ZEARALENONE IN PASTURE AND ITS EFFECTS ON REPRODUCTION IN EWES

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ABSTRACT

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Zearalenone is an oestrogenic mycotoxin which has the potential to cause reproductive disorders in sheep. Zearalenone-producing Fusarium species are present in New Zealand pasture and it is likely that the amount of zearalenone present during the mating period may be sufficient to cause reproductive dysfunction in the grazing sheep.
This study consisted of three trials which aimed to measure zearalenone levels in the pasture and sheep, and determine the subsequent effects on reproductive performance. The first trial investigated the levels of zearalenone during April in various components of the ryegrass plant at various pasture sites, which included urine-patch, dung-patch and inter-excreta sites. Zearalenone taken up by the ryegrass plant was also determined. The second trial comprised of 6 groups of ewes (n=10), and compared levels of zearalenone and related metabolites in the blood and urine of ewes grazed on pasture or chicory and either orally (5 mg/ewe)or intravenously dosed (2 or 0.5 mg/ewe) daily with zearalenone. The subsequent effects on ovulation rate, conception rate, and number of lambs carried was also determined. The third trial comprised of 4 groups (n=l 10) of ewes, of which two groups were grazed on grass-dominant pasture and the remaining 2 groups were grazed on chicory for two weeks prior to mating at which time one of the groups on each grazing treatment was interchanged and the ram introduced. The levels of free and conjugated zearalenone in the blood and urine were determined and the subsequent effects on ovulation rate, conception rate and the number of lambs carried were measured.

In the first trial it was shown that zearalenone concentration within sites was highly variable at that time of the year, however, urine-patch and dung patch sites yielded significantly higher quantities of zearalenone. Zearalenone appeared to be readily taken up by the ryegrass plant through the roots and translocated into the young growing tissue of the plant. The distribution of zearalenone in the pasture and the plant are discussed with regards to zearalenone intake by the animal.
The zearalenone dosing trial showed that significant levels of zearalenone, α-and β-zearalenol, zeranol and taleranol were present in the blood and urine of dosed ewes and that levels of all compounds analysed were higher in ewes grazed on pasture. Ewes grazing pasture had a significantly lower ($P<0.05$) ovulation rate than ewes grazed on chicory.
The third trial showed that chicory was effective in reducing the levels of free zearalenone present in the ewe around the time of mating with levels in ewes grazed on chicory being significantly lower ($P<0.05$) in both the urine and blood, than in ewes grazed on grass pasture. There were no significant differences in reproductive performance. Zearalenone levels in the pasture were generally lower in 1995 than in previous years and might have reduced possible differences in reproductive performance between ewes on the different feed types.
The implications of higher zearalenone concentrations in the pasture are discussed with regards to reproductive performance and the use of chicory as a feed prior to mating.

Further research is required to identify and clarify links with zearalenone and metabolites produced in pasture and reproductive dysfunction in ewes.

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LIST OF ABBREVIATIONS AND DEFINITIONS

kg	**Kilograms**
g	**Grams**
mg	**Milligrams**
µg	**Micrograms**
ng	**Nanograms**
ppm	**Parts Per Million**
ml	**Millilitres**
mmol	**Millimols**
Z/Cr	**Zearalenone:Creatinine ratio**
CIDR	**Controlled Internal Drug Release**
°C	**Degrees Celsius**
GC-MS	**Gas chromatography-Mass spectroscopy**
HPLC	**High performance liquid chromatography**
IgG	**Immunoglobulin**
LH	**Luteinizing Hormone**
FSH	**Follicle Stimulating Hormone**
N	**Nitrogen**
Fusarium	**Used when describing a particular species**
Fusarium	**Used when description is non-specific**
fusaria	**Used when referred to collectively**

CHAPTER I

Introduction

The fertility of sheep flocks in New Zealand is well below their potential (Knight, 1990). Even in sheep flocks where ewe condition and management are very good, lambing percentages are well below that which would be expected. For example, lambing percentages in Northland are often below 100% and very rarely above. With progression from North to South in New Zealand there is an upward trend in lambing percentages indicating that the possible reproductive problems are greater in northern regions of the country (Quinlivan and Martin, 1969).

There are several compounds which have been found in New Zealand pastures, which have been shown to exhibit oestrogenic effects in grazing animals. It has long been appreciated that many plants and some fungi are able to produce compounds which possess oestrogenic activity in animals (Miksicek, 1994).The majority of oestrogens produced by plants appear to be secondary metabolites which are nonsteriodal in nature. The main pasture plant constituents isolated and found to have oestrogenic activity are isoflavones and coumestans from legume species.

The saprophytic fungi belonging to Fusarium species and including., *F. crookwellense*, *F. culmorum*, and *F. semitectum* are common in New Zealand pasture herbage (di Menna *et al.*, 1991). It is known that these fungi are capable of producing zearalenone and its metabolites α- and β-zearalenol which belong to a rare class of natural products, the β-resorcyclic acid-lactones, which are capable of binding to estrogen receptors because of their chemical similarity to oestradiol (Hurd, 1977).

The effects of zearalenone have been documented with pigs and include hyperestrogenism which was initially termed "vulvo-vaginitis" and symptoms such as swelling of the vulva and uterine enlargement (Maryamma, *et al.*, 1992).

To date the majority of studies have been concerned with the effects of zearalenone on swine fed grain contaminated with fusaria and very few studies have examined the effects of zearalenone and its metabolites in sheep grazing pasture where zearalenone-producing species of fusaria are present. Smith *et al.*, (1986) showed that reproductive performance was markedly reduced in ewes treated with 25 mg of zearalenone daily for 10 days prior to mating.

In a later study by Smith *et al.*, (1990) there was a linear decline in ovulation rate with increasing dose of zearalenone and there were reductions in conception rates. The results obtained in these trials show that zearalenone, ingested in large enough quantity, will disturb reproductive function in sheep. However, the question remains as to whether zearalenone-producing Fusarium species is pasture is a factor in reducing reproductive performance in sheep grazing that pasture.

Zearalenone, in concentrations of 0.4 - 4.0 mg/kg dry weight of herbage, have been found in pasture samples collected from January to April at sites near Pukekohe, Wanganui and Gisborne (di Menna *et al.*, 1987). The presence of the various Fusarium species in pasture and their ability to produce significant quantities of the oestrogenic metabolite zearalenone, could mean that grazing animals would ingest potent quantities of zearalenone.
It seemed possible that fusaria populations, which peak at the time most ewes are mated, might produce sufficient zearalenone in some pastures to affect reproduction (Jagusch *et al.*, 1986; di Menna *et al.*, 1987). Given that up to 4 mg/kg dry matter of zearalenone can be present in the pasture around the time of mating and that Smith *et al.*, (1990) concluded that intakes of zearalenone of 3 mg/ewe/day or more during the mating period would be reflected as depressed ovulation rates and lower lambing percentages, it is likely that zearalenone and its metabolites may be factors in reducing reproductive performance in sheep.

CHAPTER II

Review of literature.

1. The mycotoxin zearalenone

Zearalenone [6-(10-hydroxy-6-oxo-trans-1-undecenyle)-β-resorcylic acid lactone] is a naturally occurring mycotoxin synthesised by Fusarium mould species endemic to temperate climates. Zearalenone and α- and β-zearalenol, which are metabolites of zearalenone, belong to a rare class of natural products, the β-resorcylic acid lactones, which have the capability of binding to oestrogen receptors (Hurd, 1977; Fitzpatrick *et al*., 1990; Miksicek, 1994). While these fungal oestrogens are less potent on a molar basis than 17β-oestradiol, they can stimulate the activity of the oestrogen receptor to the same maximal extent as the natural hormone (Miksicek, 1994). Zearalenone is 200-fold less active on a molar basis than 17β-oestradiol in stimulating a transcriptional response through the human oestrogen receptor (Miksicek, 1994). The relative binding affinity to the oestrogen receptor differs between metabolites and the species of animal. Fitzpatrick *et al*., (1990) determined the relative binding affinity of zearalenone, α-zearalenol and β-zearalenol for oestrogen receptors in the pig, rat and chicken. That study found that α-zearalenol exhibited greater binding affinity than zearalenone and β-zearalenol the least binding affinity in all species examined. The relative binding affinity of α-zearalenol was greater in the pig, than the rat and significantly greater than in the chicken. Data obtained by Diekman *et al*., (1989) indicated that zearalenone suppressed concentrations of serum follicle stimulating hormone (FSH) and luteinizing hormone (LH) in a similar way to oestradiol benzoate, although the biphasic stimulatory affect of oestradiol benzoate for LH was not manifested by zearalenone.

α-zeralenol or zeranol is also a nonsteriodal veterinary drug permitted for use as an anabolic agent for cattle and sheep (Bories *et al*., 1990).

Fusarium species produce many different types of secondary metabolites, which include zearalenone and related alcohols. However, the ability to produce mycotoxins varies between species and also between strains of the same species.

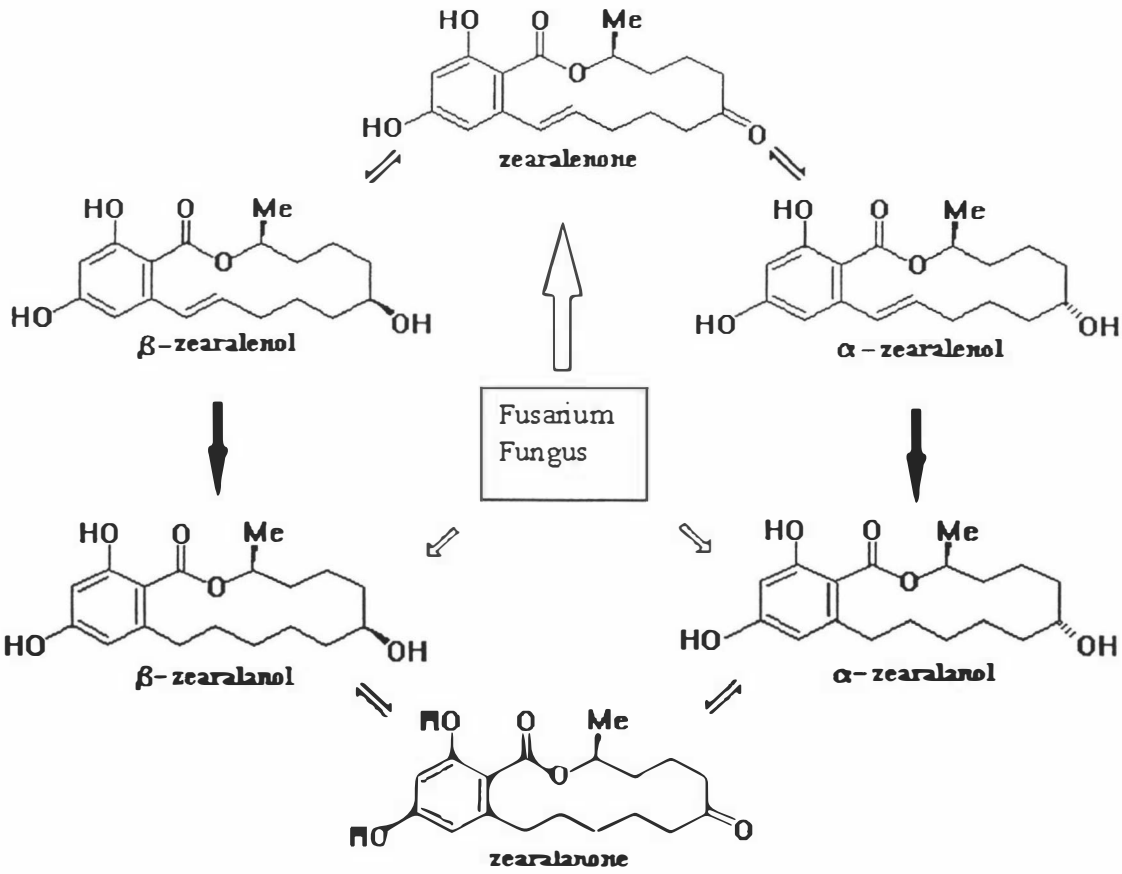
To date, most research on fusaria mycotoxins has been concerned with contamination and toxin production in cereal products, and the effects on animals which consume the contaminated products (Lauren *et al.*, 1988).
The feeding of cereal products in the animal production industry is largely confined to pig and poultry farming and therefore there is more information on the effects of zearalenone in these animals, in particular the pig, than in grazing animals such as sheep and cattle.

2. Zearalenone chemistry and metabolism

Laboratory-grown fusarium produces zearalenone as a major metabolite but also produces double-bond alkene metabolites α- and β-zearalenol and single-bond alkane analogues zearanol (α-zearalanol) and taleranol (β-zearalanol).
There are reports on the effects of resorcylic lactones such as zearalenone in cattle; however, compared to swine, cases of zearalenone mycotoxicoses are limited (Sundolf and Strickland, 1986). In practice zearalenone has only moderate effects on bovine fertility in comparison to the profound effects exhibited in swine (Mirocha *et al.*, 1980). The difference may be partially caused by the dissimilarity of the feed of pigs and cows and partially by the different effects zearalenone has on these animals. The distribution of urinary and faecal metabolites is different in cattle and swine which suggests variation in metabolism of zearalenone between the two species (Mirocha *et al.*, 1980).
The most essential difference between the metabolism of the swine and that of the bovine is caused by rumination. Kallela and Vasenius, (1982) found that rumen fluid had a decreasing effect on the amount of zearalenone. This would suggest that the reason why the effects of zearalenone are not as severe as that found in pigs is due to detoxification in the rumen. However, Kiessling *et al.*, (1984) showed that the decrease in zearalenone was the result of a reduction to zearalenol, mainly α-zearalenol. This product has three to four times more oestrogenic activity than the parent compound and the reduction of zearalenone to zearalenol also increases the polarity, which may influence not only excretion but also uptake from the digestive tract into the blood stream. Therefore, the suggestion by Kallela and Vasenius, (1982) that zearalenone degradation in the rumen was a first line defence against the toxic compound present in the diet is doubtful and the ruminant may be at a disadvantage if substances such as zearalenone become more toxic as a result of the action

of ruminal microbes. Kiessling *et al.*, (1984) found that the metabolism of zearalenone in the bovine rumen is not significantly different from that in the ovine rumen.
In addition to transformation of zearalenone in the rumen, recent studies have shown that the transformation of zearalenone into its various metabolites is also carried out within the animal (Fig. 2.1.). Miles *et al.*, (1996) found labelled α- and β-zeralenol and α- and β-zearalanol glucuronides in the urine of sheep orally and intravenously dosed with labelled zearalenone. Zeranol and taleranol have also been found in the urine of pasture fed animals (Erasmuson *et al.*, 1994).

Figure 2.1. Zearalenone and related metabolites and the metabolic pathways which link them.



3. Effects of zearalenone in laboratory animals

The effects of zearalenone have also been examined in laboratory animals such as rats, mice, rabbits and guinea pigs. Zearalenone and related metabolites have uterotrophic activity in rats (Christensen, 1979.; Brooks *et al.*, 1971 and Mirocha *et al.*, 1978). Zearalenone has been shown to prevent pregnancy in rats if given on the second day after mating (Brooks *et al.*, 1971). Zearalenone inhibited the growth of mouse embryos in vitro and induced morphological changes in the endometrium (Long *et al.*, 1989). The major effect of exogenous oestrogens, including zearalenone, administered during early pregnancy in rats and mice, is the accelerated embryo migration in the uterine tubes, with a lesser effect of interfering with implantation (Greenwald, 1967). Zearalenone dosed to rabbits resulted in the alteration of several trace elements and amino acids known to be of critical importance in early embryonic development although anomalies in embryonic development were not observed (Osborn *et al.*, 1988). This study concluded that zearalenone or its associated metabolites affect factors that influence fertility during the early preimplantation period.

Guinea pigs responded to the oestrogenic effects of zearalenone in early pregnancy in a manner similar to that seen in other rodents although, unlike many other rodents, the guinea pig has a protracted oestrous cycle similar to that of larger species and implantation occurs during midcycle (Long, & Diekman, 1989).

4. Effects of zearalenone in pigs

The reproductive effects of mouldy corn in pig diets were first reported by McNutt *et al.*, (1928), and included the symptoms which have since collectively been referred to as the "oestrogenic syndrome" or "hyperestrogenisim". The induced hyperestrogenism was initially termed "vulvo-vaginitis" and included symptoms such as swelling of the vulva, uterine enlargement, ovarian atrophy, mammary development, and in some cases, vaginal or rectal prolapse (Aucock *et al.*, 1982). These clinical effects were largely observed in prepubital gilts and they appeared to more susceptible to effects of ingested zearalenone. Immature gilts given 1 mg of zearalenone/day developed tumefacation of the vulva, and 5 mg/day also caused an increase

in uterine weight (Long *et al.*, 1982). Dietary levels of 1-5 mg/kg zearalenone are sufficient to produce clinical signs of hyperoestrogenisim in young gilts (Aucock *et al.*, 1982 ; Ruhr *et al.*, 1983).

Large amounts of zearalenone in the feed (100 mg/kg of feed) have profound effects on cycling sows, including nymphomania, pseudopregnancy, ovarian atrophy, and morphological changes in the endometrium (Long *et al.*, 1982). Mouldy feed in the diet of pregnant sows has been associated with abortion, weak pigs, stillbirths, decreased litter size, and foetal mummification, although concentrations of zearalenone in the feed were not determined in most of these cases (Long *et al.*, 1982).

Aside from the obvious clinical effects of ingested zearalenone in swine, the effects on various hormone profiles, luteal function and early pregnancy have also been documented in the pig. It was shown that feeding zearalenone at concentrations of 5-10 ppm from day 5 to 20 of the oestrous cycle causes luteal maintenance and extended inter-oestrous intervals (Edwards *et al.*, 1987). This could be explained by the fact that oestrogens, given at the appropriate time during the oestrous cycle, are luteotrophic in the swine (Kidder *et al.*, 1955).

Diekman and Long, (1989) found that zearalenone administered on days 7 to 10 after breeding altered secretory patterns of serum LH during days 10 and 14 after breeding. Studies by Long *et al.*, (1982) and Diekman and Long, (1989) have shown that ingesting zearalenone during early pregnancy has adverse effects on blastocyst development and embryo survival in the swine. Diekman and Long, (1989) found that blastocysts from sows given a diet containing purified zearalenone (1 mg/kg of body weight) from days 7 to 10 after breeding, were fragmented and contained foci of necrosis, whereas blastocysts from control sows were normal. In an earlier study by Long *et al.*, (1982), gilts that were fed Fusarium-contaminated feed or purified zearalenone from days 2 to 15 after breeding lost their embryos and retained their corpora lutea.

Ruhr *et al.*, (1983) examined the effect of zearalenone on the reproductive potential in the boar. This study found that zearalenone, administered at 200 mg per kg of the feed ration, did not permanently block or adversely affect spermatogenesis in the mature boar nor was the reproductive potential of the boar reduced. Berger *et al.*, (1981) found a reduction in plasma testosterone concentration and a subsequent reduction in libido after prepubital boars were given 40 mg/kg of feed from 14 to 18 weeks of age. The results of Ruhr *et al.*, (1983) and Berger *et al.*, (1981) would suggest that the effects of zearalenone in boars are

similar to sows in that prepubital animals appear to be more susceptible. However, it would also appear that zearalenone has minimal effects on boars at levels where clinical signs would be observed in sows.

5. Effects of zearalenone in poultry

The effects of zearalenone have also been documented in production birds such as chickens and turkeys. Chickens given zearalenone at 10mg/kg body weight, had cystic changes in the oviducts of the females and atrophy of the seminiferous tubules and an increase in connective tissue proliferation in the testis in the males (Maryamma, *et al.*, 1992).Allen *et al.*, (1981) showed that the weight of male broiler comb and testes were reduced by high levels of dietary zearalenone. Chji *et al.*, (1980b) found that zearalenone administered orally or intramuscularly increased the oviduct weight of growing female White Leghorn chickens. However, Marks and Bacon, (1976) fed Fusarium-infected corn to provide 25 and 100 ppm of zearalenone or purified zearalenone at these levels and did not adversely influence the reproductive performance of laying hens.

Female turkeys fed pure zearalenone (100 ppm) produced less eggs which also weighed less than eggs from turkeys with no zearalenone in the diet (Allen *et al.*, 1982). Although these observations would suggest that zearalenone was responsible for the significant drop in egg production and weight, it is likely that other mycotoxins present in the Fusarium infected feed were responsible for the reduction. Chi *et al.*, (1980) and Allen *et al.*,(1981) concluded that the effects of zearalenone in growing broiler chicks and turkey poults is minimal. Allen *et al.*, (1981) showed that male turkey poults fed 400 and 800 mg zearalenone/kg of diet had increased development of dewlaps and caruncles and exhibited pronounced strutting behaviour. It would appear that turkeys are more susceptible to the effects of zearalenone then chickens. A possible reason for this is that turkeys metabolise zearalenone mainly to α-zearalenol which is more oestrogenically active, whereas chickens produce roughly equal amounts of α– and β–zearalenol (Olsen *et al.*, 1985)

The effects of zearalenone on some other species of birds have also been documented. Palyusik and Koplic-Kovacs, (1975) reported that dietary *F. culmorum*-contaminated corn produced in female geese a non-significant reduction of egg production and fertility. Vanyi and Szeky, (1980) noted a cessation of spermatogenesis in Guinea-cocks fed for 2 to 3 weeks with grain containing 30 to 40 mg/kg zearalenone.

The effects of zearalenone in poultry are in many cases unclear due to conflicting results however, the incidence of some reported oestrogenic effects means that zearalenone cannot be discounted as an oestrogenic agent in poultry.

6. Effects of zearalenone in cattle

Fusaria fungi and the mycotoxin zearalenone were identified in suspect mouldy feed implicated in dairy herd health problems, including decreased fertility. Zearalenone fed at dosages of 14 to 75 mg/kg of ration may have caused a high artificial insemination index, vaginitis, prolonged oestrus, swollen vulvas, decreased milk production, and precocious mammary gland development in dairy cows and heifers (Bugeac and Berbinsch, 1967; Danko and Aldsay, 1969; Miller *et al.*, 1973; Shreeve and Patterson, 1975). Mirocha *et al.*, (1968) reported decreased fertility in a 150-cow dairy herd fed mouldy hay with a zearalenone concentration of 14 mg/kg of feed. The artificial insemination index returned to an acceptable value after the feeding of the heavily fungus-infected hay was stopped. A dairy herd being fed between 5 and 75 mg of zearalenone/kg were affected with unexplained swollen vulvas, decreased milk production and partial anorexia (Vanyi *et al.*, 1973). Kallela and Ettala, (1984) found that zearalenone content of the hay fed to cows was related to the occurrence of abortions.

In dairy heifers given zearalenone at 250 mg daily,there was a reduced conception rate Weaver *et al.*, (1986a) although it was concluded that zearalenone, by itself, was not a major factor in bovine infertility. Weaver *et al.*, (1986b) found that dairy cows which were administered zearalenone had corpora lutea which were reduced in size but there was no change in serum progesterone concentration, red and white blood cell count, haemoglobin, and in oestrous cycle length. This study also concluded that zearalenone by itself does not seen to be an important factor in dairy cow health.

7. Effects of zearalenone in sheep

Because of its oestrogenic actions zearalenone is likely to influence oestrus, ovulation and fertilisation if administered pre-mating in the ewe. The properties of dosed oestrogens observed in sheep are a prolongation of oestrous behaviour (Fletcher and Linsay, 1971), failure of ewes to ovulate, most probably due to an interference with LH release from the

pituitary (Scaramuzzi *et al*., 1971; Smith *et al*., 1987)) and a reduction in fertilisation rate through a possible oestrogenic effect on sperm transport (Croker *et al*., 1975)

In a trial by Smith *et al*., (1986) where ewes were dosed with 25 mg daily for 10 days prior to mating, there was a marked reduction in reproductive performance with only 9.1% of ewes yielding fertilised eggs compared to 57.6% of the control ewes. In the same trial 46% of the treated ewes were anovular compared to 12% in the controls. The major reduction in ewes ovulating and in ovulation rate, coupled with the markedly lower fertilisation rates, produced an almost complete failure of the reproductive process. A later trial by Smith *et al*., (1988) showed a decline in ovulation rate in ewes which were dosed with 6 mg of zearalenone for 10 days prior to mating. A third trial by Smith *et al*., (1990) in which ewes were dosed at different rates for 10 days prior to mating showed a linear decline in ovulation rate with increasing dose rate, also cycle length decreased and the duration of oestrus increased with increasing dose levels. There was no effect of zearalenone treatment after mating on pregnancy rate or embryonic loss which is in contrast to the findings of Mitton *et al*., (1975) that zearalenone caused abortions in sheep. In the trial of Smith *et al*., (1990) the zearalenone dose had significant effects on liver weight, ovary weight and the weight of uterus and oviducts but not on the carcass weight. The fertilisation rate in this trial was unaffected which is in contrast to the findings of Smith *et al*., (1986). The maximum amount of zearalenone administered to ewes in these trials was 25 mg per day which represents approximately 16 ppm of the dietary dry matter intake. This is considerably lower than the zearalenone intakes reported in pigs for a similar degree of depression in reproductive performance (Diekman and Long, 1984) and thus the ewe would appear to be more sensitive than the sow to zearalenone (Smith *et al*,. 1986).

Given that zearalenone can cause reproductive dysfunction in sheep, it is necessary to determine whether sheep grazing pasture would ingest sufficient amounts of zearalenone around the time of mating to affect reproduction. This would clearly depend on the suitability of the climate and environment for Fusarium species to colonise pasture material and to produce zearalenone, and in the grazing behaviour of the sheep.

8. Production of zearalenone

Levels of zearalenone produced on maize grown in Manawatu and Waikato, New Zealand, infected with different Fusarium species, have ranged between 1 and 16 mg/kg (Hassan *et al.*, 1987). Zearalenone levels from wheat samples grown in Manawatu, New Zealand have ranged from 0.04 to 0.35 mg/kg (Agnew *et al.*, 1986).

Seven Fusarium species., *F. acuminatum, F. avenaceum, F. crookwellense, F. culmorum, F. graminum, F. oxysporum* and *F. semitectum* are the predominant species found in New Zealand pastures (di Menna *et al.*, 1987). Of these the most common species *F. culmorum* and *F. crookwellense* produce zearalenone consistently and in greatest amounts (di Menna *et al.*, 1987 and 1988). *F. culmorum* from pasture isolates was also found to produce α-zearalenol and β-zearalenol (di Menna *et al.*, 1988).

The presence of the various fusaria in pasture and their ability to produce significant quantities of the oestrogenic metabolite zearalenone indicates that animals grazing these pastures might ingest significant quantities of zearalenone to cause reproductive disorders. Pasture fusaria populations increase when the combination of grass minimum temperature, humidity and day length are favourable for fungal growth. They are present in greatest numbers in late summer and autumn (February to April) when counts of 104 to 106 Fusarium macroconidia/g wet weight of leaves have been recorded (diMenna *et al.*, 1969.; Smith *et al.*, 1990). There are however more than just macroclimatic conditions to be considered in determining the suitability of an environment for Fusarium proliferation in pasture. The characteristics of a pasture are influenced by plant species and the grazing animal which create separate microclimates within the pasture. These different site types within the pasture provide conditions which may or may not be suitable for fusaria colonisation. The plant species in the pasture affect the amount of dead material which is important as fusaria populations are largely restricted to the dead material in the herbage (di Menna *et al.*, 1991). Keogh, (1973b) found more fusaria macroconidia on dead ryegrass blade and sheath than on live ryegrass leaf. di Menna *et al.*, (1969) found that fusaria numbers were slightly higher on the sheath than on the blade, and much higher on the dead litter than on the sheath or blade. Pasture length could be another determining factor of within pasture macroconidium counts, as the sporulation of Fusarium species. is stimulated by light. This may account for the findings of di Menna *et al.*, (1991) which found higher

macroconidia counts in shorter pasture where light penetration is better. The major effects of grazing animals on pasture are herbage removal, treading and the deposition of excreta. Most of the soil N is taken up by plants during spring growth; during summer and into autumn, further growth in the absence of pasture legumes depends on recycling of N by the animal. During the season the ryegrass dominant pasture will become a patchwork of excreta and inter-excreta areas each with characteristic growth patterns. In the excreta areas such as urine-patch and dung patch sites where there is a high N status the ryegrass grows rapidly and becomes densely tillered. Conversely the inter-excreta areas, which are generally N-deficient, have sparse tiller populations and are slow growing. The high N sites also offered favourable conditions for the proliferation of many fungal species which include those known to produce zearalenone. A study by Keogh, (1973a), on the influence of the grazing animal on distribution patterns of Fusarium species in ryegrass pastures, found that spore loads were higher on herbage from urine patches than on herbage from inter-excreta sites.

It is, however, difficult to predict when zearalenone production will be at its greatest as zearalenone is not formed with the spores and, therefore, spore counts cannot be used as indicators of zearalenone levels. Zearalenone peaks often appear following peaks in counts of Fusarium macroconidia, but after variable time intervals (di Menna *et al.*, 1991). Factors determining zearalenone production in pasture seem to be site-related as no correlation between zearalenone concentration and Fusaria numbers has been observed (di Menna *et al.*, 1987). It would appear that the production of zearalenone may have some relationship with changing climatic conditions. Mirocha *et al.*, (1969) found that maximum zearalenone production in stored maize was obtained when the temperature was reduced to 12 °C to place a 'stress' on the fungus. Mirocha *et al.*, (1977) stated that zearalenone production needed alternating low and moderate temperatures which fluctuated over a range of 10-25°C. Findings by Miller *et al.*, (1983), suggested that zearalenone production is associated with senescent fusaria populations on field corn inoculated with *F. graminearum*. A study by di Menna *et al.*, (1987) found that zeralenone appeared at two sites 2 weeks after the peak fusaria counts which is consistent with Miller's findings. However, zearalenone was not found at other sites after fusaria numbers had dropped. Wolf and Mirocha, (1973) consider that zearalenone is a fungal sex regulating hormone after observing that perithecia formation was stimulated at low zearalenone concentration and inhibited at high concentration. Windels *et al.*, (1989) disputed this as there was a lack of correlation

between zearalenone and perithecium formation and suggesting instead that factors such as nutrition, light quality, and photoperiod are stronger influences on perithecium formation. So far the formation of zearalenone has proven difficult to characterise as different zearalenone producing species may form the metabolite under slightly different conditions. No particular weather pattern has yet been associated with high zearalenone levels, nor has the aspect of pasture sites had a consistent effect (Smith *et al.*, 1991).

9. Purpose and scope of the study

The purpose of this present study was to investigate the presence of the oestrogenic mycotoxin zearalenone in grazed pasture and determine the effects of ingested zearalenone on reproductive performance in sheep. In addition the metabolism of zearalenone and related compounds was investigated and the potential use of low zearalenone feeds such as chicory to reduce zearalenone intake and the risk of reproductive dysfunction.

The aims of the experiments in this study were:
1) To determine the distribution of zearalenone in ryegrass-dominant pasture and to demonstrate that zearalenone can be taken up via the roots of ryegrass plants.

2) To examine the metabolism of zearalenone in ewes dosed orally and intravenously with zearalenone and determine the subsequent effects on reproductive performance.

3) To determine the effects of zearalenone ingested from pasture on the reproductive performance of ewes and assess the efficacy of the low zearalenone forage chicory in reducing zearalenone levels in the ewe.

CHAPTER III

The distribution of zearalenone in ryegrass pasture and uptake by the ryegrass plant.

1. Introduction

Zearalenone-producing Fusarium species are common in New Zealand pasture herbage (di Menna *et al.*, 1991). At times the zearalenone concentration in the pasture can be great enough to affect the reproductive performance of grazing ewes (Jagusch *et al.*, 1986). It is not yet possible to predict when these high concentrations of zearalenone may occur. Making regional or seasonal predictions has been difficult as it is affected by variation in both zearalenone concentration and fusaria numbers in a pasture site on any one day. di Menna *et al.*, (1991) monitored the zearalenone levels and fusaria macroconidia in green and dead herbage fractions and on bulk herbage from long and short pasture at a site near Gisborne, in an attempt to identify factors which might be responsible for within-site variation. It was found that zearalenone concentrations were higher on dead than on green material and higher in bulk samples from short pasture than long pasture. The study concluded that, as zearalenone and fusaria populations producing it were largely restricted to the dead material in the herbage, it is this fraction which in the pre-mating period presents a potential hazard to the reproductive performance of grazing ewes.

To better understand the variation in zearalenone concentration between sites and within the plant it is necessary to differentiate further between sites in the pasture with different characteristics and between components in the plant. The grazing animal has a large influence on the pasture characteristics. During the summer months, distinct patchwork areas in the pasture are formed by dung and urine from the grazing animal. These areas provide substrate and a high N site which suits the development of fusaria populations. Keogh, (1973a) found that fusaria spore loads were markedly higher on herbage from urine patch than on herbage from inter-excreta sites. To date the differences in these sites has been determined for fusaria populations, and possible differences in zearalenone levels have not been investigated. di Menna *et al.*, (1987) found that there was no correlation between zearalenone concentration and fusaria numbers. This lack of correlation is not suprising given the different zearalenone-

producing potential of the various Fusarium species and variability in conditions suitable for these species to produce zearalenone.

Another important aspect of urine patch sites is the rapid growth and tillering of the grass around the patch of dead material. During late summer the rapidly growing areas around the urine patch are preferentially grazed by sheep and often provide most of the diet (Keogh, 1984). Given the high proportion of the diet contributed by the urine patch areas, it is the production and distribution of zearalenone in these ares which are of most importance when determining the zearalenone intake of the grazing animal. The characterisation of zearalenone levels within these sites is important in understanding the movement of zearalenone into plant tissues and determining the likelihood that the grazing animal will ingest sufficient quantities of zearalenone to affect reproduction. It is also necessary to investigate the zearalenone distribution within the plant in order to characterise movement of the compound to different parts of the plant and the eventual transfer to the grazing animal. The aims of this investigation were:

1. To characterise zearalenone levels in dung patch, urine patch and inter-excreta sites within a ryegrass dominant pasture and the distribution of zearalenone within the ryegrass plant.
2. To determine the uptake of zearalenone by the plant and the subsequent transport to different components of the plant.

2. Methods and Materials

2.1. Distribution of zearalenone in ryegrass pasture

Herbage samples were collected in late April 1995 from three perennial ryegrass (*Lolium perenne* L.) dominant pastures at AgResearch Aorangi lowland research station consisting of a newly sown pasture (Pasture 1), and two established pastures, one containing endophytic (*Acremonium lolii*) fungi (Pasture 2), and the other being free of endophytic fungi (Pasture 3). The samples consisted of complete ryegrass plants and the soil supporting the roots. Three to five dung patch (DP), urine patch (UP), and inter-excreta (IE) sites (as described by Keogh, 1986) were sampled in each of the three pastures. The samples were kept in sand boxes until dissection. The soil was removed from each sample and a small amount kept for zearalenone analysis. Six tillers from each sample were divided into tiller tops and roots. A further twenty

ryegrass tillers from each sample were taken and dissected into leaf blade (Bl) and leaf sheath (Sh) which were grouped according to the age of the leaf (Bl 1-4 and Sh 2-4). Dead leaf material was also divided into blade (DBl) and sheath (DSh). Roots (Rt) were separated into young and old tissue. Flowering stem (St) and daughter tillers (DT) were also dissected out of the twenty tillers. The components were then freeze dried and weighed and ground prior to zearalenone analysis.

2.1.1. Zearalenone determination

Zearalenone levels in the herbage were determined by an indirect competitive ELISA immunoassay using partially purified zearalenone binding antibodies (di Menna *et al.*, 1991). Zearalenone analyses was carried out in the Plant and Fungal Toxins Lab, Agreasearch, Ruakura Research Centre (see Appendix 2 for full details of assay).
Zearalenone yield was determined by multiplying zearalenone concentration by the dry herbage weight.

2.2. Uptake of zearalenone by the ryegrass plant

Eighty ryegrass tillers were collected from the same site in a pasture which had not been recently grazed. The soil was washed from the roots and the entire plant was washed with distilled water. The plants were kept overnight with the roots submerged in distilled water. The tillers were divided into four treatment groups (n = 20 tillers) and each group of tillers was supported in a container with nutrient solution (see appendix 3 for details of nutrient solution). In three containers, 50 µg of pure zearalenone was added to 6 litres of nutrient solution (2 litres per container). The fourth container was filled with 2 litres of nutrient solution without zearalenone.
The ryegrass tillers were grown in the containers for 7 days at which time they were removed and dissected into blade (Bl), sheath (Sh), mature blade(MBl), mature sheath (MSh), stem (St), daughter tillers (DT), young roots (YR) and old roots (OR). Each component of the twenty tillers was freeze dried, weighed, ground and analysed for zearalenone concentration.
Samples of the nutrient solutions were taken at the beginning and end of the treatment period for zearalenone assay.

Plate 3.1. Ryegrass tillers in nutrient solution

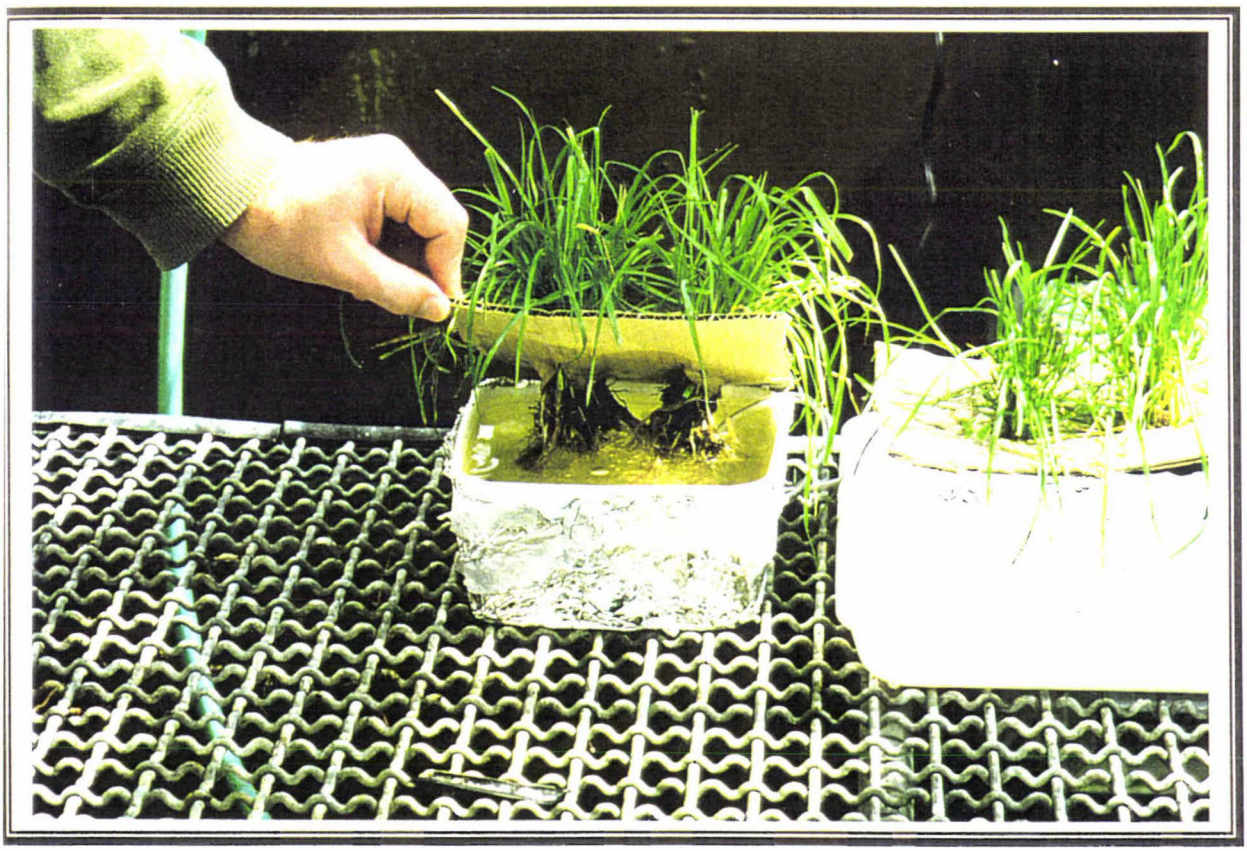


Plate 3.2. Dissection of ryegrass tillers into components

2.3. Statistical analyses

Statistical analysis was carried out using Graph-pad Prism 2.0 software: 10855 Sorrento Valley Rd #203, San Diego, CA 92121 USA.

One and two-way analysis of variance was used to compare zearalenone concentrations and yields between pastures and between dung patch, urine patch and inter-excreta sites within a pasture. The reported probability (P) values were calculated from 'Type III' sums-of-squares, after fitting generalised linear model. Sources of variation due to pasture, site type and interactions between site type and pasture were determined. Generalised linear model procedures were also used to compare zearalenone concentration and yield between plant components and the interaction between site type and component.

Zearalenone levels are given as means $\pm$ standard error of the mean (SEM).

3. Results

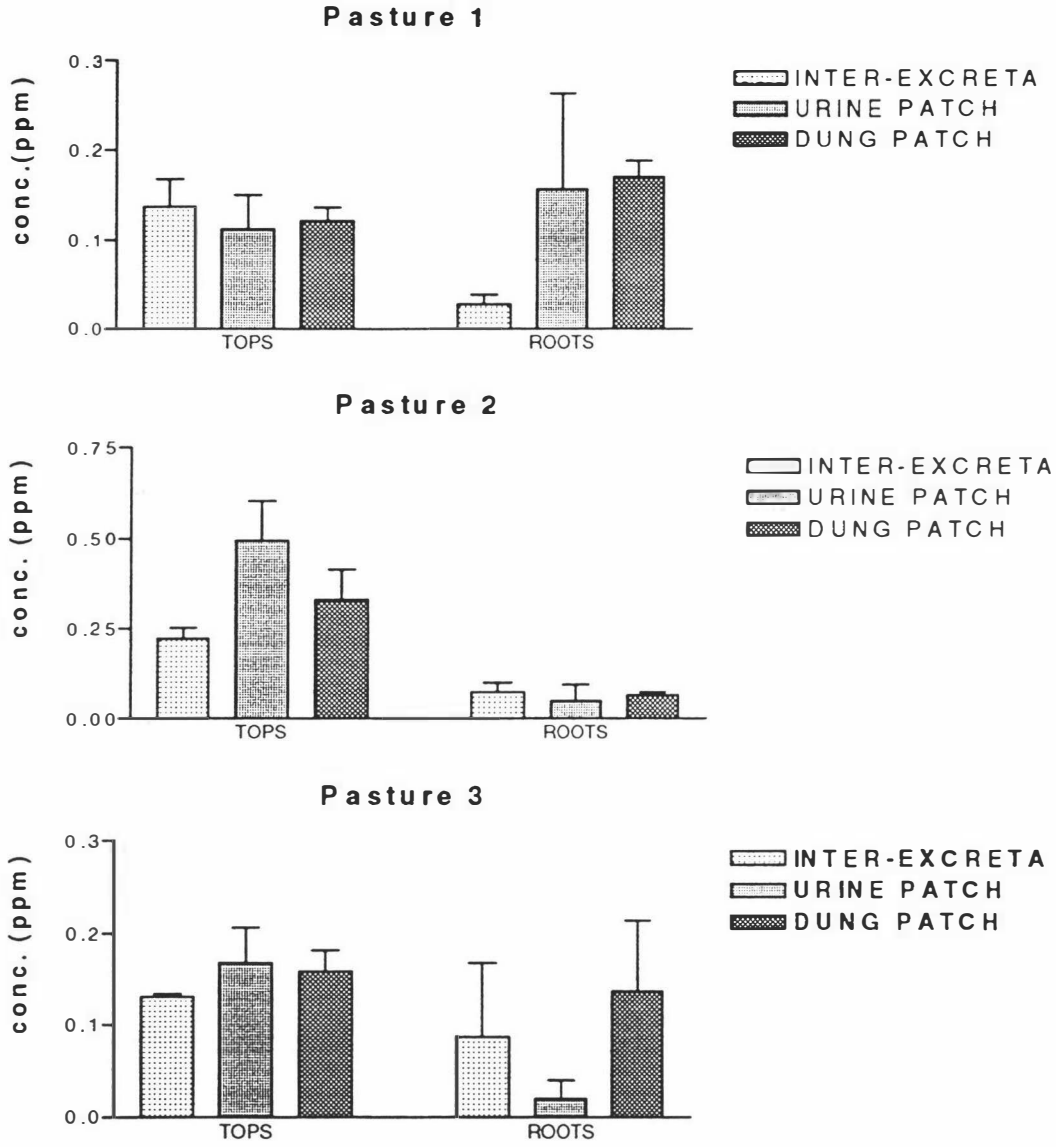
3.1. Zearalenone concentration in pasture sites

The difference in mean zearalenone concentration in the tiller tops between the three pastures was found to be statistically very significant ($P<0.001$) with levels in pasture 2 less than half those in pastures 1 and 3. There was no significant difference in zearalenone concentration in the roots between the three pastures.

Levels of zearalenone were significantly ($P<0.05$) higher in the tiller tops in pasture 2 than in the roots. There was no significant difference in zearalenone concentration between tiller tops and roots in pastures 1 and 3.

There was no significant interaction between the pasture and the site types within a pasture.

Figure 3.1. Zearalenone concentrations (mean + SEM) in tiller tops and roots between inter-excreta, urine patch and dung patch sites in the three pastures .



In pasture 1 zearalenone concentrations were 0.137 ± 0.031 ppm (mean ± SEM), 0.112 ± 0.038 ppm and 0.137 ± 0.015 ppm in the tiller tops and 0.028 ± 0.010 ppm, 0.156 ± 0.106 ppm, and 0.170 ± 0.017 ppm in roots for inter-excreta, urine patch and dung patch sites respectively. There was no significant difference in zearalenone concentration between the dung patch, urine patch and inter-excreta site types in the tiller tops. Zearalenone concentration in the dung patch roots were significantly higher ($P<0.05$) than inter-excreta roots. There was no difference in zearalenone concentration between urine patch and dung patch roots and between urine patch and inter-excreta roots.

In pasture 2 zearalenone concentrations were 0.223 ± 0.03 ppm, 0.495 ± 0.109 ppm and 0.331 ± 0.084 ppm in the tiller tops and 0.044 ± 0.027 ppm, 0.047 ± 0.047 ppm, and 0.064 ± 0.009 ppm in the roots for inter-excreta, urine patch and dung patch sites respectively. There was no significant difference in zearalenone concentration between the three site types in either the tiller tops or roots.

In pasture 3 zearalenone concentrations were 0.131 ± 0.003 ppm, 0.168 ± 0.038 ppm and 0.159 ± 0.023 ppm in the tiller tops and 0.045 ± 0.041 ppm, 0.020 ± 0.020 ppm, and 0.089 ± 0.077 ppm in the roots for inter-excreta, urine patch and dung patch sites respectively. The was no significant difference in zearalenone concentration between the three site types in either the tiller tops or roots.

Zearalenone concentrations were significantly ($P<0.05$) higher in the tiller tops than in the roots. Concentrations were on average 3.6 ± 1.3 times higher in the tiller tops than in the roots of inter-excreta plants, 6.2 ± 2.8 times higher in urine patch plants and 3.9 ± 1.3 times higher in dung patch plants.

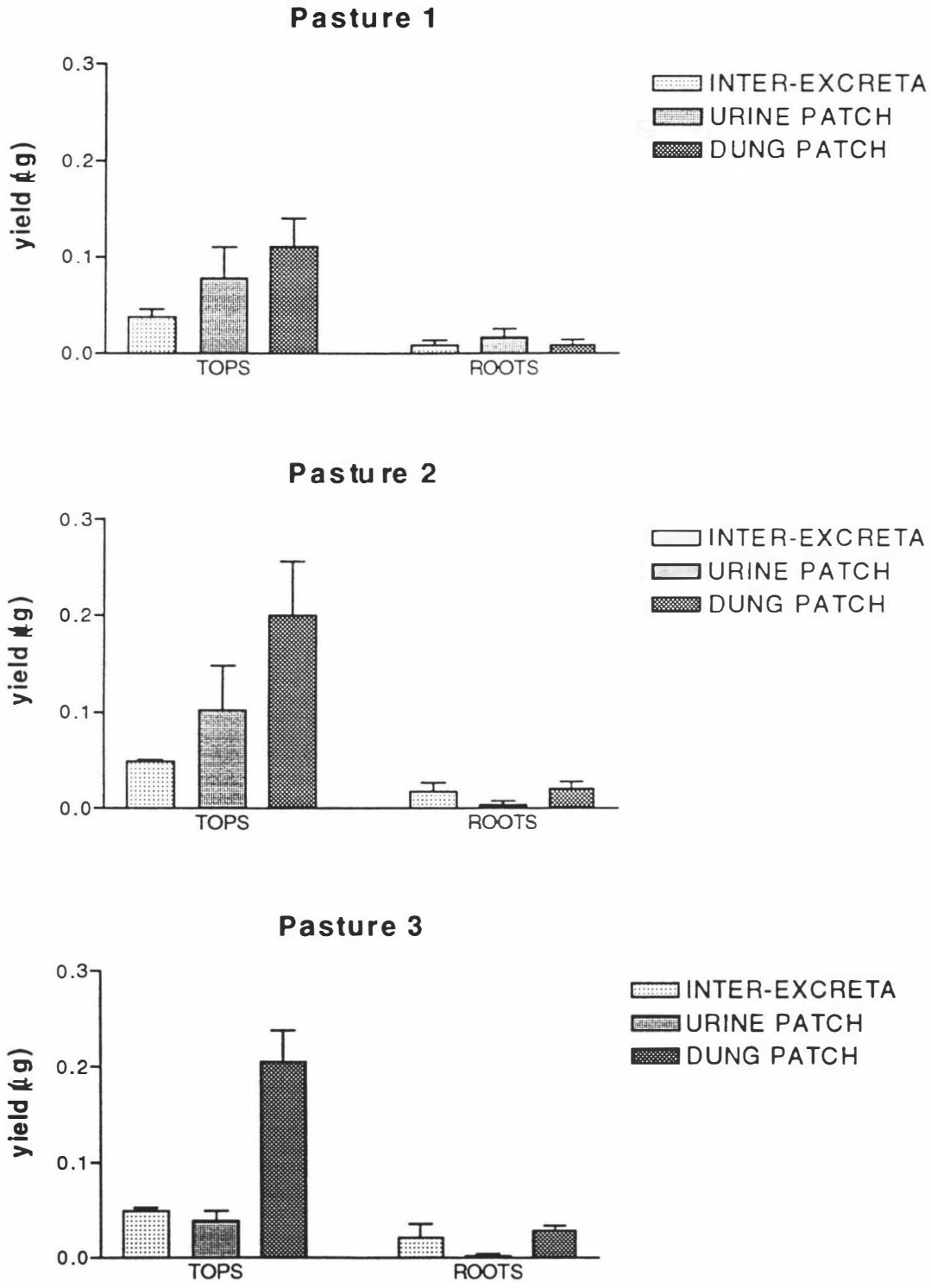
3.2. Soil samples

Zearalenone was only detected (0.01 µg/g) in two soil samples, one from a dung patch site in pasture 1 and the other from a dung patch site in pasture 2.

3.3. Zearalenone yield (Total zearalenone in the plant tissue)

There was no significant difference in the zearalenone yield between the three pastures. The effect of site type on zearalenone yield was significant ($P<0.001$). There was no significant interaction between pasture and site type.

Figure 3.2. Mean (+SEM) zearalenone yields in tiller tops and roots between inter-excreta, urine patch and dung patch sites in each pasture.



In pasture 1 zearalenone yields were 0.038 ± 0.008 µg, 0.078 ± 0.032 µg and 0.111 ± 0.029 µg in the tiller tops and 0.009 ± 0.005 µg, 0.017 ± 0.009 µg, and 0.009 ± 0.006 µg in the tiller tops and roots for inter-excreta, urine patch and dung patch sites respectively.

Zearalenone yields were not significantly different between the dung patch, urine patch, and inter-excreta sites.

In pasture 2 zearalenone yields were 0.049 ± 0.002 µg, 0.102 ± 0.046 µg and 0.200 ± 0.056 µg in the tiller tops and 0.018 ± 0.009 µg, 0.004 ± 0.004 µg, and 0.021 ± 0.007 µg for inter-excreta, urine patch and dung patch sites respectively. Zearalenone yield in the tiller tops of the dung patch sites were significantly higher than the inter-excreta sites, however, there was no difference in yield between the urine patch and dung patch sites or the urine patch and inter-excreta sites. There was no difference in zearalenone yield in the roots between the three site types.

In pasture 3 zearalenone yields were 0.049 ± 0.004 µg, 0.039 ± 0.010 µg and 0.215 ± 0.178 µg in the tiller tops and 0.021 ± 0.015 µg, 0.002 ± 0.002 µg, and 0.029 ± 0.005 µg for inter-excreta, urine patch and dung patch sites respectively.
There was no significant difference in zearalenone yield in the tiller tops between inter-excreta and urine patch sites. Zearalenone yield was significantly higher ($P<0.05$) in dung patch sites than urine patch and inter-excreta sites. There was no difference in the zearalenone yield of the roots between the three site types.

There was no significant difference in zearalenone yield between the same site types in the different pastures. Yields in the tiller tops were significantly greater than in the tiller roots.

3.4. Determination of zearalenone concentration and yield in components of ryegrass tillers from inter-excreta, urine patch and dung patch sites

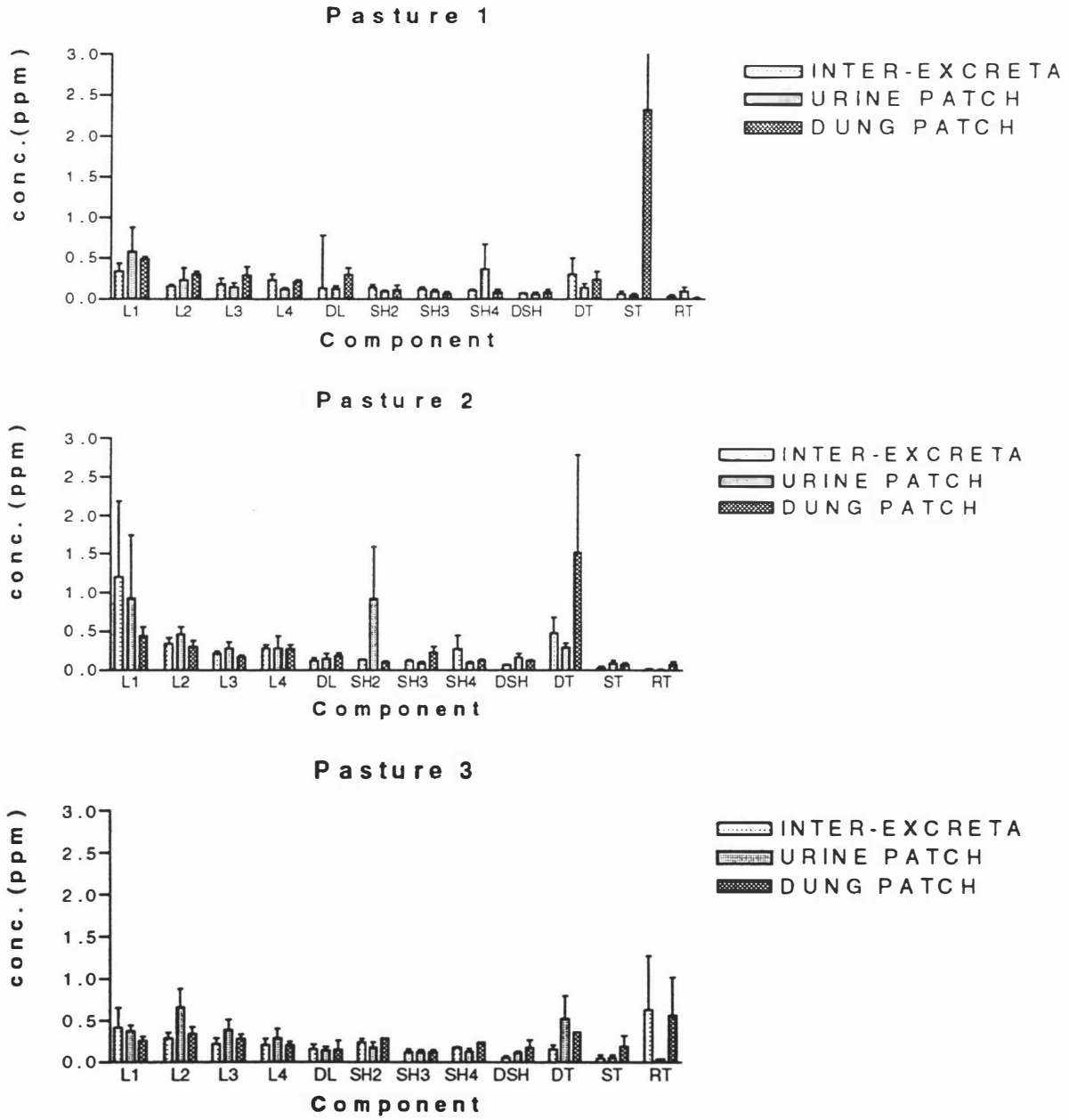
3.4.1. Zearalenone concentration

The difference in zearalenone concentration between the components was found to be highly significant ($P<0.001$) in pasture 1 and there was a significant difference ($P<0.01$) in zearalenone concentration of the components between the dung patch, urine patch and inter-excreta sites. The interaction between components and site types in pasture 1 was found to be

highly significant (P<0.001). Zearalenone concentrations were generally higher in the leaf blade than in the sheath and were highest in the younger parts of the plant.

There was no significant difference in zearalenone concentration between components or different site types in pastures 2 and 3 and there was no significant interaction between site type and component.

Figure 3.3. Zearalenone concentrations in dissected components of ryegrass tillers.



3.4.2. Zearalenone yield

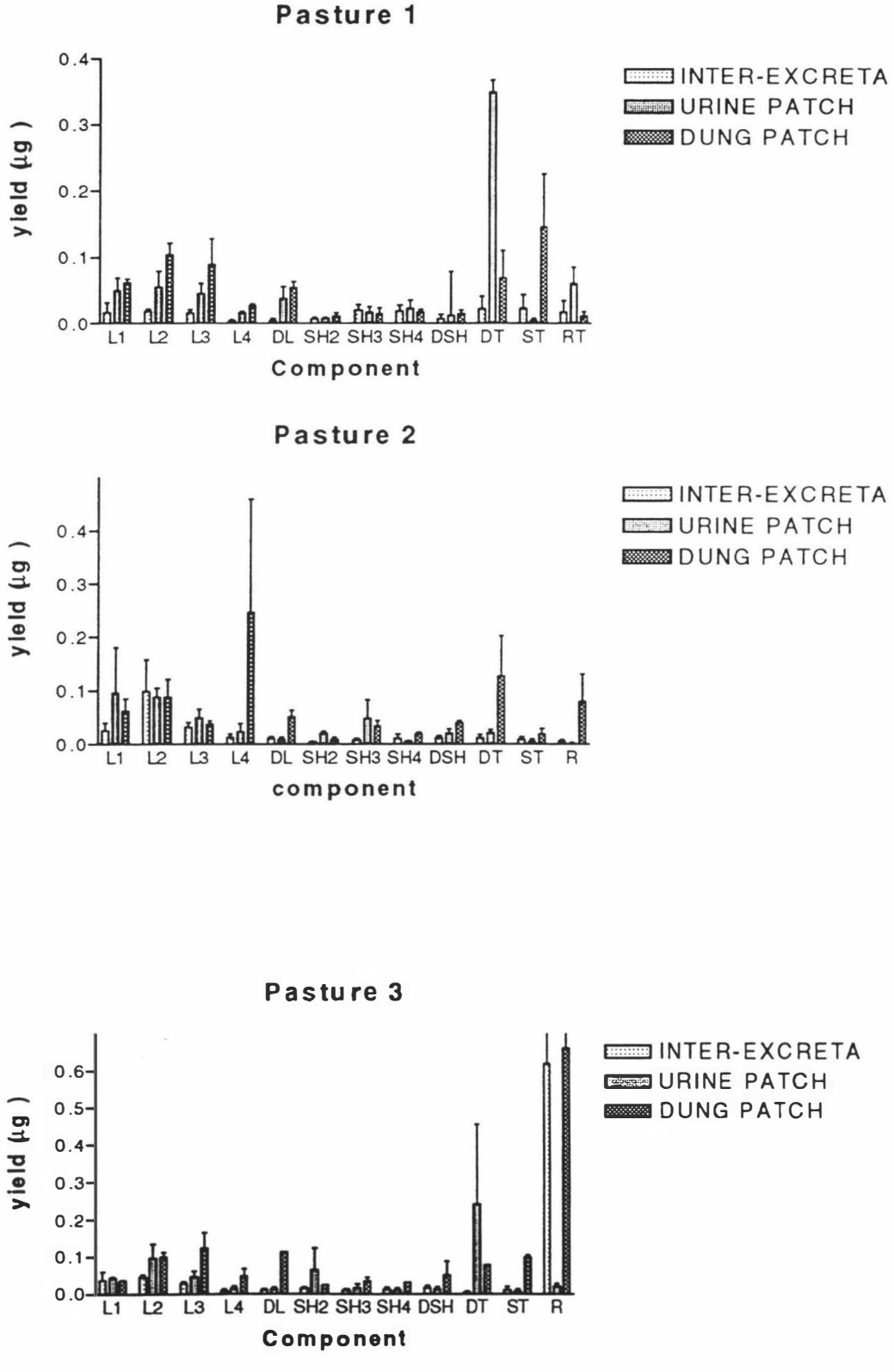
Figure 3.4. shows the zearalenone yields of the components between the dung patch, urine patch, and inter-excreta sites. Zearalenone yields of the different plant components within a site type were also significantly different ($P<0.01$) and there was a significant ($P<0.05$) interaction between site type and component.

In pasture 1 zearalenone yields were generally higher in the leaf blade than in the sheath. Yields were on average higher in components from dung patch plants than urine patch which were higher than inter-excreta. Relatively high yields were obtained from the roots of urine and dung patch plants however.

In pasture 2 there was a significant difference ($P<0.05$) in zearalenone yield between components from urine patch, dung patch and inter-excreta. There was no significant difference in zearalenone yield between components within a site type and no significant interaction between site type and component.

In pasture 3 there was no significant difference in zearalenone yield between components of each site type and between components within a site type and there was no significant interaction between site type and component.

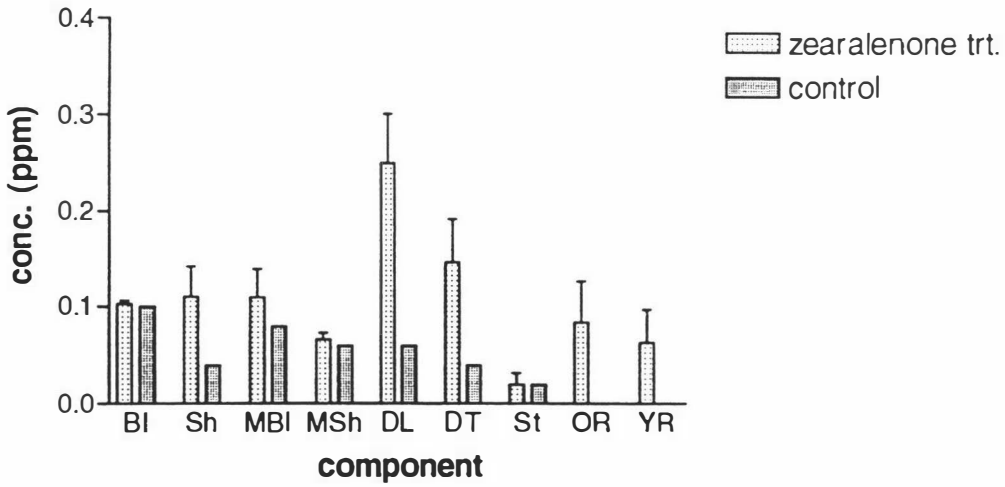
Figure 3.4. Zearalenone yields in dissected components of ryegrass tillers.



3.5. Zearalenone uptake

The ryegrass components grown in the solution containing zearalenone all had higher zearalenone concentrations than the control. The highest zearalenone concentration in the zearalenone treatment was found in the dead leaf material at 0.25 ± 0.05 ppm compared to 0.06 ppm in the control dead leaf. The highest concentration in the control plants was found in the blade (0.1 ppm) which was as high as the blade in the zearalenone treatment (0.103 ± 0.003 ppm).

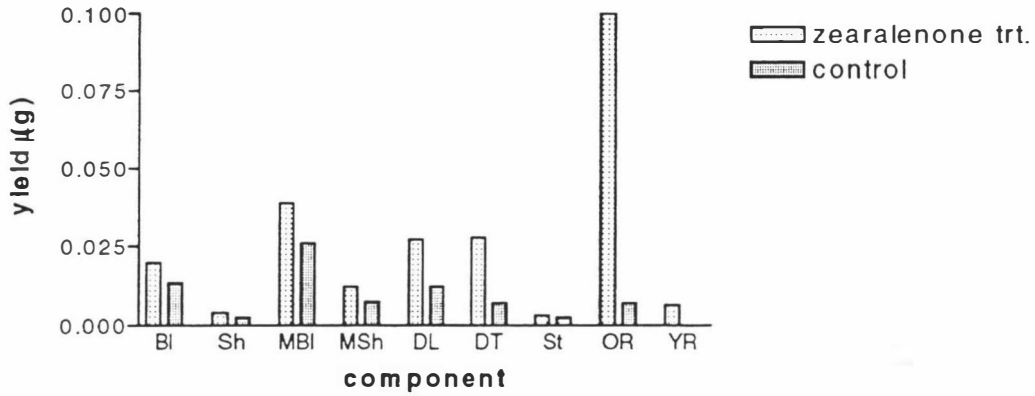
Figure 3.5. Zearalenone concentration in the blade (Bl), sheath (Sh), mature blade (MBl), mature sheath (MSh), dead leaf (DL), daughter tillers (DT), stem (St), old root (OR) and young root (YR), components in ryegrass tillers grown in a solution which contained zearalenone.



3.5.1. Zearalenone yield

The highest zearalenone yields in the zearalenone treatment (highest to lowest) were in the old root, mature blade, dead leaf and daughter tillers. These components were also highest yielding in the control plants with the exception of the old roots.

Figure 3.6. Mean zearalenone yields in the blade (Bl), sheath (Sh), mature blade (MBl), mature sheath (MSh), dead leaf (DL), daughter tillers (DT), stem (St), old root (OR) and young root (YR), components in ryegrass tillers grown in a solution which contained zearalenone.



3.5.2. Zearalenone concentration in nutrient solutions

The pre-treatment zearalenone concentration in the nutrient solutions was 0.46 ng/ml and 0 ng/ml for the zearalenone and control treatments respectively. After the tillers were removed from the solutions the zearalenone concentrations were 0.04 ng/ml and 0 ng/ml for the zearalenone and control treatments respectively. These results mean that there was approximately 1.84 µg present in solution in the 4 litres of nutrient solution which was reduced to 0.016 µg after the plants were removed. Therefore 1.82 µg of zearalenone was removed during the treatment period. The total zearalenone yield in the tillers grown in this solution was 1.81 µg.

4. Discussion

4.1. Zearalenone distribution in pasture

The results obtained in the investigation into zearalenone distribution within ryegrass pasture varied between areas and site types within an area. Zearalenone concentrations were generally lower in pasture1 which was the youngest and hence had less dead material in the sward and soil near the surface for fusaria to colonise. Zearalenone concentrations in the tiller tops were significantly higher in Pasture 2 (endophytic) which may suggest that the presence of

endophyte fungi affects zearalenone levels. In all three areas sampled, there was no significant difference in zearalenone concentration between inter-excreta, urine patch and dung patch sites. This lack of significant difference is indicative of the large variability in zearalenone concentration within each site type.

High N sites such as urine and dung patch sites offer favourable conditions for the proliferation of many fungal species which include various Fusarium species known to produce zearalenone (Keogh, 1973b). Zearalenone production has been linked to changing climatic conditions (Mirocha *et al.*, 1978) such as temperature and light, although no particular weather pattern has been associated with high zearalenone concentrations. Given the large number of variables present in zearalenone production, it is difficult to determine the conditions which result in production of zearalenone by the fusaria present in the pasture. These variables explain the lack of correlation between Fusarium numbers and zearalenone concentration observed by di Menna *et al.*, (1987). The samples in this investigation were taken on one occasion and therefore offer no information on zearalenone production resulting from changes in climatic conditions and substrate available for zearalenone-producing Fusarium colonies. The samples were taken in late April which is later than the months in which zearalenone production is considered to be at its peak. The pastures had not been recently grazed and there was new regrowth and very little dead material present. These factors and the fact that zearalenone production was found to be lower in 1995 than previous years (Keogh, pers. comm) reduces the possibility of detecting differences between the sites.

The zearalenone concentrations found in this investigation were all below concentrations found between January and April in other New Zealand pastures from Wanganui and Gisborne in an earlier study by di Menna *et al.*, (1987), which reported zearalenone levels of between 0.4 and 4.0 mg/kg dry weight of herbage.

In most cases zearalenone was not detected in the soil samples which indicates that most of the zearalenone at these sites was associated with the plant material above and below ground. It is possible that zearalenone was leached from the soil by watering during the period the plants were kept in the sand boxes prior to dissection as the soil samples containing zearalenone were among those dissected first.

In all areas sampled the zearalenone yields were highest in the tillers from dung patch sites followed by urine patch and inter-excreta sites. This result reflects the larger herbage mass of the sites with higher N levels and the lack of grazing pressure on the dung patch sites. At the

time of sampling the areas were not being grazed and some regrowth had occurred which resulted in the higher herbage masses in the sites with higher N levels. The herbage around urine patch sites is preferentially grazed (Keogh, 1984) and it is from this portion of the pasture that the grazing animal is likely to ingest zearalenone.

The ryegrass dissection results showed that zearalenone concentrations were generally higher in the leaf blade than the sheath which was most evident in the youngest leaves. The daughter tillers also had relatively high levels of zearalenone present. As was seen in the whole tiller samples there was no significant difference in zearalenone concentration between inter-excreta, urine patch and dung patch sites within a pasture and there was a large variability in zearalenone concentration within a site type. The higher zearalenone concentrations in the young parts of the plants would suggest that zearalenone taken up by the roots is translocated predominantly to these areas along with most of the nutrients required for growth. This has implications for the grazing animal as it is these parts of the plants which are selectively grazed and become the primary source of zearalenone for the animal.
Zearalenone yields were greater in the leaf blade than in the sheath which is due to the higher zearalenone concentration and dry weight associated with the blade. Yields of zearalenone were highest in the dung patch and urine patch plant components which is due to a larger herbage mass than any difference in zearalenone concentration. The measurement of zearalenone yield in the different plant components enables the quantification of zearalenone intakes by the grazing animal as it allows comparison between parts of the plant which are eaten by the animal and those which are not normally eaten and the amount of plant material in each component.

4.2. Assessment of method of estimation of zearalenone distribution

The samples taken in this investigation gave an instantaneous representation of the zearalenone distribution at the various site types and these allowed comparison between the site types and pasture types. However, the method could not show the possible changes in zearalenone concentration and distribution over time within a site type which would illustrate some of the seasonal and grazing management effects on zearalenone production. Samples were kept outside in sand boxes until dissection which was carried out over a two week period. The

length of time from sampling to dissection may have influenced the zearalenone present in the plant causing difficulty in comparing samples which were dissected days apart. Despite these limitations, this investigation provided information on zearalenone distribution within different pasture site types and within the ryegrass plant.

4.3. Zearalenone uptake by the ryegrass plant

The zearalenone uptake trial was aimed at determining whether zearalenone could be taken up by the roots and translocated in the ryegrass plant. The highest concentration was found in the dead leaf material (0.25 $\pm$ 0.05 ppm) which was not expected considering there would be no active transport to this tissue. The next highest concentration was found in the daughter tillers (0.147 $\pm$ 0.045 ppm). The daughter tillers, and other new growth areas on the plant, are the sites of most of the nutrient transport and therefore nutrients taken up by the root would be transported and deposited in these tissues along with other compounds such as zearalenone which may have been taken up by the roots. The relatively high concentration of zearalenone in the old and young roots of the tillers grown in the solution containing zearalenone may be as a result of zearalenone adhering to the root surface rather then deposition of zearalenone in the root tissue. Analysis of the zearalenone concentration in the nutrient solution showed that only approximately 1.84 µg of the 50 µg added to the 6 litres of solution stayed in solution. Zearalenone's low solubility in water and possible adherence to the container walls are the most likely reasons for the loss of zearalenone. However, it appeared that most of the zearalenone remaining in solution was taken up by the plant as the difference in total yield between the zearalenone treatment and the control plant was approximately 1.6 µg and the loss of zearalenone from the nutrient solution was 1.82 µg which leaves 0.22 µg of zearalenone unaccounted for.

4.4. Assessment of the method

The zearalenone uptake trial was a preliminary investigation into measuring zearalenone uptake via the roots and subsequent transport within the plant. The trial was a first attempt at determining zearalenone uptake in the ryegrass plant and was important in the further development of a suitable method for achieving this. Major concerns were in keeping

zearalenone in an aqueous solution in significant amounts so that uptake by the roots can be determined. Once developed fully, this method will be valuable in characterising zearalenone uptake and distribution by many species of pasture plants.

4.5. Conclusions

The main factors which affect the ingestion of zearalenone by the grazing animal are grazing behaviour and the production and distribution of zearalenone in the forage. Grazing behaviour can be easily determined and is well understood, however, the difficulty in characterising zearalenone production and distribution within the pasture and plant does not allow accurate quantification of zearalenone intake. It is necessary to further investigate the distribution of zearalenone in different pasture sites and within the plant so that the relationship between zearalenone production in the pasture and subsequent ingestion by the animal can be determined.

The characterisation of zearalenone transport in the ryegrass plant is an important part in understanding how zearalenone, once produced, is ingested by the grazing animal. If we assume that zearalenone is more likely to accumulate in the youngest parts of the plant once taken up by the roots it is likely that a large proportion of this will be ingested by grazing animals. Further work needs to be done to better understand the transport of zearalenone in the plant and the implications to the grazing animal.

CHAPTER IV

Zearalenone and related compounds in the blood and urine of ewes intravenously and orally dosed with zearalenone and the effects on reproductive performance.

1. Introduction

Zearalenone is a naturally occurring mycotoxin produced by Fusarium species which are present in New Zealand pasture (di Menna *et al* ., 1987; di Menna *et al.*, 1991 and Lauren *et al.*, 1988). Zearalenone and its metabolites α- and β-zearalenol have been shown to have oestrogenic properties (Miksicek, 1994). The adverse effects of zearalenone on reproduction have been extensively reported in pigs (Aucock *et al.*, 1982; Long *et al.*, 1982 and Ruhr *et al.*, 1983), poultry (Allen *et al.*, 1981 and Maryamma *et al.*, 1992) and cattle Mirocha *et al.*, 1968; Danko and Aldsay., 1969, and Miller *et al.*, 1973). To date only a few studies have examined effects of zearalenone on ewes. Reproductive performance was markedly reduced in ewes dosed daily with Fusarium cultures containing 25 mg zearalenone for 10 days prior to mating (Smith *et al.*, 1986). Later Smith *et al.*, (1988) observed a significant reduction in ovulation rate in ewes dosed daily for 10 days with Fusarium cultures containing 6 mg zearalenone. Intakes of zearalenone of 3 mg/ewe/day or more during a ten day period prior to mating have been reflected as depressed ovulation rate and lower lambing percentages (Smith *et al.*, 1990). In these trials investigating the effects of zearalenone on sheep reproduction, the zearalenone was dosed as part of a Fusarium culture which is likely to contain other compounds, some of which will also have bioactive properties, which may add to the effect on reproductive performance. In the studies where pure zearalenone was dosed orally, only the reproductive parameters were examined and no account was taken of how much of the dosed zearalenone entered the blood stream and in particular the amount of unconjugated or free zearalenone which is the oestrogenically active portion. The conversion of zearalenone into other metabolites was also not taken into account. Kiessling *et al.*,(1984) showed that zearalenone was reduced to α-zearalenol and to a lesser extent β-zearalenol in rumen fluid. It is likely that a large proportion of ingested zearalenone is converted in the rumen to α-zearalenol which is of higher oestrogenic potency than zearalenone (Miksicek, 1994). Fusarium species present in New Zealand pasture are capable of producing α- and β-zearalenol which may result in sheep

ingesting these metabolites with the forage (di Menna *et al* ., 1987). There is also evidence that zearalenone is converted to zearalenols and zearalanols in the animal (Miles *et al*., 1996) Therefore, it is necessary to consider the other metabolites of zearalenone in addition to zearalenone when determining effects on reproductive performance. The aims of this study were:

1. To determine free and conjugated zearalenone and other zearalenone metabolites in the blood and urine of ewes dosed orally or intravenously with pure zearalenone for ten days prior to mating.
2. To measure the effects of zearalenone on the reproductive performance in sheep.

2. Methods and Materials

2.1. Animals and treatments

Sixty mixed-age Romney ewes were divided into two groups (n=30) which were either grazed on perennial ryegrass (*Lolium perenne.* L.) pasture or on chicory (*Chicorium intybus* L. *cv* Grasslands Puna), which has low levels of zearalenone (<0.1 μg/g). Within each grazing treatment the ewes were divided into three sub-groups (n = 10) which were allocated to either oral (O) or intravenous (IV) dosing with zearalenone, and a control (C). The oral dosed group received a dose of 5 mg of pure zearalenone in 10% ethanol solution daily for 10 days prior to mating. Half of the ewes in the intravenous dosed group received 2 mg of zearalenone (IVH) in 10% ethanol injected intravenously daily for 10 days prior to mating and the remaining half received 0.5 mg of zearalenone (IVL) in the same manner.

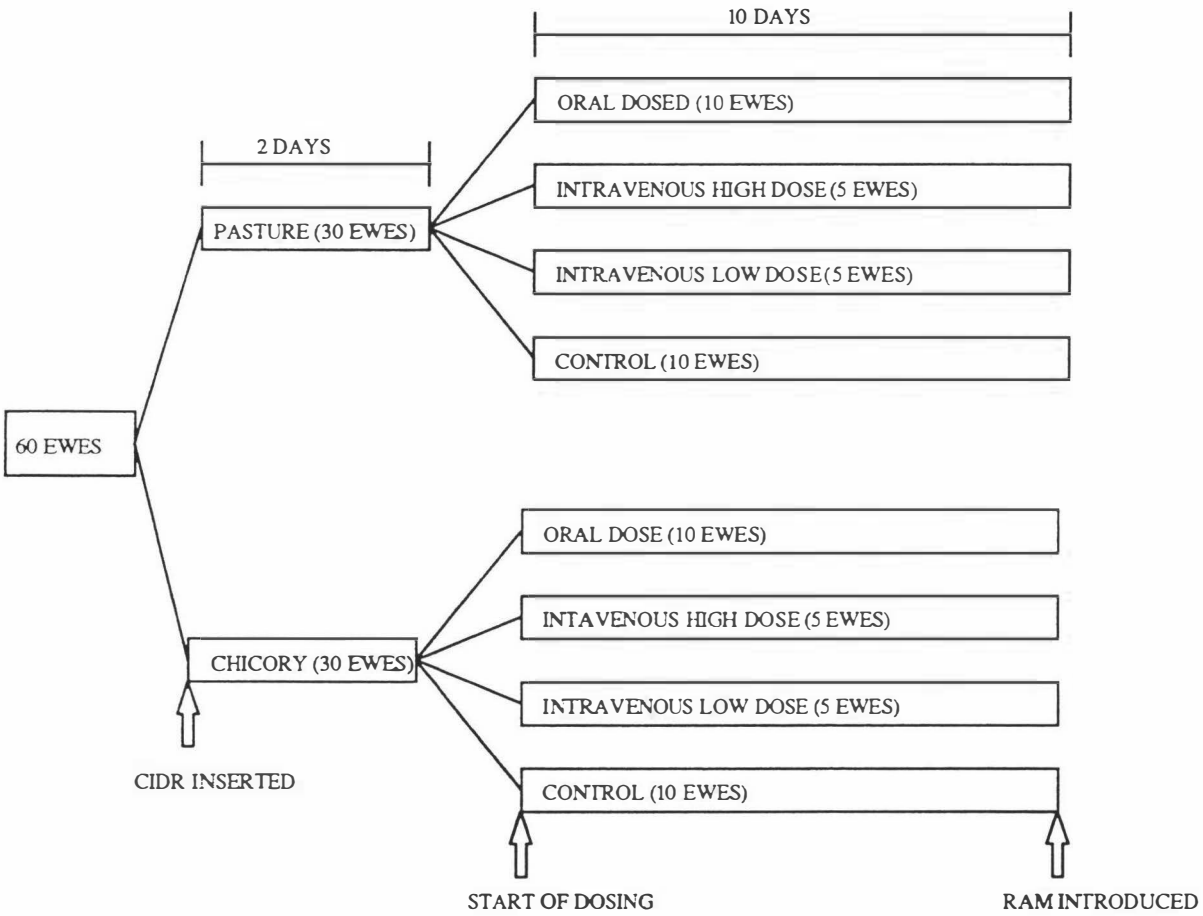
Ewes were treated with synchronisation devices (CIDR, type G, containing 0.3 g progesterone) for 14 days before mating (see Fig. 4.1.).

All ewes were blood sampled pre-treatment by jugular venipuncture with hypodermic needle and evacuated collection tube (vaccutainer 10 ml draw). Subsequent blood samples were taken daily before dosing. On day 5 of dosing blood and urine samples were collected at 2, 4 and 24 hours after dosing in the pasture group and 1, 4 and 24 hours after dosing in the chicory group. Samples of chicory and the ryegrass pasture were taken for zearalenone determination.

After the 10 days of treatment, the CIDRs were removed and the ewes were run on their respective forages with an entire ram fitted with harness and crayon and inspected daily for

mating marks. After all ewes had shown oestrus, the crayon colour was changed to identify returns. Ovulation rates were determined 7 days after mating by laparoscopic examination.

Figure 4.1. Diagram showing treatment groups



2.2. Determination of zearalenone and its metabolites in blood and urine

Whole blood and urine samples were analysed for conjugated and free "zearalenone" equivalents by enzyme-linked immunoassay (ELISA) (di Menna et al., 1991(appendix II)).The ELISA recognises zearalenone (100%), but also has appreciable cross-reactivities with α-zearalenol (220%), β-zearalenol (60%), α-zearalanol (110%),Taleranol (35%) and zearalanone (46%).

Blood and urine taken at 24 hour intervals was subsampled and combined to make composite samples representing each treatment group at each sampling and were analysed for total zearalenone, zearalanone, α-zearalenol, β-zearalenol, zeranol and taleranol by a multiresidue assay for a number of anabolic and oestrogenic compounds and fungal metabolites (Erasmuson *et al*,. 1994). Aliquots (5ml) were analysed by glucuronidase re-formation of the alcohols, extraction with hexane/tBME (70:30), and then HPLC cleanup of the extracts with programmed fraction collection . A fraction was evaporated under nitrogen, then trimethylsilyl derivatized with MSTFA, and subject to GC-MS analysis (see appendix I for full details of assay).

2.3. Statistical analyses.

Statistical analyses were done using Graph-pad Prism 2.0 software: 10855 Sorrento Valley Rd #203, San Diego, CA 92121 USA.
Differences in ovulation rate between oral, intravenous and control groups were compared by the Kruskal-Wallis test which is a non-parametric test to compare three or more unpaired groups. Difference in ovulation and conception rate between groups grazed on chicory and groups grazed on pasture were compared by Wilcoxon signed rank test which is a nonparametric test that compares two paired groups.
Levels of zearalenone and related metabolites in the blood and urine were compared by one-way analysis of variance (ANOVA) techniques.

3. Results

3.1. Liveweight

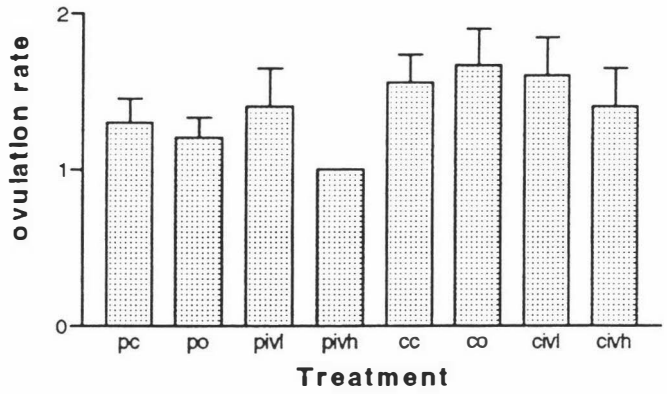
Ewes grazed on chicory lost an average of 0.5 $\pm$ 0.72 kg and ewes grazed on pasture lost an average of 1.0 $\pm$ 0.43 kg. There was no significant difference in the weight loss between the grazing treatments.

3.2. Ovulation rate

There were no significant differences in ovulation rate between oral dosed (O), intravenous high (IVH) and low (IVL) dosed and the control (C) groups within either the pasture or the chicory treatments (Fig 4.2.).

However, a significant difference ($P<0.05$) was found in mean ovulation rate between the group grazed on chicory (1.57 ± 0.11) and the group grazed on pasture (1.23 ± 0.08).

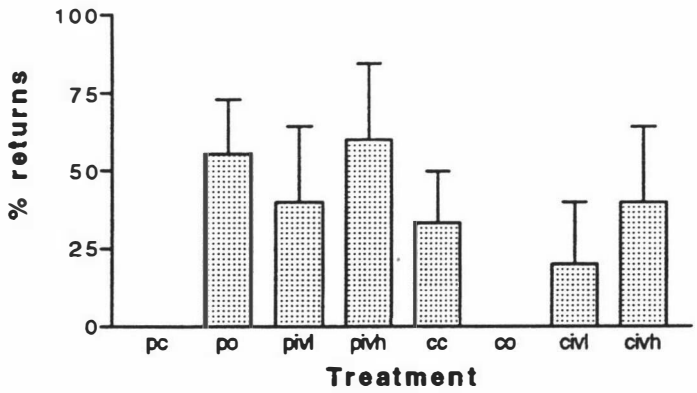
Figure 4.2. Mean ovulation rate (+ SEM) for each treatment group.



3.3. Conception

There was no significant difference in the number of returns to oestrus between the groups of ewes grazed on chicory and those grazed on pasture (Fig. 4.3.). There were no returns in the pasture control or chicory oral groups. The number of returns were 33% and 20% for the groups grazed on pasture and chicory respectively however, they were not significantly different.

Figure 4.3. Number of returns in each treatment group.



3.4. Zearalenone in the forages

The zearalenone concentrations in the forages were 6.0 μg/g and 0.18 μg/g for the ryegrass pasture and chicory respectively

3.5. Zearalenone and its metabolites in blood

Free zearalenone concentrations (Fig 4.4.) in the intravenously dosed pasture groups 2 hours after dosing in the IV groups were 0.20 ± 0.11 ng/ml and 0.858 ± 0.47 ng/ml for the low and high dose respectively. The highest concentration recorded (0.69 ± 0.20 ng/ml) in the low IV group was at 4 hours after dosing. The free zearalenone concentration in the oral dosed pasture group was greater than the IV groups with a concentration of 1.56 ± 0.14 ng/ml at 4 hours after dosing. By 24 hours the levels of free zearalenone in the blood of all the dosed pasture groups had fallen to 0.09 ± 0.05 ng/ml, 0.046 ± 0.05 ng/ml for the oral and high IV dose respectively and were not detected in the low IV dose group. The free zearalenone concentration in the groups grazing chicory were on average higher that the levels in the pasture groups. The highest free zearalenone concentrations recorded were 0.67 ± 0.29 ng/ml, 1.35 ± 0.16 ng/ml and 0.75 ± 0.19 ng/ml for the oral, low IV and high IV dose groups respectively, 1 hour after dosing. By hour 4 the free zearalenone concentrations had decreased to 0.43 ± 0.05 ng/ml, 0.28 ± 0.08 ng/ml, 0.28 ± 0.12 ng/ml for the oral, low IV and high IV dosed groups respectively.

The conjugated zearalenone concentration in the pasture groups follows a similar pattern after dosing and was at similar levels to the free zearalenone. Conjugated zearalenone concentrations were 1.50 ± 0.29 ng/ml, 0.81 ± 0.33 ng/ml and 0.82 ± 0.30 ng/ml for the oral, low IV and high IV dosed groups respectively 4 hours after dosing. The oral and low IV dosed groups had highest conjugated zearalenone levels at 4 hours after dosing whereas the high IV group had similar concentration for all samples.

The levels of conjugated zearalenone (Fig 4.5.) in the groups grazing chicory follow a similar pattern to the concentrations of free zearalenone. The conjugated zearalenone concentrations were highest 1 hour after dosing being 1.18 ± 0.44 ng/ml, 0.98 ± 0.56 ng/ml and 1.61 ± 0.46 ng/ml for the oral, low IV and high IV groups receptively. Levels of free and conjugated zearalenone appeared to be very high in the chicory control group 1 hour after dosing (1.65 ±

0.23 ng/ml and 2.62 ± 0.31 ng/ml for free and conjugated respectively) which cannot be explained.

Figure 4.4. Mean levels of unconjugated (free) zearalenone in the blood of ewes in the OD, IVH, IVL and CON groups grazed on either pasture or chicory, at four times during a 24 h period after dosing.

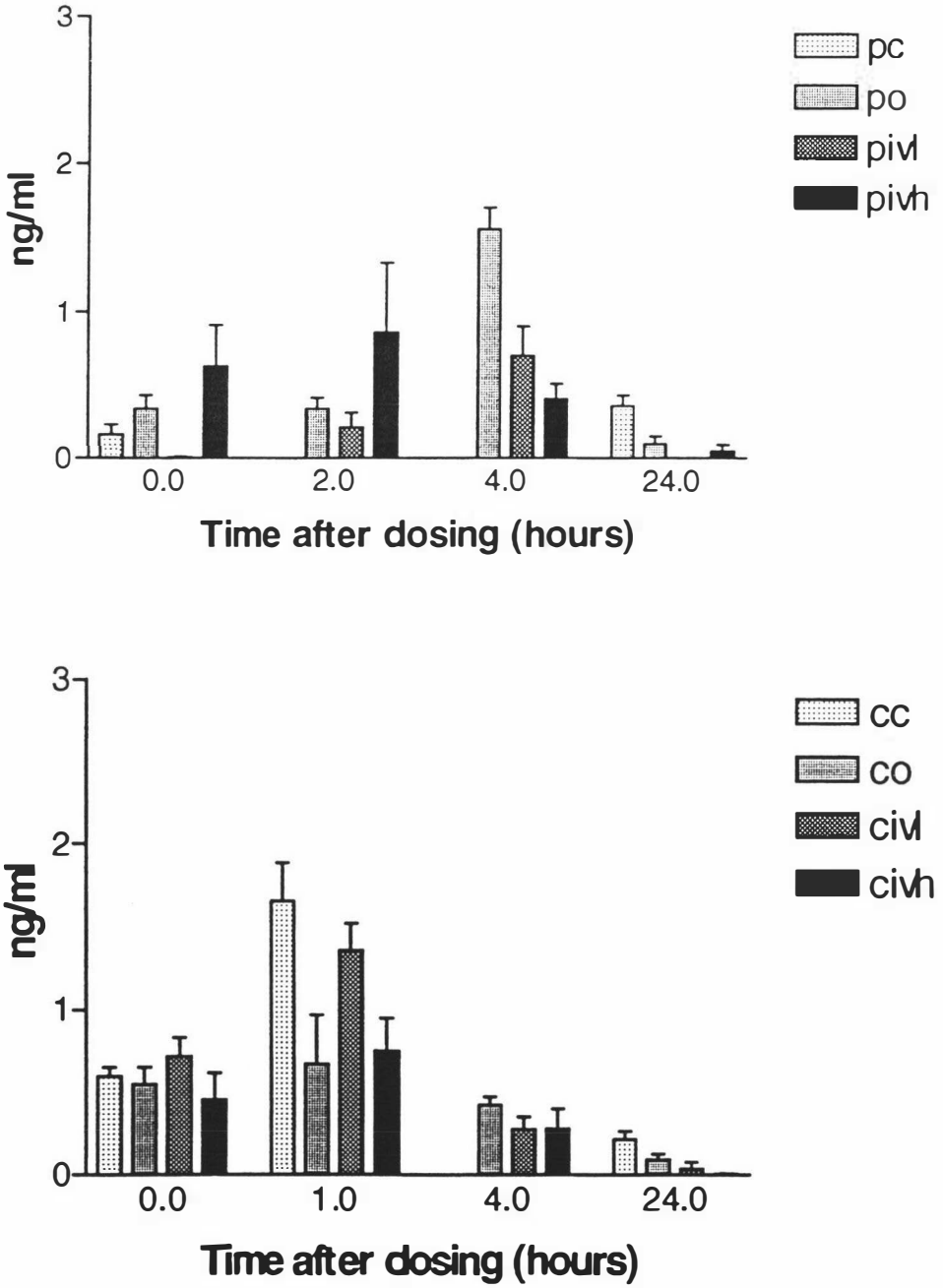
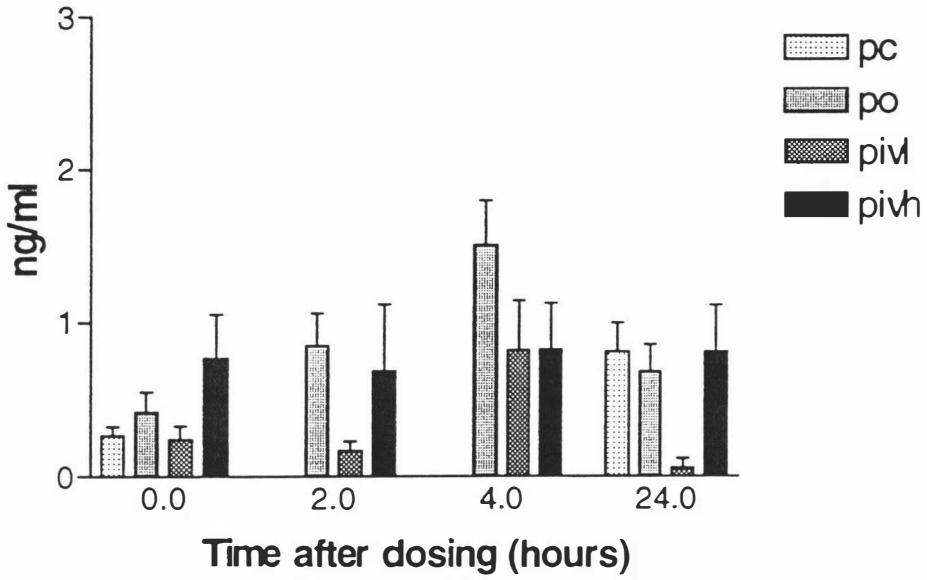
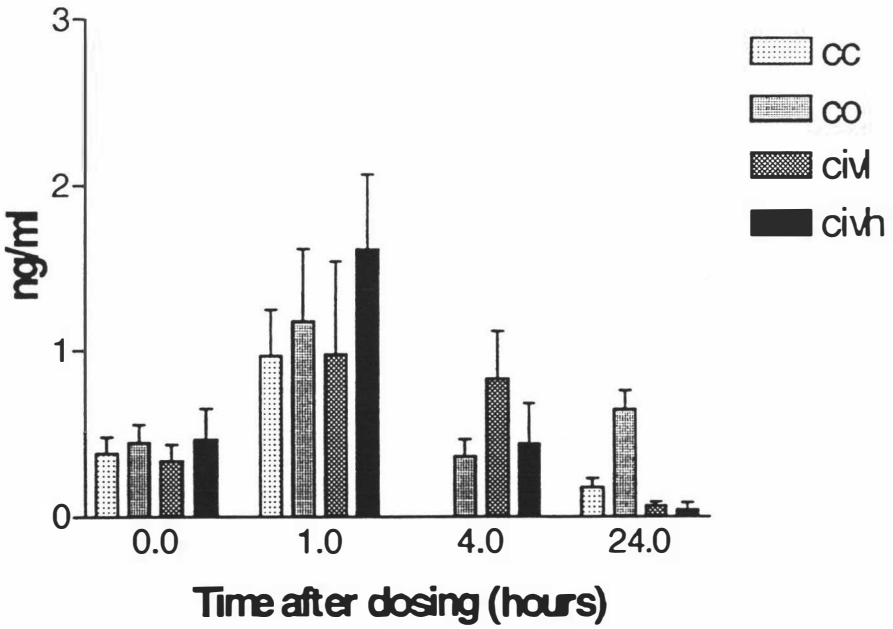


Figure 4.5. Mean levels of conjugated zearalenone in the blood of ewes in the OD, IVH, IVL and CON groups grazing either ryegrass pasture or chicory, at various times during a 24 h period after dosing.





Levels of alkane metabolites were very small (<1%) compared to alkene metabolites. Zearalenone was the major metabolite present (16.7 ng/ml) in the oral dose ewes. Alpha and beta zearalanol and zearalenol were found in both control and oral dosed groups.

Figure 4.6. Levels of alkene zearalenone metabolites in the blood of oral dosed ewes and control ewes grazed either pasture or chicory.

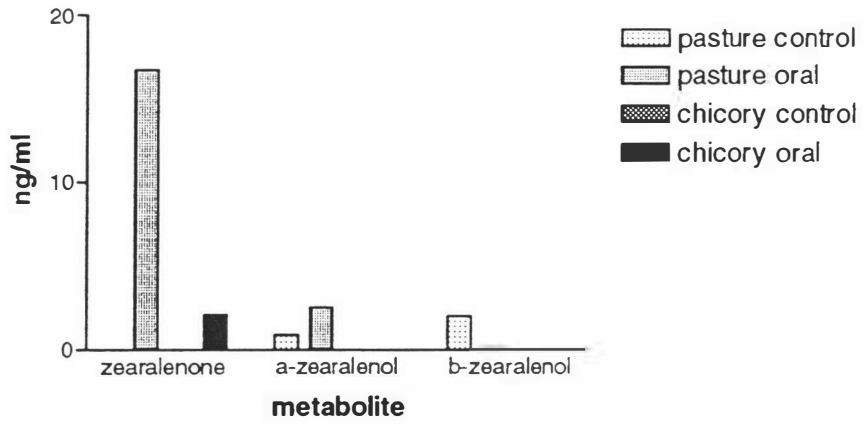
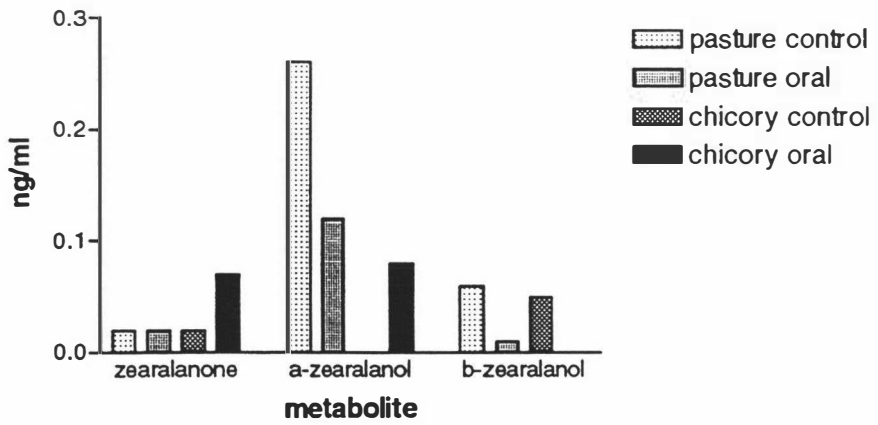


Figure 4.7. Levels of alkane zearalenone metabolites in oral dosed and control ewes grazing either pasture or chicory.



Zearalenone was also the predominant metabolite present (2.1 ng/ml) in the oral dosed group grazing chicory. Levels of zearalenone in the oral dosed group grazing pasture were eight times higher than levels in the oral dosed group grazing chicory. Zearalenone, α-zearalenol, β-zearalenol and α-zearalanol were not detected in the chicory control group. Levels of alkane metabolites were <1% of the alkene metabolites where both alkene and alkane metabolites were present.

Levels of both alkene and alkane metabolites were higher in the groups grazing pasture than those grazing chicory (Fig 4.7.).

3.6. Zearalenone and its metabolites in the urine

After two days of grazing the different forages the levels of free and total zearalenone were significantly higher ($P<0.05$) in the urine of ewes grazed on the pasture treatment (Fig 4.8.). The free zearalenone:creatinine ratios were 0.055 $\pm$ 0.009 mmol/mol and 0.027 $\pm$ 0.005 mmol/mol for the pasture and chicory groups respectively. The conjugated zearalenone:creatinine ratios were 0.389 $\pm$ 0.037 mmol/mol and 0.134 $\pm$ 0.020 mmol/mol for pasture and chicory treatments respectively. Levels of free zearalenone in the urine of ewes grazing chicory were approximately half those in the urine of ewes grazing ryegrass pasture after two days of grazing.

Figure 4.8. Free and conjugated zearalenone/creatinine ratios in the urine of ewes which have been grazed on either ryegrass pasture or chicory for two days before dosing treatments started.

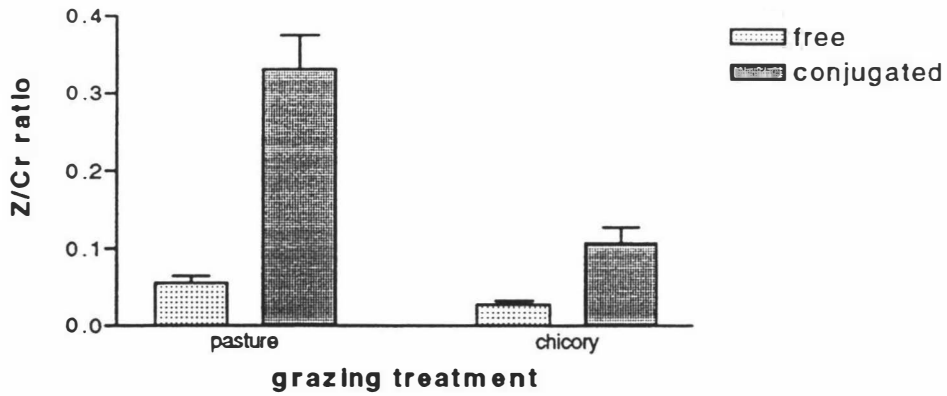
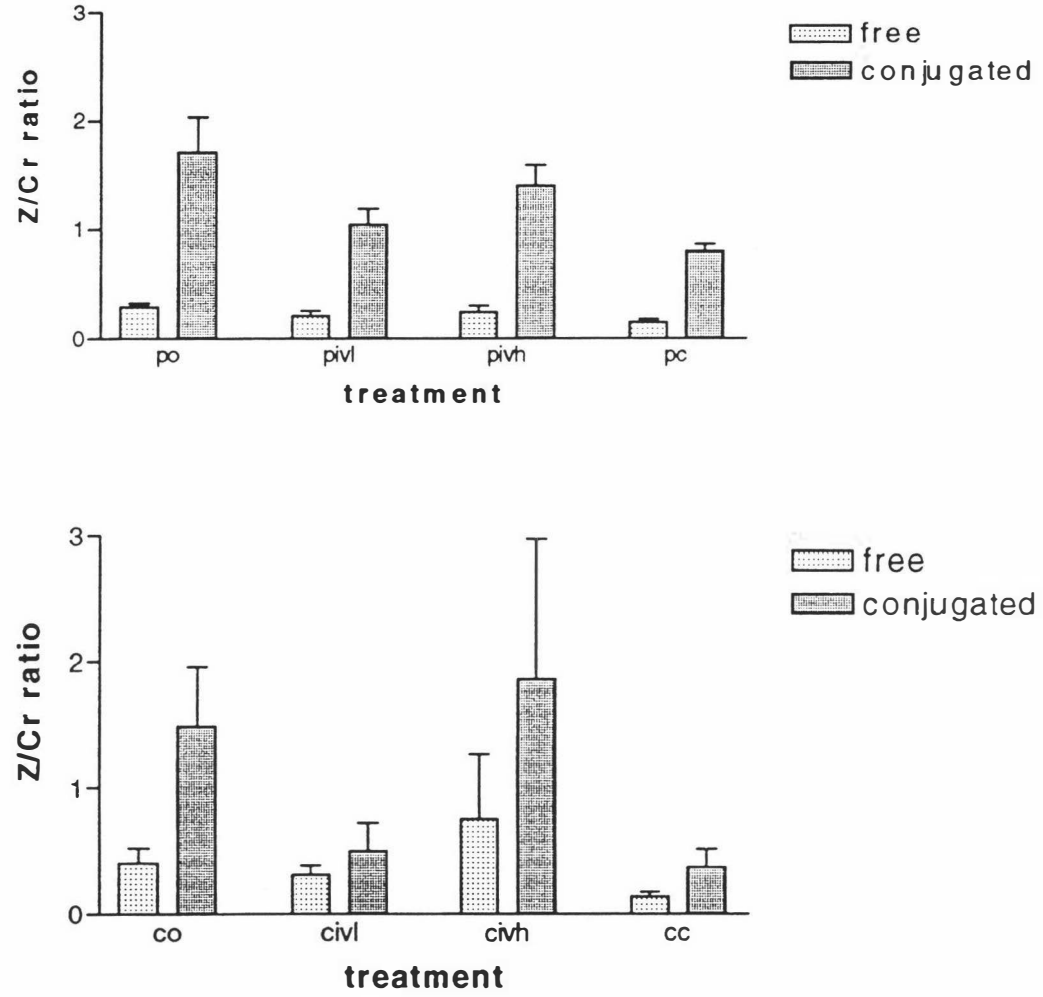


Figure 4.9. Free and total zearalenone:creatinine ratios on day 6 of dosing in the urine of the OD, IVL, IVH and control (C) ewes grazed on either ryegrass pasture or chicory.

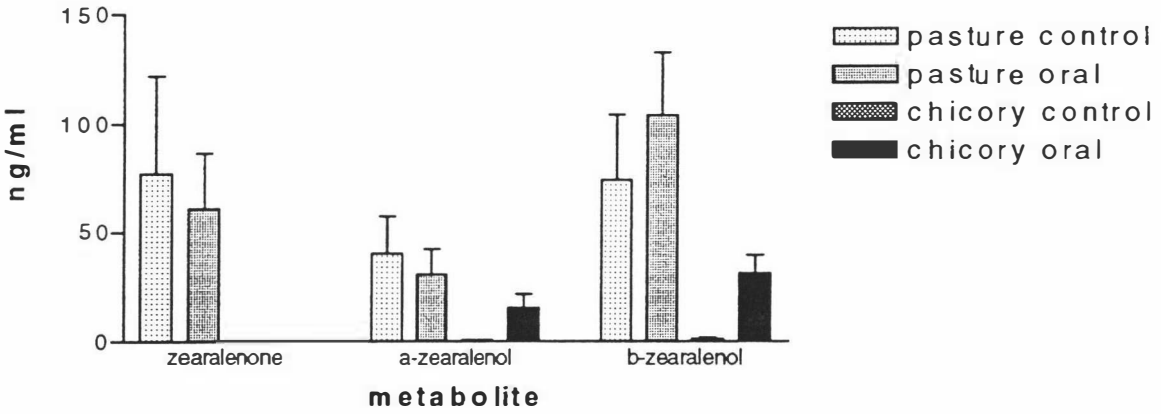


Conjugated zearalenone levels in the pasture control group were significantly higher ($P<0.05$) than in the chicory control group, however there was no significant difference in free zearalenone. The oral dosed groups were significantly higher ($P<0.05$) in both free and total zearalenone than their respective control groups. There was no significant difference in either free or conjugated zearalenone concentration between oral and intravenously dosed groups within or between the chicory and pasture groups (Fig 4.9.).

There was no significant difference in the levels of any of the metabolites in the urine (Fig 4.10.)between the dosed and non-dosed groups grazed on pasture, however, there was significantly ($P<0.05$) more of the metabolites in the groups grazed on pasture than those on chicory. Zearalenone and β-zearalenol were the most abundant metabolites in both dosed and

non-dosed ewes. The zearalenone related metabolites were predominantly present in alkene form.

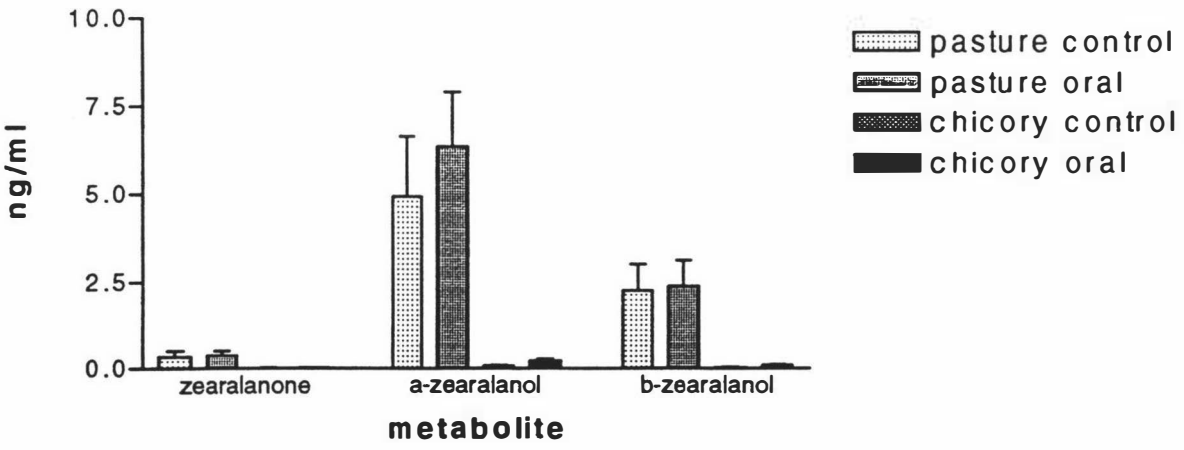
Figure 4.10. Levels of alkene zearalenone metabolites in the urine of zearalenone dosed and non-dosed ewes grazed on either ryegrass pasture or chicory.



Concentrations of α-zearalanol and β-zearalanol (Fig 4.11.)were significantly greater ($P<0.05$) in the urine of the group dosed with zearalenone. No zearalenone was detected in the non-dosed group.

Concentrations of all metabolites in the dosed and non-dosed groups grazed on chicory were significantly lower ($P<0.05$) than the groups grazed on pasture.

Figure 4.11. Levels of alkane zearalenone metabolites in the urine of ewes which are either dosed or non-dosed with zearalenone and grazed on either ryegrass pasture or chicory.



4. Discussion

4.1. Animal measurements

The weights of the ewes fell in both the chicory and pasture treatments but there was no significant difference in the mean liveweight loss between the two grazing treatments.
The results obtained in the zearalenone dosing trial showed no significant difference in ovulation rate between the type of dosing or the amount dosed within the pasture or chicory groups. However, there was a significant difference ($P<0.05$) observed in ovulation rate between the groups grazed on pasture and the groups grazed on chicory. Because of the relatively small number of animals used in each dosing treatment it is likely that the differences in ovulation rate between oral dosed, intravenous dosed and the control ewes within each of the grazing treatments, and in particular the chicory treatment, would not be significant. The difference in mean ovulation rate between the ewes grazed on chicory and the ewes grazed on ryegrass pasture could be an indication of the difference in zearalenone intake between the ewes grazing the ryegrass pasture and those on chicory or could be a reflection of the different nutritional properties of the two feed types. Although chicory has been shown to have nutritional qualities which are superior to ryegrass pasture (Fraser *et al.*, 1988), the change in liveweight between ewes grazed on chicory and ewes grazed on pasture was not significantly different which reduces the possibility that the difference in ovulation rate was due to a difference in nutrition.
Smith *et al.*, (1988) found that conception rate was only reduced in ewes which were ingesting more than 12 mg of zearalenone per day. There were no significant differences in returns to service in the groups grazed on chicory or pasture which may be due to the small number of animals in each treatment and/or insufficient free zearalenone to affect conception rate.
In addition to better defining the relationship between zearalenone ingestion and reproductive performance in ewes, the other important aspect of this trial was to characterise the metabolism of free and conjugated zearalenone and its various metabolites in the sheep and attempt to relate this to the reproductive data obtained. The intravenous groups were dosed at rates of 0.5 mg and 2.0 mg for the low and high dose rates respectively, and the oral dosed groups received 5 mg at each dosing. Although the zearalenone concentrations of the chicory and the ryegrass pasture were determined, it would be difficult to know how much zearalenone

was ingested by the ewes as the daily intakes were not determined. However, assuming that the intakes were similar for the pasture and chicory treatments and given the relative zearalenone concentrations at approximately 6.0 μg/g and 1.3 μg/g of plant tissue for pasture and chicory respectively, it is likely that the zearalenone intake in the ewes grazing the ryegrass pasture was considerably higher than in ewes grazing chicory. Smith *et al.*, (1990) concluded that intakes of 3 mg/day and above were sufficient to reduce ovulation rates which would suggest that the levels of zearalenone dosed in this trial, and in particular the pasture groups, were more than sufficient to evoke a response in ovulation rate

4.2. Zearalenone in the blood

Blood samples taken at 24 hour intervals, directly before the daily doses of zearalenone were administered, showed the relative free and total zearalenone concentration in each of the dosing groups and grazing treatments. It was only possible to compare the groups grazed on pasture with the groups grazed on chicory at 0, 4 and 24 hours after dosing. The levels of free and conjugated zearalenone in the blood are representative of the net effect of dosing free zearalenone and the influences of ingestion, conjugation, excretion and recycling of zearalenone. Conjugation and deconjugation of zearalenone by the liver and recycling via the bile are important factors that may have a large contribution to changes in free and conjugated zearalenone concentrations in the blood in addition to the effect due to the amount ingested or dosed. No blood samples were taken between 4 and 24 hours after dosing so determination of zearalenone concentrations between these times was not possible.

4.3. Zearalenone in the urine

Analysis of free and conjugated zearalenone in the urine provided a better indication of the relationship between zearalenone intake and passage through the animal because once in the urine, zearalenone was not recycled back into the blood. Urine samples taken before dosing began and when the ewes had been on the grazing treatments for two days showed clear differences in zearalenone concentration in the animals grazing chicory from those grazing pasture. The results showed that both free and conjugated zearalenone levels were significantly higher in ewes grazed on pasture than on chicory.

The urine samples taken on the sixth day of dosing also had concentrations which were indicative of the zearalenone intake. There were significantly ($P<0.05$) higher levels of free and conjugated zearalenone in the dosed groups than in the control groups on both chicory and pasture treatments. These results reflect the larger amounts of zearalenone present in the dosed animals. The difference in zearalenone intakes between ewes grazed on pasture and those grazed on chicory was also illustrated by the zearalenone concentration in the urine samples. Levels of free zearalenone were not significantly different in the pasture and chicory control groups, however, the amounts of conjugated zearalenone were higher in the pasture control. The lack of difference in the free zearalenone concentration is largely due to conjugation of most of the zearalenone before passing into the urine. Unlike levels of free zearalenone in the blood, which often represented more than 50% of the total zearalenone present, the proportion of free zearalenone in the urine was generally lower at around 20% of the total zearalenone present. However, the reduction in the proportion of free zearalenone from the blood into the urine observed in this trial indicates that the process of conjugation may not be very efficient. Many other toxic compounds exist solely in the conjugated form once they reach the urine and the proportion of free compound in the blood is much lower than that seen with zearalenone. The inefficient conjugation of zearalenone by the ewes means higher proportions of the oestrogenically active or free zearalenone may be present in the system. However, the levels of free and conjugated zearalenone in the urine and blood are not conclusive as they often don't reflect the level of zearalenone intake by the animal.

A urinary total zearalenone/creatinine (as measured by ELISA) marker for zearalenone intoxication in sheep has been established by Sprosen *et al.* (1995) which indicates that levels in excess of 12.5 mmol zearalenone per mol creatinine may be associated with significant reductions in lambing percentages. The urine results obtained in this dosing trial were significantly lower than this marker level. However, urine samples were taken later in the morning when most of the ewes had already urinated thereby removing the zearalenone excreted during the previous night and possibly explaining the lower levels measured in the urine.

4.4. Metabolism of zearalenone

Previous studies have regarded zearalenone alone when considering reproductive dysfunction which takes no account of the likely oestrogenic effects of other zearalenone metabolites. Kallela and Vasenius, (1982) made the assumption that ruminants would be less affected by the oestrogenic effects of zearalenone because it was degraded in the rumen and, therefore, rendered inactive. The fact that it may be degraded to metabolites, of which some are more potent in oestrogenic activity than zearalenone (Fitzpatrick, 1989; Miksicek, 1994) was unknown at that time. The reduction of zearalenone by rumen protozoa and bacteria to α-zearalenol and to a lesser degree to β-zearalenol was demonstrated by Kiessling *et al.*, (1984). In addition recent findings by Erasmuson *et al.*, (1994) and Kennedy *et al.*, (1995) have found zeranol, taleranol α-zearalenol and β-zearalenol in the bile and urine of grazing animals and that the source of these compounds could be intrinsic i.e. resulting from transformation of zearalenone in the animal, or extrinsic i.e. being produced by Fusarium species prior to ingestion by the animal. Fitzpatrick *et al.*, (1986) found that the relative order of oestrogenic potency for zearalenone and its major metabolites in order of strongest to weakest was α-zearalenol, zearalenone and β-zearalenol. Zearalenone metabolised into α-zearalenol in the pasture or in the animal, will result in a higher risk of reproductive dysfunction than if zearalenone intake alone was considered. The presence of zearalenone metabolites in the urine of zearalenone dosed sheep has been examined subsequent to this investigation, by Miles *et al.*, (1996). The relative proportions of the alkane and alkene metabolites were similar to those found in the urine of orally dosed ewes in this trial with the major metabolites present being zearalenone, α- and β-zearalenol and relatively small levels of the alkane metabolites i.e. zearalanone, α- and β-zearalanol. The study by Miles *et al.*, (1996) also determined free and conjugated zearalenone concentrations relative to time after dosing. Urine samples in this investigation were not taken in a sequence after dosing, however, the decline of free zearalenone in the blood samples after intravenous dosing and the increase after oral dosing are similar to urine samples taken after dosing by Miles *et al.*, (1996) The similarities found in relative zearalenone metabolite levels indicate that there are specific metabolic pathways involving zearalenone within the sheep which must be examined further. Therefore further investigations are required into the presence of these zearalenone metabolites in sheep and their role in reproductive dysfunction.

4.5. Zearalenone related metabolites in the urine

In all the metabolite concentrations determined, the levels in the pasture groups were significantly ($P<0.05$) higher than those in the chicory groups.

This finding provides some interest in light of results of Kiessling *et al.*, (1984) where α-zearalenol was the predominant metabolite remaining after the reduction of zearalenone in the rumen suggesting that α-zearalenol would be the predominant metabolite absorbed into the blood stream of the sheep after zearalenone was ingested. This, however, does not account for any further transformations which may take place once in the blood stream. However, the relative proportions of the alkane metabolites determined in the urine differ from the alkenes in that the proportion of α-zearalanol is twice that of β-zearalanol and up to ten times that of zearalanone. This result follows more closely the findings of Kiessling *et al.*, (1984) which may be due to the fact that the alkane metabolites obtained from the forage were already in the α- and β-zearalanol forms and that all ingested alkane forms would be further metabolised in the rumen.

4.6. Conclusions

Blood results were not correlated to the level of zearalenone intake, however the urine results provided a good indication of differences in zearalenone levels and metabolism in sheep in relation to zearalenone intake.

The results obtained in this trial showed no difference in ovulation rate between dosed and non-dosed animals despite differences in zearalenone intake. The lack of difference may have been due to the small group sizes. There was, however, a difference in ovulation rate between groups grazed on chicory and those on pasture which could be due to the higher zearalenone intake of ewes grazed on pasture given that there appeared to be no nutritional differences between the groups. Further work is required dosing zearalenone to a larger number of animals to identify effects on reproductive performance.

CHAPTER V

Zearalenone in ewes grazed on pasture or chicory and subsequent effects on reproductive performance.

1. Introduction

Lambing performance on many New Zealand farms is often below the expected level despite adequate ewe condition, management and absence of disease.

Zearalenone-producing Fusarium species are common in New Zealand pasture herbage (di Menna *et al.*, 1991). The oestrogenic effects of zearalenone have been reported in swine (Long *et al.*, 1982; Diekman & Long, 1989) and cattle (Mirocha *et al.*, 1968; Danko & Aldsay, 1962; Miller *et al.*, 1973). Recent findings have indicated that zearalenone present in pastures may be sufficient to reduce reproductive performance in ewes (Smith *et al.*, 1986; Smith *et al.*, 1987; Smith *et al.*, 1988; Smith *et al.*, 1990; Jagusch *et al.*, 1986). These studies found the exposure of ewes to zearalenone around the time of mating reduced ovulation rate and the subsequent lambing percentage. Numbers of fusaria are greatest in late summer and autumn when suitable environmental conditions and substrate exist for proliferation of the fungi. This seasonal increase in fusaria activity coincides with mating on many New Zealand sheep farms and it seems possible that zearalenone produced by the Fusarium fungi at this critical time of the year may be sufficient to reduce reproductive performance in grazing sheep. Smith *et al.*, (1990) concluded that intakes of 3 mg/ewe/day or more during the period around mating would be reflected as depressed ovulation rates and lower lambing percentages. Levels of zearalenone between 0.4 and 4.0 mg/kg dry weight have been reported in some New Zealand pastures (di Menna *et al.*, 1987) which could result in sufficient zearalenone intakes by the ewes to reduce reproductive performance. However, these zearalenone levels were determined from pasture samples which took no account of the various site types present in pasture during the late summer and autumn. High N sites such as urine patch areas, which can represent as much as 40 % of the pasture, are often associated with the highest numbers of fusaria (Keogh, 1986) and, therefore, could be expected to contain higher levels of zearalenone. Urine patches are grazed more frequently and intensively than other sites in the pasture and can, therefore, contribute disproportionately to the acquisition of fungal toxins by livestock (Keogh, 1986).

The grazing behaviour of the ewe increases the likelihood that sufficient zearalenone will be ingested around mating to reduce reproductive performance.

The aims of this trial were:

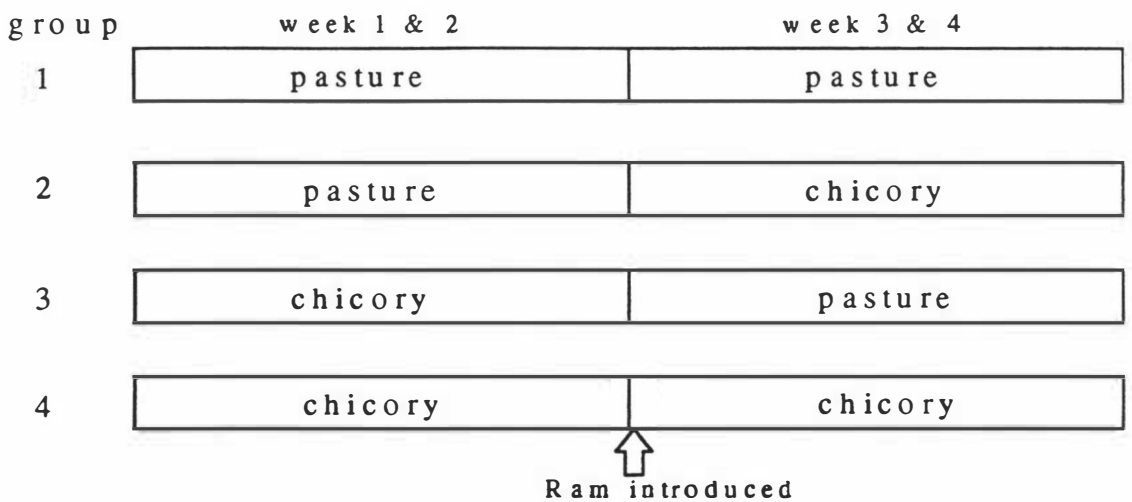
1. To measure free and conjugated zearalenone in the blood and urine of ewes grazing grass-dominant pastures or chicory.
2. Determine any subsequent effects on reproductive performance.

2. Methods and materials

2.1. Animals and treatments

Finish Landrace x Romney 2-tooth ewes were ear tagged and allocated to four groups (n=110). Thirty ewes in each group were treated with synchronisation devices (CIDR, type G, containing 0.3g progesterone) inserted into the vagina for 14 days prior to mating and the remaining 80 ewes were not synchronised. Two groups were grazed on chicory (*Chicorium intybus* L. *cv* Grasslands Puna), which is a forage herb with very low zearalenone concentrations (< 0.1 µg/g), and the remaining two groups were grazed on grass-dominant pasture. The groups remained on these treatments for two weeks at which time one group on each forage type was interchanged (Fig. 5.1.). Entire rams, with harnesses and crayons, were introduced to all groups after the first two weeks and checked daily for mating marks.

Figure 5.1. Grazing treatments for each group of 30 synchronised + 80 non-synchronised ewes.



Seven days after the beginning of mating all synchronised ewes were examined by laparoscopy to determine ovulation rate. After all ewes were mated for the first time the crayon colour was changed and the ewes were checked twice weekly for returns to service. The number of lambs carried per ewe was determined by ultrasound scanning 90 days after the end of mating.

2.2. Sampling

All the ewes were weighed pre-treatment, at the beginning of mating and after the first cycle with the ram. Blood samples were taken from synchronised ewes by jugular venipuncture with evacuated collection tubes (10 ml draw vaccutainer™) and hypodermic needle on the days the ewes were weighed. Urine samples were taken from synchronised ewes at the beginning of mating.

Samples of chicory and the various pasture species were taken weekly during the trial period for zearalenone analysis.

2.3. Zearalenone determination in herbage, blood, and urine

Zearalenone concentrations determined by an indirect competitive ELISA immunoassay using partially purified zearalenone binding antibodies (di Menna *et al.*, 1991) (see appendix II for full details of the assay).

2.4. Statistical analyses

All statistical analyses were done using Graph-pad Prism 2.0 software: 10855 Sorrento Valley Rd #203, San Diego, CA 92121 USA.

Ovulation rate and pregnancy scanning data for each treatment group were compared using Friedman test and Dunn's multiple comparison test. Differences in weight change were compared by analysis of variance (ANOVA) and Tukey's multiple comparison test. Free and conjugated zearalenone concentration in the blood and urine were compared by t-test. Results are displayed as mean values $\pm$ standard error of the mean (SEM).

Plate 5.1. Laparoscopic examination of ewes

Plate 5.2. Ewes grazing grass-dominant pasture

Plate 5.3. Ewes grazing chicory

3. Results

3.1. Weight change and reproductive performance

Tables 5.1. and 5.2. shows reproductive data and weight change for synchronised and non-synchronised ewes.

Table 5.1. Weight change (over 44 day trial period), ovulation rate, returns to service and number of lambs carried in synchronised ewes in each treatment group.

	Treatment groups			
	1	2	3	4
Weight Change (kg)	1.25 ± 0.34^{a}	1.15 ± 0.37^{a}	-0.35 ± 0.49^{b}	-0.87 ± 0.34^{b}
Ovulation rate (corpora lutea/ewe)	2.40 ± 0.14	2.31 ± 0.15	2.23 ± 0.1	2.29 ± 0.14
Returns to service (%)	17	30	30	40
Pregnancy scanning (N° lambs carried)	1.43 ± 0.15	1.63 ± 0.12	1.63 ± 0.10	1.87 ± 0.10

Means (+ SEM)in each row with different superscript letters differ significantly ($P<0.01$)

Table 5.2. Weight change (over 44 day trial period), returns to service, and number of lambs carried in non-synchronised ewes in each treatment group.

	Treatment groups			
	1	2	3	4
Weight Change (kg)	7.06 ± 0.21^{a}	5.80 ± 0.27^{b}	5.48 ± 0.23^{b}	5.69 ± 0.31^{b}
Returns to service (%)	7	6	10	13
Pregnancy scanning (N° lambs carried)	1.71 ± 0.08	2.22 ± 0.28	1.76 ± 0.08	1.91 ± 0.07

Means (+ SEM)in each row with different superscript letters differ significantly ($P<0.01$)

3.2. Zearalenone levels in the herbage

Forage samples taken from the pasture including the various site types and species. Zearalenone concentrations per dry weight of herbage, were 3.01 ± 2.50 μg/g in grass pasture and 0.25 ± 0.08 μg/g. in the chicory.

3.3. Zearalenone in the blood

The levels of conjugated and free zearalenone in the blood of the ewes pre-treatment were 0.163 ± 0.035 ng/ml and 0.219 ± 0.034 ng/ml respectively.

There was no significant difference in the levels of conjugated zearalenone after two weeks between the ewes grazed on pasture (0.170 ± 0.023 ng/ml) and those grazed on chicory (0.238 ± 0.030 ng/ml). The level of free zearalenone was significantly greater in the blood of ewes grazing pasture (0.295 ± 0.027 ng/ml) than in the ewes grazing chicory (0.122 ± 0.014 ng/ml). Free zearalenone represented 67% of the total zearalenone present in the ewes grazed on pasture and 40% of the total zearalenone present in the ewes grazed on chicory.

There was no significant difference in the levels of conjugated zearalenone in the blood after six weeks of grazing between any of the treatments, however the levels of conjugated zearalenone in the blood had increased significantly ($P<0.05$) in all the grazing treatments since the pre-treatment sampling and two week sampling, and were often up to ten times the concentration (see Figure 5.2.).

There was no significant difference in the levels of free zearalenone after six weeks in the blood of ewes on the pasture or chicory treatments, although levels of free zearalenone were significantly lower ($P<0.05$) than at the pre treatment and week 2 samplings. Free zearalenone represented between 2% and 3% of the total zearalenone present (see Figure 5.3.).

Figure 5.2. Conjugated zearalenone in the blood of ewes grazed either on chicory or pasture.

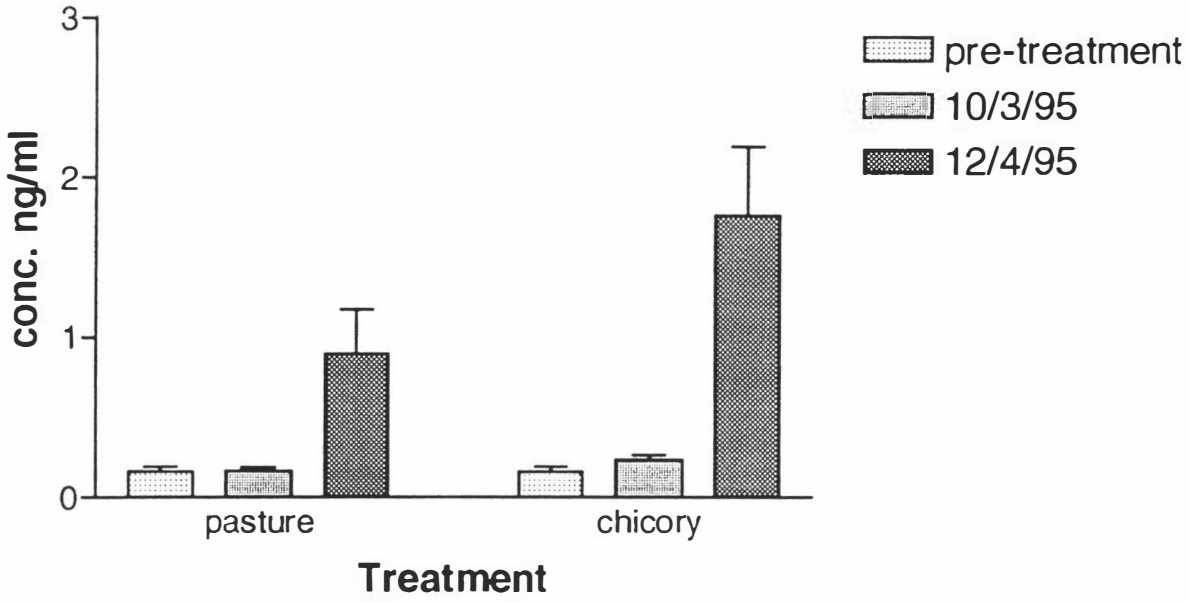
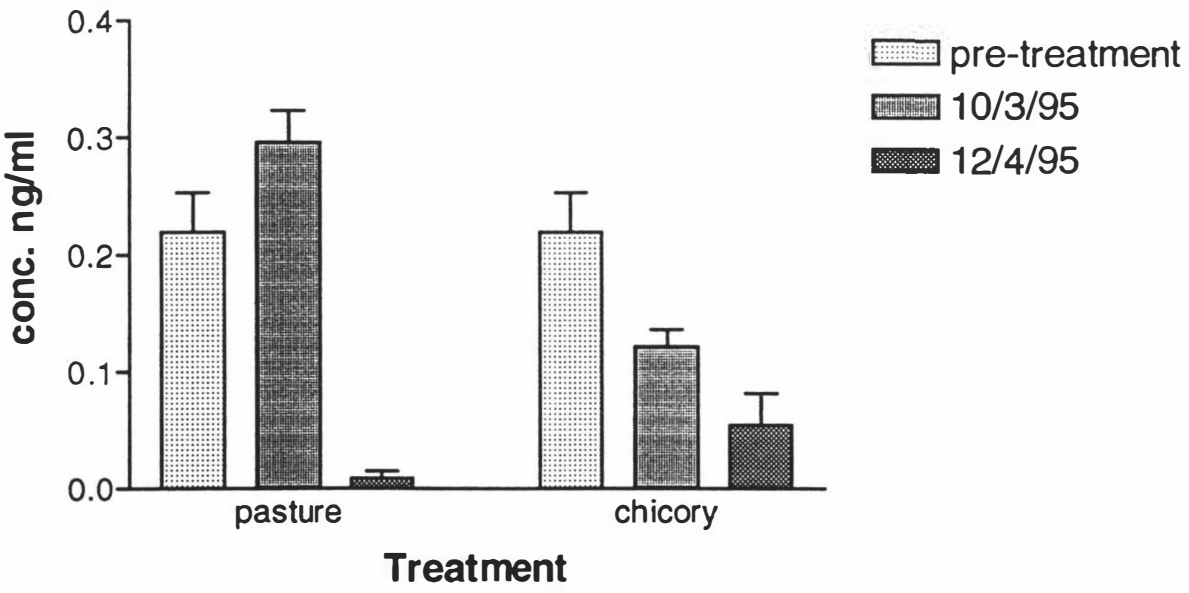


Figure 5.3. Free zearalenone in the blood of ewes grazing either on chicory or pasture.

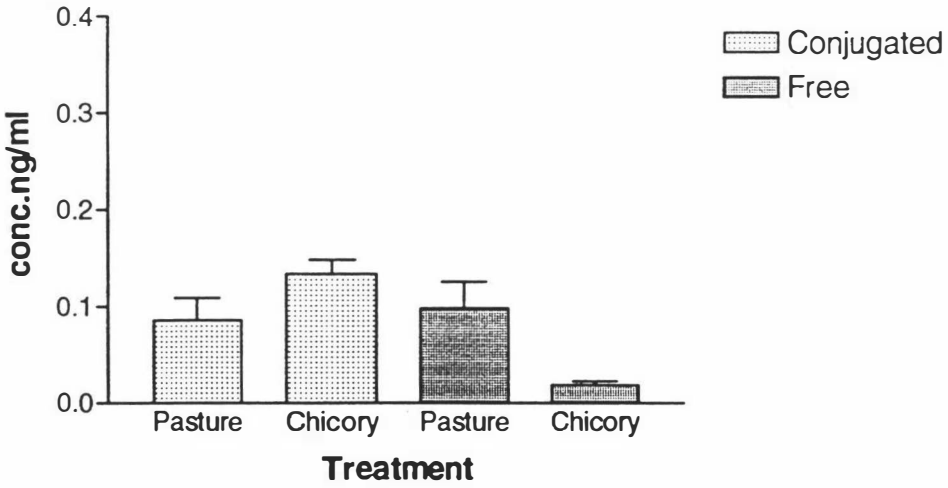


3.4. Zearalenone in the urine

There was no significant difference in the levels of conjugated zearalenone in the urine between ewes grazing chicory (0.099 ± 0.027 ng/ml) or pasture (0.086 ± 0.024 ng/ml). There was a significantly higher concentration of free zearalenone in the urine of ewes grazed on pasture (0.134 ± 0.015 ng/ml) than in ewes grazed on chicory (0.019 ± 0.004 ng/ml). (See Figure 5.4.)

The proportion of the total zearalenone in the urine present in free form was 61% for the pasture group and 16.7% for the chicory group.

Figure 5.4. Free and conjugated zearalenone in the urine of ewes either grazing pasture or chicory for two weeks prior to mating.



4. Discussion

4.1. Animal performance

Results obtained in this trial showed no significant difference in ovulation rate, conception rate or the number of lambs carried per ewe between any of the grazing treatments. However, several factors may have contributed to a lack of difference being observed. The live-weight data showed that ewes grazing the chicory prior to mating lost weight on average whereas those grazing the pasture gained weight. It is possible that the loss of weight in the groups grazing the chicory treatment prior to mating would have reduced ovulation rates. Therefore, any positive effects on ovulation rate which may have resulted from grazing the lower zearalenone feed would have been diminished by the poorer feed availability in the chicory groups. The liveweight data showed that, although the synchronised and non-synchronised ewes in each treatment group were run together, the synchronised ewes were on average 4-5 kg lighter than the non-synchronised ewes despite having similar average liveweights at the start of the trial. The reasons for the lighter live-weights of the synchronised ewes are not clear although, possibilities include the synchronisation treatment and/or the laparoscopy procedure and generally the animals were handled more often than the non-synchronised ewes.

4.2. Zearalenone in the herbage

Levels of zearalenone in the herbage were highly variable depending on the species sampled and from where the sample was taken. Pasture samples taken include species such as ryegrass, brown top, and Yorkshire fog. Levels were generally lower than recorded in previous years (Keogh, pers. comm.). Despite the lower levels of zearalenone recorded this year, the levels of zearalenone in the chicory were still well below those in the grass species.

The relatively low levels of zearalenone recorded in the pasture during this trial could be another reason why no difference was observed in reproductive performance between ewes grazing pasture and those grazing chicory. Zearalenone intakes above 3 mg/ewe/day are

sufficient to depress ovulation rate and lower lambing percentages (Smith *et al.*, 1990). The average levels of zearalenone in the pasture are often below 3 mg/kg of dry plant tissue which would mean that ewes harvesting an average of 1 kg dry matter per ewe per day would be ingesting levels of zearalenone below 3 mg/day. The low pasture zearalenone levels were reflected in the urine and blood samples taken pre-treatment and on the 10th March which were more critical times when considering likely effects on reproductive performance.

4.3. Zearalenone in the blood

Analysis of the blood for free and conjugated zearalenone showed that there were clear differences between ewes grazed on chicory and those grazed on pasture. After introduction to the grazing treatments the levels of conjugated zearalenone remained relatively similar and after two weeks of grazing there was no significant difference in the levels of conjugated zearalenone between the groups grazed on pasture or on chicory. A possible reason for the lack of change in the conjugated zearalenone levels is that much of the conjugated zearalenone may be recycled by the liver via the bile and therefore remains in the body much longer (Smith, J.F., pers. comm.). The possible recycling of conjugated zearalenone has made the interpretation of blood results difficult particularly when results have been in terms of total zearalenone and have not discerned between the conjugated portion and the oestrogenically active free zearalenone. The blood analysis showed clear differences in the levels of free zearalenone by the second week of grazing with levels in the pasture group increasing significantly ($P<0.05$) whereas the free zearalenone levels in the ewes grazing chicory had decreased. The differences in free zearalenone levels in the blood and the lack of significant change in the levels of conjugated zearalenone in both the chicory and pasture groups resulted in a higher proportion of the total zearalenone present in the group grazed on pasture existing in the free 'active' form, compared to the group grazed on chicory.

The final set of blood samples taken four weeks after the start of mating showed up to ten-fold increases in the amount of conjugated zearalenone present since the two week and pre-treatment sampling and a reduction in the amount of free zearalenone present in all treatment groups. This change in the conjugated and free zearalenone levels cannot be easily explained as the levels of zearalenone determined in the blood pre-treatment and at the start of mating were

normally associated with background levels of zearalenone which are much lower than levels associated with increasing zearalenone levels in the pasture.

4.4 Zearalenone in the urine

The urine samples taken at two weeks showed a similar pattern to the blood samples. As with the blood levels, there was no significant difference in the levels of conjugated zearalenone between the groups grazed on pasture and those on chicory. The difference between the chicory and pasture treatments is clearly expressed in the levels of free zearalenone present in the urine and the proportion of the total zearalenone present in free form. The difference in the levels of free zearalenone in the urine between the chicory and pasture groups is greater than in the blood which indicates that a larger proportion of the free zearalenone present in the blood of the chicory group is in the conjugated form by the time it has entered the urine. The high proportion of free zearalenone in the urine of the pasture group could result from an inability of the ewe to conjugate the greater amounts of zearalenone in the blood or could indicate a large amount of conjugated zearalenone being recycled and remaining in the body.

4.5. Conclusions

The lack of a significant difference in reproductive performance between ewes grazing chicory and those grazing pasture may be due to lower feed availability in the chicory treatment and the comparatively low levels of zearalenone in the pasture during the trial period.

Zearalenone levels in the blood were difficult to interpret because of recycling which occurs in the body, however levels of free zearalenone were significantly lower in the ewes grazing chicory at the start of mating. Urine analysis gave a better indication of zearalenone intake compared to blood as the urine is the major route of excretion of zearalenone from the body.

Chicory was effective in reducing zearalenone intake and it is likely that this would be the case in years where conditions for zearalenone production are better. More work, especially in years when higher zearalenone levels prevail, will be necessary to establish if an important difference exists.

CHAPTER VI

General discussion and conclusions

The main objective of this study was to characterise zearalenone distribution in pasture and its possible role in reducing reproductive performance in sheep grazed on pasture. The distribution of zearalenone in different pasture sites was examined and a preliminary investigation into zearalenone uptake by the ryegrass plant was conducted. The distribution of zearalenone in pasture is a major piece of information in understanding the relationship between zearalenone and the grazing animal. The dosing trial was conducted to add evidence to previous studies which found that zearalenone causes reductions in reproductive performance in sheep. The grazing trial was aimed at linking zearalenone produced in pasture to reductions in reproductive performance in the grazing animal.

The presence of zearalenone-producing Fusarium species in New Zealand pastures has been demonstrated (di Menna *et al.*, 1987) and under certain favourable conditions zearalenone is produced in appreciable amounts. The distribution of zearalenone within the pasture is of great importance in determining the amount ingested by the grazing animal. Certain areas in the pasture, such as high N areas caused by animal excreta, have significantly higher populations of fungal saprophytes, which include fusaria (Keogh, 1986). It follows that zearalenone levels will generally be greatest in these areas. This characteristic of zearalenone distribution is of greatest consequence to the grazing animal as pastures are not uniformly grazed and defoliated by livestock during the summer and autumn. Herbage in urine patch sites, which can cover up to 40% of the pasture, is grazed in preference to other areas of the pasture (Keogh, 1984; Keogh, 1986). Acquisition of fungal toxins is, therefore, dependant on suitable conditions for fungal growth and toxin production, and grazing behaviour of the livestock.

The results (Chapter 3) showed that zearalenone concentrations varied greatly within sites and between site types indicating possible differences in conditions suitable for zearalenone production. However, the samples were taken in late autumn when zearalenone levels would have been falling and in addition the zearalenone levels in pasture during 1995 were lower on average than in previous years. Regular sampling during the summer and autumn from the different pastures and site types is required to better characterise zearalenone distribution in the pasture. The analysis of the components from the pasture samples and in the uptake trial

showed high concentrations in the youngest parts of the plant and in particular the daughter tillers. It appeared that zearalenone in solution was taken up by the plant and translocated to the youngest parts of the plant (Fig 3.5.) with nutrients required for growth.
Further investigations into the uptake of zearalenone using improvements to the method developed in this investigation, will enable characterisation of zearalenone uptake, not only in the ryegrass plant, but in other common pasture species. This will give further insight into the relationship between zearalenone in the pasture plant and the grazing animal.

In the animal the importance of levels of free and conjugated zearalenone were difficult to interpret (Chapter 4, section 4.2.) because in many cases there appeared to be no correlation between the amount of zearalenone ingested or dosed and the levels in the blood. Dosed zearalenone and its metabolites have been detected in the bile (Kennedy *et al.*, 1995; Hewitt *et al.*, 1996) which offers a route for excretion and subsequent reabsorption of zearalenone into the blood. In addition to this recycling, it is likely that zearalenone is constantly being conjugated and deconjugated in the liver and metabolised to other related compounds (Erasmuson *et al.*, 1994). All these factors affect levels of free and conjugated zearalenone in the blood and are responsible for much of the variability. Urinary levels of free and conjugated zearalenone could be correlated with zearalenone intake (Chapter 4 section 4.3.) and were the best indication of zearalenone levels in the animal. Urine is the major excretory pathway for zearalenone and once in the urine zearalenone cannot re-enter the blood stream which is the reason why urinary zearalenone levels are a good indication of zearalenone present in the animal. Levels of free zearalenone were of most interest as it is this portion of the zearalenone present in the animal which has the oestrogenic effects.
Although zearalenone was the major compound considered in this investigation, other related compounds with significant effects on the grazing animal, were also examined. The metabolism of zearalenone to several related compounds by fusaria in the pasture (di Menna *et al.*, 1987), by micro organisms in the rumen (El-Sharkawy and Abdul-Hajj, 1988) and once in circulation in the animal (Miles *et al.*, 1996) has been shown and therefore the effects of these compounds cannot be ignored. Aside from oestrogenic properties common to many of the zearalenone related metabolites, some metabolites, namely zeranol, have been shown to have anabolic properties and have been used widely as a means of improving animal growth rates. Now banned as an anabolic drug for farm animals in European countries, the issue of zeranol

In conclusion, the results obtained in the three trials were unable to show a direct link between zearalenone in pasture and reproductive performance in ewes, however, there were clear indications that these links may exist and given appropriate conditions for zearalenone production in pasture, it is likely that the zearalenone concentration in the plant and therefore the amount of zearalenone ingested by the animal will be sufficient to affect reproductive performance. It was also found that several other zearalenone-related metabolites were present in sheep grazed on pasture which were produced both within the animal by metabolism of zearalenone and within the pasture to be subsequently ingested by the animal. Further studies into the effects of zearalenone on ewe reproduction will also have to consider more closely the other compounds present which may also have oestrogenic effects.
Finally, this study was successful in evaluating the forage herb chicory as a feed which significantly reduces the risk of free zearalenone in ewes when fed during mating.

There is a need for further research to identify and clarify links between pasture zearalenone and the reproductive performance of New Zealand ewe flocks.

Appendix 1

GC-MS analysis of zearalenone carried out by Dr Anton Erasmuson at National Chemical Residues Analytical Laboratory, MAF, Wallaceville Animal Research Centre, Upper Hutt, New Zealand.

Sample treatment . Blood and urine samples were stored frozen. Aliquots (5ml) were analysed by glucuronidase re-formation of the alcohols, extraction with hexane/tBME (70:30), and then HPLC cleanup of extracts with programmed fraction collection. A fraction was evapoprated under nitrogen, then trimethylsilyl derivatized with MSTFA, and subjected to GC-MS analysis.

Chromatography. The normal phase HPLC separation used cyanopropyl Whatman PAC column (4.6 mm x 100 mm) in a Varian Vista 5500 HPLC, isocratically pumping hexane/tBME/methanol/acetic acid (695:250:50:5) at 0.9 mL/min. An azo dye (phenylazohomovanillyl alcohol) was used to monitor for drift in retention times. Samples were automatically injected by a Shimadzu SCL-6B and fractions collected by a Pharmacia FRAC-300.

Residue quantitation used a Hewlett-Packard 5890 gas chromatogragh interfaced to a low- resolution mass selective detector (MSD 9570).

Appendix 3

Nutrient stock solution used for zearalenone uptake trial (Chapter 3.).

Stock solutions used to make nutrient solution.

Stock A $CaNO_3$ (159.3 g/l), NH_4NO_3 (80.1g/l), $FeCl_3$ (10.82g/l) and DTPA (15.74g/l);

Stock B KH_2PO_4 (3.12g/l) and K_2HPO_4 (1.33g/l);

Stock C KNO_3 (7.99g/l), $MgSO_4.7H_2O$ (3.36g/l) and $Na_2SO_4.10H_2O$ (10.07g/l);

Stock D $CuSO_4.5H_2O$ (0.010g/l), hydrated HnCl (0.409g/l), H_3BO_3 (0.350g/l), $Na_2MoO_4.2H_2O$ (0.002g/l) and $ZnSO_4.7H_2O$ (0.055g/l)

The relative concentrations of the stock solutions in the nutrient solution were 1 ml/l, 4 ml/l, 8 ml/l and 1 ml/l for stock solutions A, B , C, and D respectively.

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