Zearalenone challenge in sheep: urine sampling to measure response

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ABSTRACT

Zearalenone is a fungal toxin produced by *Fusarium* species commonly found on autumn pastures in New Zealand. It may interfere with reproductive functions in sheep. In a search for a genetic measure of susceptibility, ewe and ram lambs were dosed orally with a suspension of zearalenone at a series of rates, at 8 and 11 months of age. Urine samples were taken at intervals from 2 to 50 hours later. The concentration of urinary zearalenone and its metabolites (Zen) was assayed using an ELISA. Adjustment for urinary volume was made by measuring the urinary concentration of creatinine (Cr), and results were expressed as Zen/Cr ratio ($\log_e$-transformed). In a separate experiment, breeding ewes were dosed with zearalenone at a single rate, and Zen/Cr response was measured. The following effects were evaluated in lambs: a) sex difference (ewe versus ram lambs), b) responses to three dose rates, c) time-series response data. The heritability of Zen/Cr was determined in lambs and breeding ewes; results were analysed after standardising for each treatment and sampling group. Urinary Zen/Cr, 6 h after dosing, differed between ewe and ram lambs by a factor of 1.75 (ewe lambs higher; $P < 0.01$). Zen/Cr values for three dose rates (3, 6 and 9 mg zearalenone per lamb), averaged over three sampling time points after dosing, were 23.5, 43.2 and 59.3, respectively ($P < 0.0001$). Zen/Cr values for three sampling time points (2, 4 and 6 h after dosing), averaged over three dose rates, were 58.0, 35.8 and 29.0, respectively ($P < 0.0001$). By 50 h after dosing, the Zen/Cr average had fallen to 4% of its 2-h value. The interaction between dose rate and time of urinary sampling was not significant for log Zen/Cr. A preliminary estimate of the heritability for standardised Zen/Cr was 0.32 ± 0.10 ($P < 0.01$). The dosing regime reported here could be used for a test of animal response to zearalenone in ram breeders' flocks.

Keywords: sheep; zearalenone; dose response; urine; heritability**.**

INTRODUCTION

Zearalenone is a fungal toxin produced by *Fusarium* species commonly found on autumn pastures in New Zealand. The toxin may interfere with reproductive functions in sheep because of its structural similarity to oestrogens. Examples of the potential reproductive cost of zearalenone in the diet of sheep have been reported (Jagusch *et al*., 1986, Smith *et al.,* 1988, 1990; Towers & Sprosen, 1993). We have recorded animal responses to zearalenone dosing, in a series of studies with lambs and ewes at Ruakura. The purpose of the present study was to determine a dose-response relationship to zearalenone challenge in sheep, along with the time-course and heritability of the urinary response.

MATERIALS AND METHODS

Animals

All animal manipulations in this study were approved by the Ruakura Animal Ethics Committee. The main source of animals consisted of Coopworth ewes and lambs from a self-replacing, pedigree-recorded Zearalenone flock at Ruakura (Morris *et al*., 2005). It was established in the autumn of 2000, and was derived from a flock used in studies at Ruakura in 1997 by J. F. Smith into the effects of zearalenone and Androvax™ on reproductive function. Breeding ewe numbers ranged from 31 in 2000 to 56 in 2003. Replacement female stock and some service sires were also bred within this flock. A second source for this study was Romney ewe lambs from three facial eczema (FE) breeding lines, which were either Resistant, unselected (Control) or Susceptible to FE (Morris *et al*., 1995).

Dosing

The primary dose-response information was collected from lambs born in 2003, following an autumn season in 2004 when no zearalenone was found on the Ruakura pastures. An oral dosing procedure with zearalenone was therefore developed. Zearalenone was extracted from a dried and ground *Fusarium* isolate grown on rice, by soxhlet extraction. The resulting material was purified by liquid-liquid partitioning, flash column chromatography and crystallization to yield zearalenone as a pale coloured solid (92% pure). Zearalenone was dosed orally as a fine suspension in a single dose (25% aqueous ethanol, 12 mL dose), at various rates (see below), using an anthelmintic drench gun. A feasibility study was carried out in May 2004, followed by a dose-rate trial in August 2004.

- *Pilot Lamb Dosing Trial (May 2004)*: Forty 8-month-old ewe and ram lambs from the Zearalenone flock were dosed at average live weights of 38.6 kg (ewes, n = 19) and 38.8 kg (rams, n = 21). Urine samples were collected from all animals 6 h after dosing, by the respiratory occlusion method (Divers, 1992). The two sexes were dosed separately but on the same day and with the same material. The sex difference in lambs (ewe vs ram response to 6 mg zearalenone) is reported here.

- *Lamb Dosing Trial (August 2004)*: Ninety six 11-month-old ewe lambs from the Zearalenone flock (n = 17) and the FE lines (n = 79) were dosed orally with zearalenone. Average live weights were 41.5 kg in the Zearalenone flock and 36.9 kg in the FE lines. Three dose rates were tested, 3, 6 and 9 mg zearalenone per animal. Six lambs were urine-sampled as base-line controls. Urine-sampling times after dosing were as follows: at 2 h, using 50% of animals, pre-selected at random; at 4 h, using the remaining animals; and at 6 h (all animals). Urine samples from 18 animals in the 9 mg dose group were also collected at 26 h and again at 50 h after dosing (the highest dose-rate group alone being used in order to minimise analysis costs).
- *Facial eczema monitoring*: Because the May experiment was conducted near the end of the season when animal protection against FE is normally necessary, serum samples were taken in May from all lambs involved in both the May and August zearalenone dosing trials, in order to test for any carryover effect of natural challenge with FE. These samples were analysed for gamma-glutamyltransferase (GGT), for which an elevated activity level indicates liver injury (Towers & Stratton, 1978).
- *Dosing Trials with Breeding Ewes (March 2002 and March 2003)*: In order to provide more data for a heritability estimate on urinary zearalenone concentrations, dose-response data were included from breeding ewes recorded in 2002 and 2003. The primary objective in the ewes was to record ovulation rates after zearalenone dosing (Morris *et al.,* 2005). Each autumn, oestrus-synchronised ewes were dosed daily for 6 days with 10 mg zearalenone per animal (average live weight 56.2 kg in 2002; 55.0 kg in 2003); urine samples from individual ewes were obtained on the third day of dosing, and again two days after dosing had terminated. Statistical models for heritability estimates are described below.

Sample analyses

An ELISA assay for urinary zearalenone and its metabolites was carried out at Ruakura (Garthwaite *et al*., 1994). The intra-plate and inter-assay coefficients of variation were 5.5 and 7.8%, respectively. The concentration of urinary creatinine was determined in a commercial laboratory, so that an approximate adjustment for urinary volume could be made by use of the ratio, Zen/Cr.

Data analyses

Urinary Zen/Cr data from the May and August trials on lambs were processed using analyses of variance (SAS, 1995), after transformation to natural logarithms. The fixed effect in the May trial was sex of lamb. The two sexes had not grazed together, but their pre-dose Zen/Cr values were both very low, averaging < 1 unit. In the August trial, fixed effects were: dose rate, sampling interval after dosing, differences between the responses of Zearalenone and FE lines, and the interaction between dose rate and sampling interval.

In order to obtain heritability estimates, restricted maximum likelihood (REML) procedures were applied to urinary Zen/Cr using an animal model (Gilmour, 1997), standardising for each contemporary group. Contemporary groups were defined as animals in the same dosing round, dosed at the same rate, and sampled at the same interval after dosing. There were four dosing rounds (May-trial lambs, August-trial lambs, 2002-trial ewes and 2003-trial ewes), comprising records of 243 lambs and ewes in total. It was assumed that the urinary Zen/Cr data from lambs and ewes could be combined into a single 'trait'. More extensive data collection will be required to test the validity of this assumption.

RESULTS

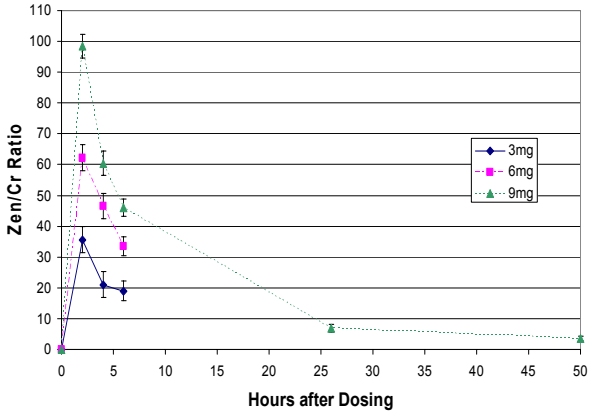
Mean Zen/Cr ratios (back-transformed) from ewe lambs and ram lambs in the May trial were 25.9 and 14.8, respectively, a factor of 1.75 ($P < 0.01$).

TABLE 1: Effects of zearalenone dose rate and sampling interval after dosing on the concentration of urinary zearalenone and metabolites (Zen) in lambs (August 2004 trial). Results are shown after adjustment for urinary volume by expressing Zen as a ratio with creatinine concentration (Cr), transformed to a natural logarithmic scale.

Dose rate (mg per head)	Log_e Zen/Cr	Interval after dosing (h)	Log_e Zen/Cr
3	3.16	2	4.06
6	3.76	4	3.58
9	4.08	6	3.37
Significance	P < 0.0001		P < 0.0001
Average SED	0.05		0.05

Effects of zearalenone dose rate and sampling interval on ewe lambs in the August trial are shown in Table 1. Pooled over the 2-, 4- and 6-h time intervals after dosing, the dose rate effects were significant ($P < 0.0001$), and back-transformed means for Zen/Cr were 23.5, 43.2 and 59.3 at 3, 6 and 9 mg, respectively. Pooled over the three dose rates, the effects of three time intervals after dosing were significant ($P < 0.0001$), and back-transformed means were 58.0, 35.8 and 29.0 at 2, 4 and 6 hours after dosing, respectively. Overall results are plotted in Figure 1. For the 9 mg treatment group, 26 and 50 h after dosing, treatment means were 7.3 and 4.2, respectively, and the 50 h sample mean had fallen to only 4% of the 2 h sample mean.

FIGURE 1: Mean urinary Zen/Cr ratios (± SE bars) in ewe lambs dosed orally with 3, 6 or 9 mg zearalenone (August 2004 trial), with samples taken at 0, 2, 4, 6, 26 and 50 h after dosing. [Zen refers to urinary zearalenone and its metabolites measured by ELISA; Cr refers to the concentration of urinary creatinine].



There were significant differences in urinary Zen/Cr among lines ($P < 0.05$): the Zearalenone line and the FE Resistant, Control and Susceptible lines had back-transformed means of 39.8, 38.5, 35.4 and 43.5, respectively. Only one Zearalenone-line animal out of 40 had an elevated GGT enzyme level before dosing in May. In the FE-line animals, no Resistant-line lambs, 27% of Control-line lambs and 65% of Susceptible-line lambs had elevated GGT enzyme levels ($P < 0.01$). This was in spite of the protection provided to Control- and Susceptible-line sheep over part of the autumn season by the use of Time Capsules™ (Munday *et al*., 1997). However, further analyses of $\log_e$ Zen/Cr, fitting $\log_e$ GGT enzyme level as a covariate in addition to the main effects, showed that there was no significant carry-over effect onto the August zearalenone dosing trial (regression coefficient = 0.20 ± 0.24 $\log_e$ units of Zen/Cr per $\log_e$ i.u./l GGT; $P = 0.40$). The range of serum GGT values was narrow, 31 to 103 i.u./l. However there were also ewe lambs from the FE lines dosed with zearalenone in the May trial (not reported in detail here), and analyses of variance revealed a $\log_e$ GGT covariate which approached significance (coefficient = 0.84 ± 0.48 $\log_e$ units of Zen/Cr per $\log_e$ i.u./l GGT; $P < 0.09$), and a line difference for GGT-adjusted $\log_e$ Zen/Cr where the line means were 3.86 units for the FE-Resistant line and 3.53 units for the FE-Susceptible line (0.33 ± SED 0.19 units; $P < 0.10$). This suggested that if challenged with zearalenone, the FE-Resistant line tended to be more sensitive to zearalenone than the FE-Susceptible line (after adjustment for recent history of FE exposure).

The heritability estimate for standardised urinary Zen/Cr in dosed sheep was 0.32 ± 0.10 ($P < 0.01$), with a phenotypic standard deviation of 0.98. The phenotypic standard deviation for $\log_e$ Zen/Cr was 0.38 (about 10 % of the mean).

DISCUSSION

This study has demonstrated a dose-response relationship between the intake of zearalenone and the concentration of urinary Zen breakdown products (Figure 1). In terms of concentrations, urinary Zen/Cr dropped to 4% of the maximum by 50 h after dosing, and this is consistent with preliminary data from Smith *et al.* (1991). They reported that peak levels of Zen/Cr were observed between 3 and 6 h after zearalenone dosing. Miles *et al.* (1996) found Zen/Cr in orally-dosed sheep peaked between 2 and 4 h after dosing. In the present data the peak time was probably closer to 2 h after dosing.

Our results show that ewe lambs appear to have levels of urinary Zen/Cr nearly twice as high as ram lambs. The recorded response is heritable. Therefore it should be possible to challenge animals from ram breeding flocks and rank them for zearalenone resistance/susceptibility, using the techniques described here. The ranking procedure would be analogous to the Ramguard test used in ram breeding flocks to challenge and test for resistance to FE (Morris *et al*., 1994). A question then arises as to whether a Ramguard test would interfere with a zearalenone test, if the tests were applied to the same animals. It is probably unwise to carry out a zearalenone test *after* a Ramguard FE test because of possible long-term liver injury from FE. In the current study, there was only minor liver injury from FE, and this resulted in a marginal effect on zearalenone response in May (covariate for log GGT significant at $P < 0.09$), but it was not related to the zearalenone response in August. However, if a zearalenone test is carried out *before* an FE test, there is unlikely to be any effect on the animal, or on the interpretation of FE test results. This is because the clearance of zearalenone is almost complete within 50 h, and there is no indication that low zearalenone doses significantly affect animal response to the FE toxin, sporidesmin.

All data included in the heritability analyses reported here were for sheep sampled following *dosing* with zearalenone. We also had data from sheep exposed to a *pasture challenge* with zearalenone, although the Zen/Cr means were often lower. The heritability of standardised urinary Zen/Cr under pasture-challenge conditions was also much lower (0.087 ± 0.095), presumably reflecting the variability of zearalenone intake as a result of diet selection and variability in daily pasture intake. However, it was encouraging to observe that the genetic correlation between standardised urinary Zen/Cr under dosed versus pasture-challenge conditions was positive and not significantly different from 1.0, whilst the phenotypic correlation was positive but moderate in size (0.32 ± 0.09).

In conclusion, these results show that the dosing regime reported here could be used for a test of animal response to zearalenone in ram breeders' flocks.

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