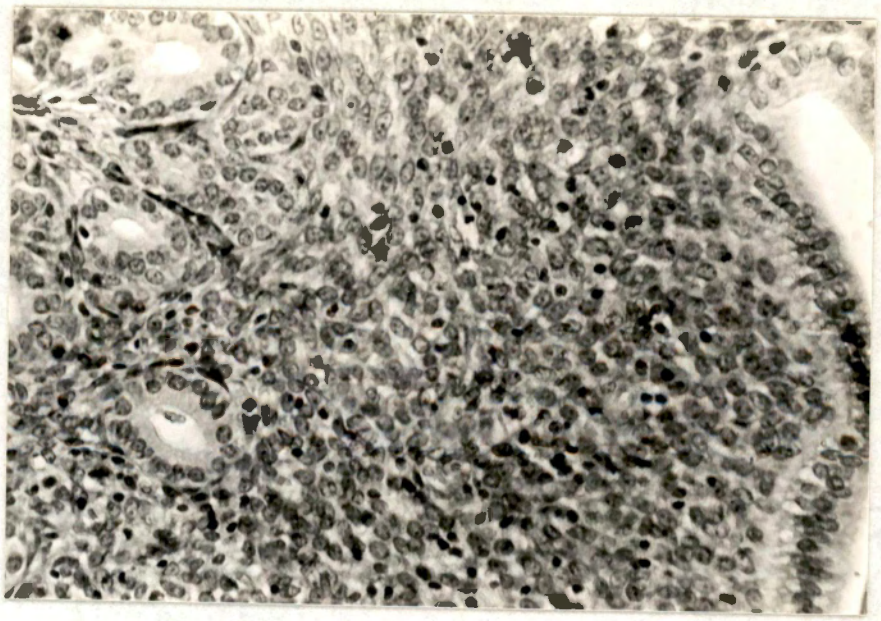
Pups stillborn

The incidence of stillborn pups was no greater in the fescue-fed dams than in the controls for trials 1, 2 or 3. Contrary to the report of Daniels, et al., (1973) there was no effect of fescue on the number of stillborn in these trials. Daniels' (1973) group reported that there was an increase in stillborn pups when dams were fed extracts of fescue and extracts of wheat. This group further reported that the incidence of uterine hemorrhage was increased and that nearly all of the rats in their study had difficult births. Again, these results are contrary. In this study only 5 of 99 dams exhibited uterine hemorrhage or dystocia,

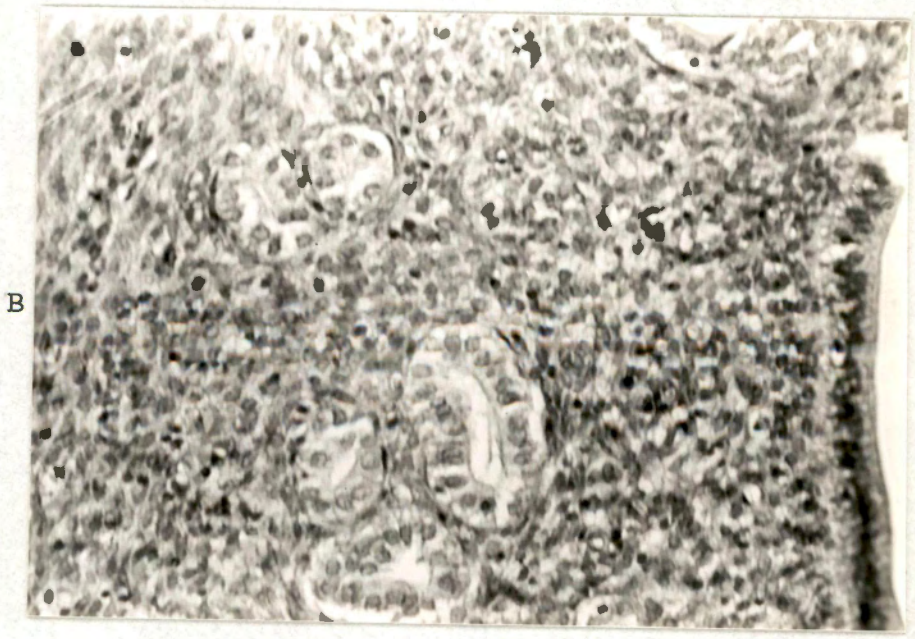
one of these dams was receiving the orchardgrass ration. Resorption of the fetuses by the dams was more prominant in this study (trial 3) than the occurrence of stillborn pups. But, neither the incidence of stillborn, uterine hemorrhage, nor resorption of fetuses was peculiar to the fescue fed groups, e.g., orchardgrass was not distinguished from the toxic fescue by these parameters.

Histologically the uteri of the dams examined showed no pathology of the endometrium, myometrium, or the blood vasculature (Figure 2). Although thickened walls were seen in some of the blood vessels of the uterus (Figure 3), this is probably not the same vascular involvement reported by Williams, et al., (1975). They found that in approximatley 50% of examined cattle afflicted by "fescue foot" the blood vessels of the hind limb had thickened walls and decreased lumen sizes. In the rat uterus, Munford (1963) has characterized the blood vessels with thick walls and decreased lumens as normal.

Perivascular hemorrhage in cattle showing signs of fescue toxicity was also described by Williams, et al., (1975). Vascular permeability studies using Evans' Blue dye in these trials failed to show an increase in vascular permeability for the fescue-fed dams or the controls. Apparently the blood-brain barrier is intact in these dams. No vascular permeability was seen in the extremities, skin, viscera, or the reproductive organs in these rats. The lack

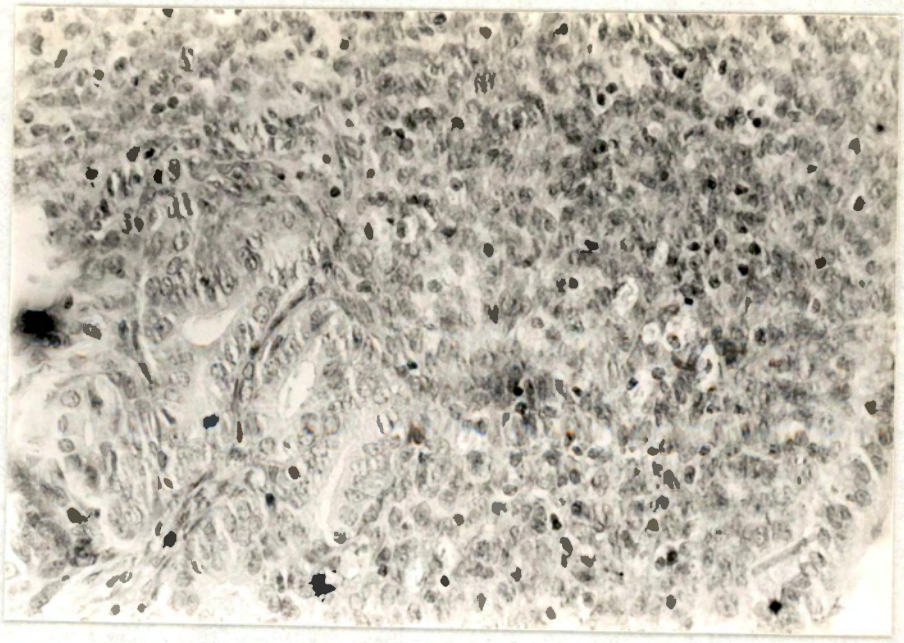


A

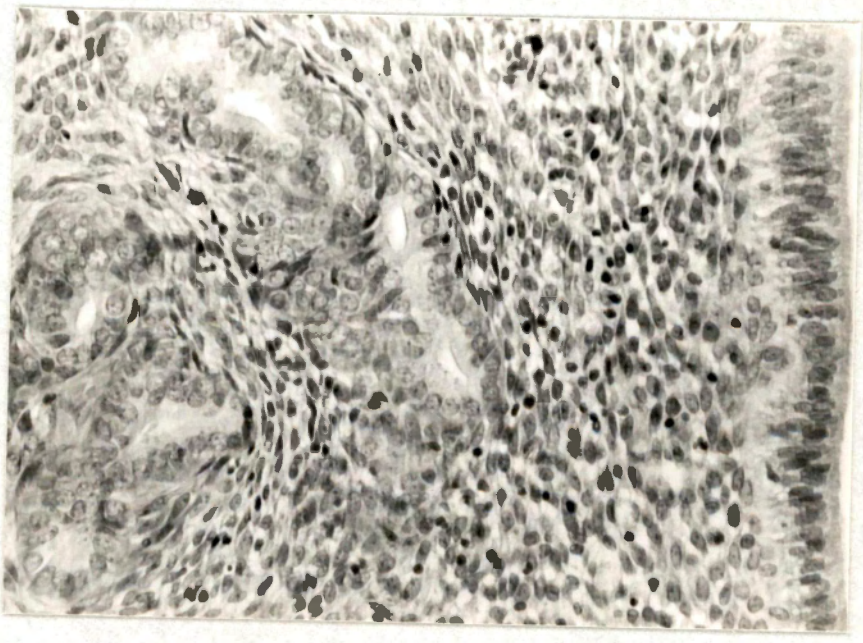


B

Figure 2. The endometrium of the rat uterus - apparently no differences exist in the mucosa, endometrium or uterine glands. A, control x 1250, B, orchardgrass x 1250, C, fescue G1-307 x 1250, D, fescue Kentucky-31 x 1250. (Hematoxilyn and eosin stain, original magnification x 312)

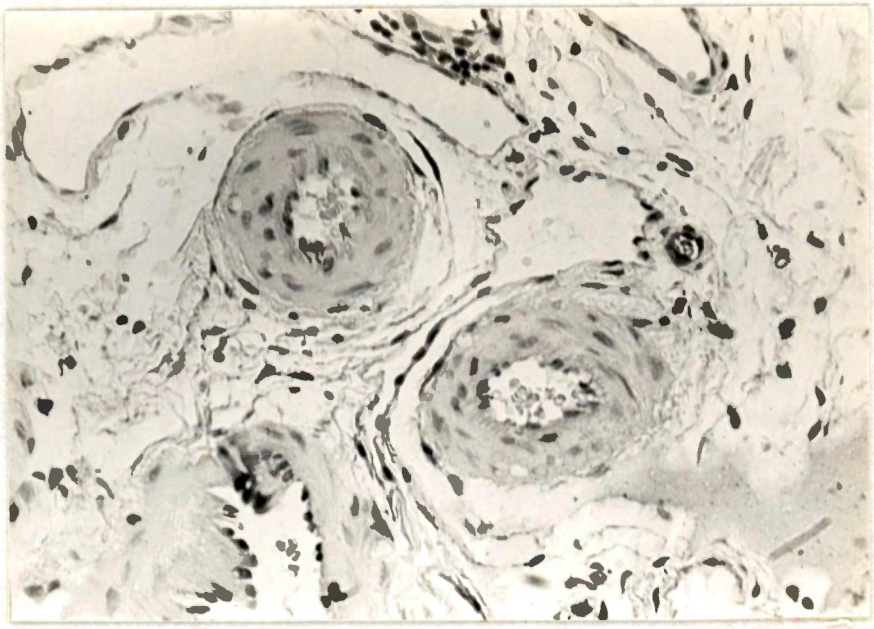


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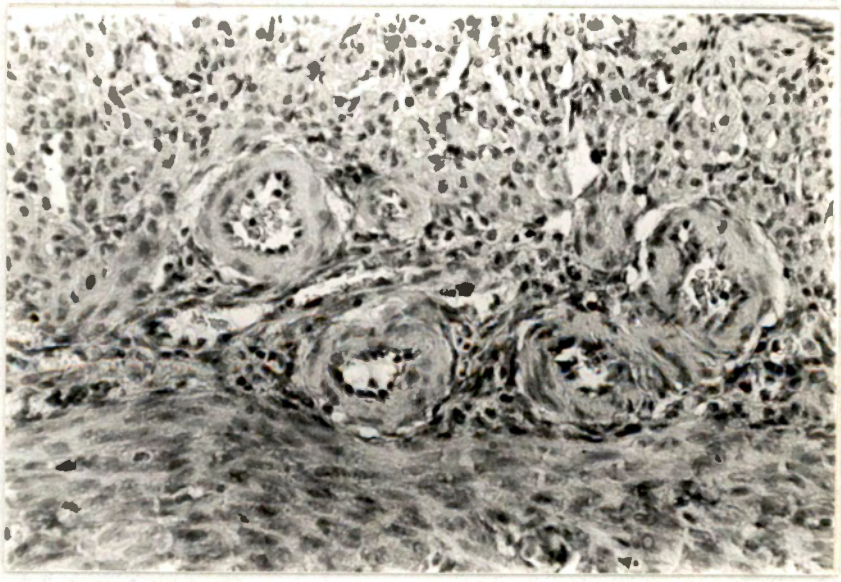


D

Figure 2. (Continued)



A



B

Figure 3. The normal thickened appearance of blood vessels in the rat uterus 5-days post-partum. A control, x 1250, B, G1-307 fescue x 1093 (Hematoxilyn and eosin stain, original magnification x 312)

of uterine pathology agrees with the results of these trials that the number of stillborn pups are not increased by feeding extracts of fescue hay or by feeding the ground hay to rats.

Hemoglobin values

Hemoglobin values obtained during trial 1 for dams given either the water or the alcoholic extracts of fescue were not lower than the controls (Table 2, page 20). However, the males receiving the water extracts of fescue were lower than the controls for hemoglobin values obtained at day 21 of treatment ($P<.01$, Table 3, page 21). This opens up the possibility of using males instead of females in a fescue toxicity assay.

The dams of trial 3 were not different in their hemoglobin values (Table 7, page 25). Pup hemoglobin values during trial 3 indicated that pups gestated on the control ration were higher than pups gestated on the G1-307 fescue ration ($P<.01$, Table 9, page 28). The highest hemoglobin values obtained were for the pups that had gestated on the Kentucky-31 (HF) ration. Both the gestation treatment and the litter/gestation ration interaction were important influences on the outcome of the data, but not the lactation ration (the ration that pups had been crossfostered to). When the error term in the analysis model was the litter/gestation interaction, the effect of gestation treatment was

diminished. This suggests that the lowered hemoglobin values obtained for the fescue pups was largely due to individual differences rather than due to the fescue. Daniels, et al., (1973) reported that the pups from dams fed fescue extracts had a tendency to have lower hemoglobins than the controls. These findings of a decreased hemoglobin value may play an important part in pup survival.

Pup survival

Of those pups that were born alive survival rate was lower in the fescue-gestated pups. Crossfostering pups to dams receiving a different ration than they (pups) had gestated on did not effect the survival rate of the pups. Thus the nursing ration in our trials was not a determining factor in pup survival. During trial 2 the percentage of pups surviving 5 days that had nursed dams fed fescue were as high as the controls in the 28.9 C environmental temperature. However, at 1.1 C there was only a 40% survival rate for the pups of the G1-307 group as compared to a 90% survival rate for the control pups ($P < .01$, Tables 5, 6, pages 23, 24). Pup survival after crossfostering in trial 3 was similar to the crossfostered pups of trial 2 maintained at 1.1 C. Pups that had gestated in the G1-307 group had a lower (32%) survival rate than did pups gestated by control dams (46%, P .05 Table 9, page 28). Chi-square analysis of pups by gestation ration and nursing ration (dam lactation ration)

indicated that the gestation ration was more a part of survival of pups than was the ration fed to dams that the pups nursed ($P<.05$). The litter/gestation ration interaction indicated an influence on pup survival. But, when the interaction was used as the error in term the analysis model, the gestation treatment effect was slightly diminished ($P<.07$). Survival rate was however, influenced by the gestation ration fed to the dams. This suggests that the pups were effected in utero by feeding fescue to the dams.

Weight gain

The average 5 day weight gain of pups during trial 2, at an environmental temperature of 28.9 C, was higher for the control pups than the pups of the Kentucky-31, G1-307, or the orchardgrass pups ($P<.01$, Table 5, page 23). During trial 3 the average 5 day weight gain was not different for the treated groups as opposed to the controls. The interaction of litter by gestation treatment ($P<.01$) was a factor in the average 5 day weight gain of pups in trial 3 (Table 9, page 28). Daniels, et al., (1973) reported similar findings, but said that this difference in weight disappeared by the age of weaning. Daniels' group (1973) speculated that pups were lighter as a result of a lack of milk production by the fescue-fed dams. They stated that this lack of production was probably due to a decreased feed intake or vasoconstriction. Crnic, et al., (1978) found that milk production in the rat

is not impaired by starvation of the dam. In Crnics' (1978) study fat, protein, and milk volume were not effected by a 40% decrease in feed intake. Although dams of our study receiving the Gl-307 fescue did consume 27% less feed than the controls, we found that milk volume, fats, and total solids were not decreased in the rats fed fescue hay (Table 8, page 27).

Milk quality factors though may still have affected the result obtained by Daniels, et al., (1973). They crossfostered only 5 pups to each dam and thought that perhaps they had given the fescue fed dams an advantage in only having to rear a small litter. But, Yagil, et al., (1976) found that litters of 2 to 6 pups decreased fat and protein production by the dam, but that litters made up of 6 to 10 pups did not effect milk production or quality factors such as fat and protein. Since the caloric value of milk is largely a function of fat content, the results of Yagils' group (1976) would suggest that the fescue dams were not given an advantage in the study of Daniels, et al., (1973).

Stomach milk content

Stomach milk content during trial 2 was observed to be less in the rat pups nursing dams fed either the Gl-307 or the Kentucky-31 than the stomach milk content of pups nursing dams fed the control ration or the orchardgrass ration, in either the 28.9 C or the 1.1 C environmental

temperature, ($P<.01$). In trial 3 no difference in stomach milk content was observed, but it was observed that the curd of pups from fescue fed dams was not as firm as the curd formed in stomachs of the control pups. Again, the litter/gestation ration interaction was an important part of this parameter ($P<.0001$), (Tables 5, 6, 9, pages 23, 24, 28), suggesting individual differences exist.

Stomach milk content is related to milk production and has been used to measure milk production in the rat by others (Cox, et al., 1976; Mayer, 1975; Morag, 1970). The apparent discrepancy that exists between trial 2 and trial 3 of this study is probably an artifact of technique. When the pups were sacrificed at the same time each morning the stomach milk content was not lower in the fescue gestated pups. Mayer (1935) similarly found that stomach milk content was related to the amount of time elapsed between nursing and sacrifice. The values that were obtained in this study of stomach milk content of 5 day rat pups are quite close to those that Mayer (1935) obtained for 7 day rat pups. The similarity of stomach milk content for the fescue-gestated pups and the control pups agree with the failure to find differences in milk production by the fescue-fed dams and the control dams. These results agree with those of Cox, et al., (1936) who observed that the stomach milk content of rat pups paralleled the milk production by the dam.

Milk samples were unobtainable in the unanesthetized rat. In the anesthetized rat we were not able to obtain milk, unless we also injected oxytocin. Even with the oxytocin injection, (5 I.U.) milk "letdown" was not readily apparent, except in 2 rats that "letdown" milk copiously within 2 minutes of the oxytocin injection. In other rats, milk was not obtainable for at least 10 minutes after suction of the teat began. This process required approximatley 1 to 1 1/2 hours to completely milk the rat. Milking by hand was found to be quicker, but the teats became inflammed and congested, which eliminated these glands for histological sections. Milk volume, fat and total solids obtained by these procedures were in accordance with the values reported by Cox, et al., (1936) and Schmidt (1971). The slow oxytocin response seen in this study was similar to earlier reports by Morag (1970), who also showed that in the rat the administration of oxytocin affects only the rate at which milk can be obtained and not the volume.

Mammary histology in our trials were similar in appearance for all treatment groups. The number bred size or shape of the alveoli of the mammary gland did not appear to be decreased in the fescue group (Figure 4). There was considerable variation among individuals of the same treatment and no glands of dams that were suckling pups appeared non-secretory. Denuccio, et al., (1978) found that the shape and size of alveoli, in the diffuse gland of the rat, were the

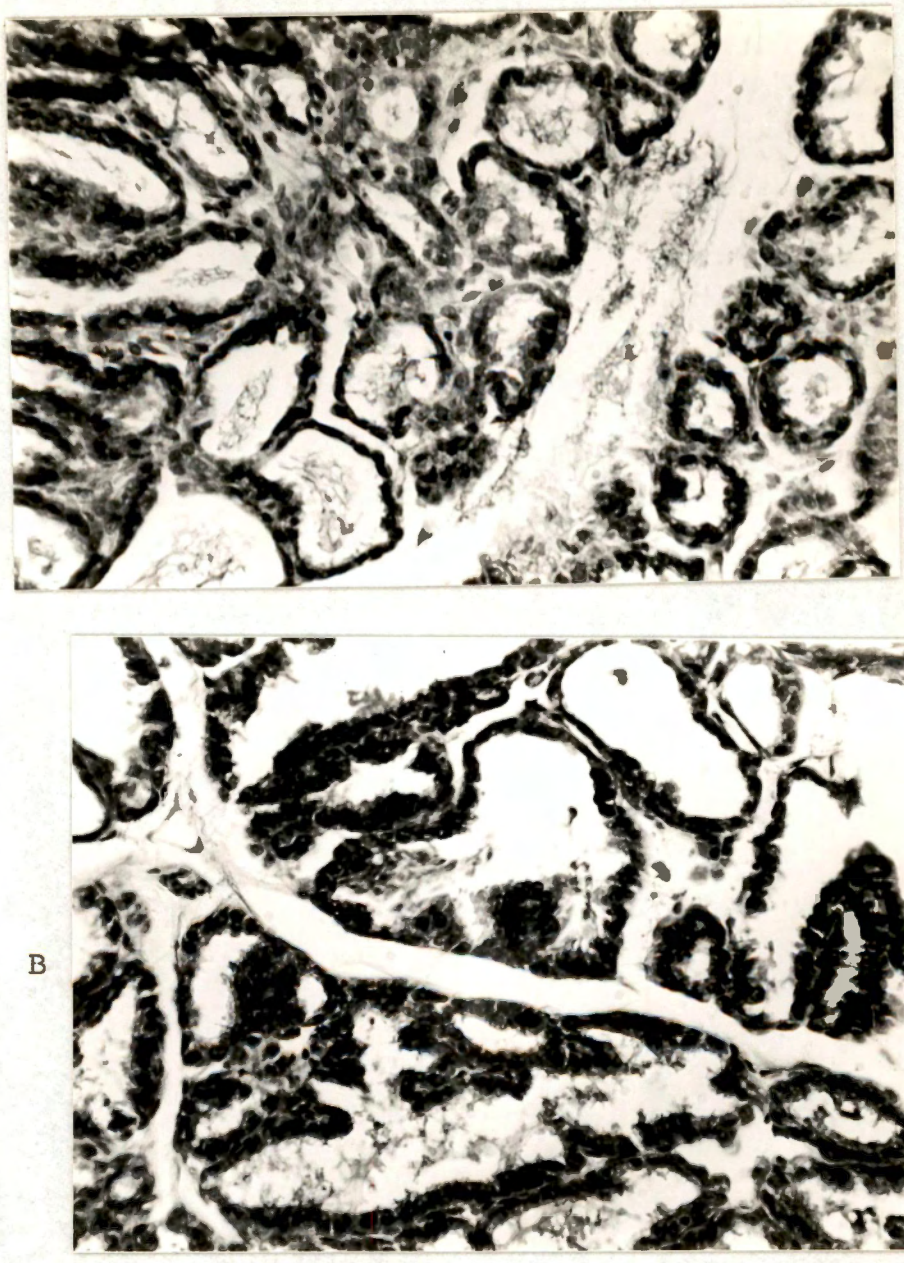
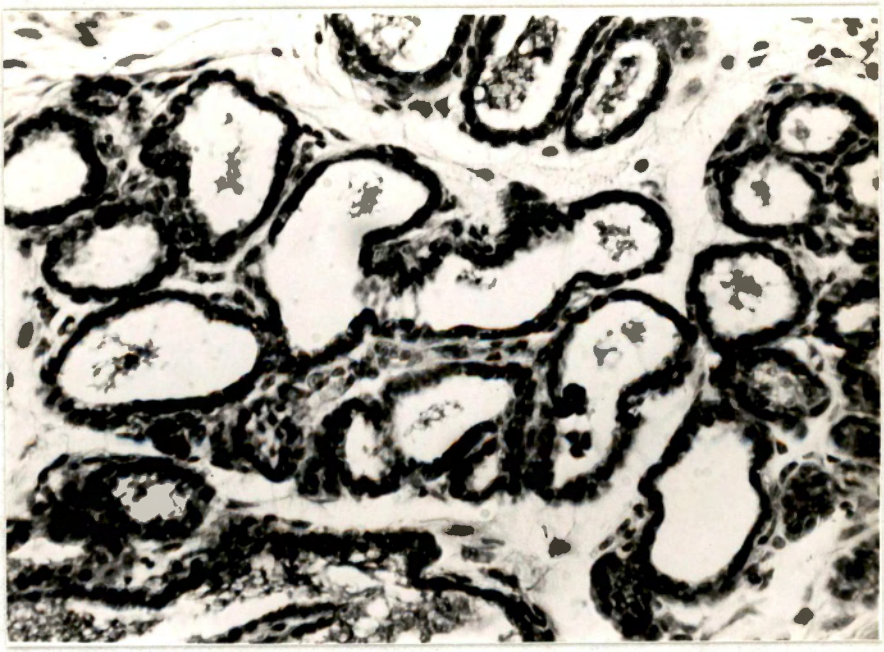


Figure 4. The mammary alveoli of the rat 5-days post partum. Note the irregular shape and size of alveoli, A, control x 1093, B, orchardgrass x 1250, C, fescue G1-307 x 1093, D, fescue, Kentucky-31 x 1093. (Hematoxilyn and eosin stain, original magnification x 312)



C



D

Figure 4. (Continued)

result of the compressive forces of the surrounding alveoli irrespective of the amount of milk fill. Furthermore, by direct microscopic examination, he found that oxytocin caused milk to move from alveoli to alveoli, and sometimes from larger ducts to smaller ducts instead of to the teat cistern for "letdown." He concluded that the histological evaluation of the rat mammary gland does not neccessarily reflect the secretory or non-secretory nature of the gland.

Bowman, et al., (1973) and Hemken, et al., (1979) have reported that the feeding of fescue resulted in a decreased milk production in dairy herds. Daniels, et al., (1973) reported that sows may also exhibit aglactia when grazing fescue pastures, and aglactia of mares grazing fescue has been reported by Garret (1980). On the basis of the results from this study, and the reports of Yagil, et al., (1976), and Crnic, et al., (1978) it would appear that the rat does not mimic the decreased lactation described for the other species. These results, plus the poor oxytocin response and the inconsistent histologies suggest that the rat is probably not an adequate model for aglactia caused by fescue toxicity.

Stomach pH

Although milk volume and composition did not effect the 5-day gain of pups, stomach pH of the pups did effect the gain of pups. Stomach pH, which was measured in trial 3, was found to be higher (more basic) in pups gestated

by the dams fed Gl-307 fescue than pups that had been gestated by dams fed the control ration ($P<.01$). The pups that had gestated in dams fed the corncob control diet or the orchardgrass diet were not different from one another in stomach pH values, (Table 9, page 28). This parameter was also governed by the gestation ration rather than the nursing ration. The 5-day weight gain of pups surviving was lower in the fescue gestated pups. As stomach pH increased weight gain decreased. The regression equations describes this phenomenon (pup weight gain = 3.15 + (-.33)(X)). We also observed that the formation of a curd in the stomach was not as firm in the fescue gestated pups as it was in the control pups. The more liquid nature of the curd suggests that digestion is not as efficient in the fescue gestated pups. Mayer, et al., (1935), in a study of the nature of rat pup stomach milk content, observed that curds that were liquid rather than solid had not been properly digested. He suggested that the pH is critical to the utilization of milk sugars in the rat stomach. These observations further suggest that changes occur in the pup pre-partum.

CHAPTER IV

CONCLUSION

Bioassay of fescue toxicity, using the laboratory rat, has been reported by others to be a reliable method (Daniels, et al., 1973); Jacobson, et al., (1973). Yet, both of these groups failed to distinguish between toxic and non-toxic forages. Daniels (1973) obtained the same level of stillbirths with extracts of wheat as he did with extracts of fescue. Jacobson (1973) on the other hand, in studying the heart rate and skin tail temperature response to extracts of fescue obtained the same responses with orchardgrass extracts. The use of ethanolic extracts of fescue and the "ethanol" control in this study showed that the number born alive was not different. This would suggest that the use of alcoholic extracts may leave an alcohol residue that is somehow responsible for the outcome of the reproductive parameters not only in this study, but that of Daniels, et al., (1973) and the results reported by Jacobson, et al., (1973). The failure to distinguish between toxic and non-toxic forages of this study and those studies reported by Daniels, et al., (1973) and Jacobson, et al., (1973) suggests that the rat is not an adequate model for fescue toxicity when reproductive and lactation records of dams are the parameters.

A valid assay for fescue toxicity using the rat may be possible through utilization of the pups. The decreased pup survival rate seen in the fescue pups, the decreased hemoglobins of these pups, the weight gain depression as a result of stomach pH increases seen in this study, and Daniels', et al., (1973) findings of lower hemoglobin values, depressed hematocrits, and lower WBC counts of rat pups from fescue fed dams suggests that perhaps further investigation of the possible in utero effects on the pups would be fruitful in developing a reliable bioassay. The results of this study show that the data were more influenced by gestation treatment rather than lactation treatment. This suggests that the uterine environment, or the longer duration of exposure to the toxin(s) via placental transfer may be how these pups are effected.

Because the results obtained by feeding ground fescue hay were similar to results obtained by using extracts of fescue, possibly a less expensive, quicker assay can be performed by feeding the forage rather than resorting to the time-consuming and expensive process of extraction.

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LITERATURE CITED

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