Session 5

Mare Reproductive Loss Syndrome and Associated Syndromes: Toxicological Hypotheses

Chairperson: Dr. Neil Williams, Livestock Disease Diagnostic Center, College of Agriculture, University of Kentucky

The Potential Role of Ergot Alkaloids in Mare Reproductive Loss Syndrome

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TALL FESCUE (*FESTUCA ARUNDINACEA* SCHREB.) IS A PERENnial, cool-season grass commonly used for forage and turf purposes (1). Fescue is characteristically more drought and cold tolerant and forms denser stands than other *Festuca* species. It is also more competitive with weeds and thrives on a wider range of soil types (1). Approximately 33 million acres of tall fescue are grown in the United States, with close to 50% of that existing east of the Mississippi River. States such as Kentucky, Tennessee, Virginia, West Virginia, and North Carolina use tall fescue as their primary grass species for forage and turf production (1). Approximately 5.5 million acres are grown in Kentucky alone and are grazed by an estimated 96,000 horses and/or ponies per year (2). Despite its good nutritive value, consumption of tall fescue by livestock results in a decrease in both reproductive and growth performance. It has been found that animal performance was depressed due to an endophytic fungus (*Neotyphodium coenophialum*) present between the cells of the plant (3). Three primary classes of alkaloids produced by the fungus and that play a potential role in affecting animal performance include the ergot (ergovaline), pyrrolizidine (lolines), and pyrrolopyrazine (peramine) alkaloids.

Cattle consuming tall fescue commonly exhibit increased respiration rates and rectal temperatures; depressed intake, subsequently resulting in lower average daily gains; and a rough hair coat, which is atypical to that usually seen during the summer grazing months (4). Contrary to depressed performance observed with cattle consuming endophyte-infected fescue, horses, most often gravid mares, typically exhibit a decrease in reproductive performance. Prolonged gestation, agalactia, increased foal and mare mortality, dystocia, tough and thickened placentas, weak and dysmature foals, reduced serum progesterone and prolactin, and increased serum estradiol-17ß are all signs commonly associated with mares consuming endophyte-infected fescue (5). Arns et al. (6) fed a fescue seed-based diet containing three levels of ergovaline (0, 0.16, and 0.31 0.39 $\mu g \cdot g^{-1}$) to determine its effect on the establishment and maintenance of early pregnancy. Mares consuming diets containing ergovaline had lower serum progesterone and prolactin levels with no effect on overall conception rates, cycles per conception, or embryonic vesicle size through 28 days of pregnancy. Conversely, Brendemuehl et al. (7) found mares grazing endophyte-infected (1.2 0.39 $\mu g \cdot g^{-1}$ ergovaline) tall fescue had prolonged luteal function (22.9 versus 15.8 days) and higher embryonic death (30 versus 7.7%) than mares grazing endophyte-free tall fescue pastures. Serum progesterone and prolactin concentrations followed similar trends observed by Arns et al. (6). Although reproductive efficiency was compromised in the two previous studies, a majority of reproductive complications occur during late-term pregnancy. Putnam et al. (8) found mares grazing tall fescue from 90 days of gestation through parturition had obvious signs of dystocia (10 of 11 mares) with foal survivability greatly reduced. Mares grazing endophyte-free tall fescue produced 11 viable foals, while those grazing endophyte-infected pastures (average 0.39 $\mu g \cdot g^{-1}$ ergovaline) produced three healthy foals. Gestation was also 20 days longer for mares consuming endophyte-infected tall fescue. Ten of 11 mares grazing endophyte-infected pastures showed no evidence of udder development or lactation prior to and during parturition. Udder development was normal in all 11 mares grazing endophyte-free tall fescue.

Because research has shown endophyte-infected tall fescue pastures, containing ergovaline concentrations as low as 0.2 $\mu g \cdot g^{-1}$, negatively affect early- and late-term pregnancies, the objectives of this experiment were to observe alkaloid concentrations in horse pastures located in the Bluegrass region of Kentucky and to determine any potential role they may play in Mare Reproductive Loss Syndrome (MRLS).

Materials and Methods

Twelve area-wide horse farms and one hay farm were monitored for alkaloid concentrations during the months of March, April, May, and June of 2002. Pastures, in which clipped forage samples were collected, contained pregnant mares or mares to be bred. Four sample types were collected on farms containing cherry trees on or near the perimeter of the farm (pure tall fescue–TF, composite weed/forage mixture–COMP, inside the cherry tree drip line–IN, and outside the tree drip line–OUT). Farms containing no cherry trees only had two sample types collected (TF and COMP). Clipped forage samples for each sample type were collected from an average of six collection sites within a field on each farm. Only fields containing pregnant mares

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were sampled, and fields were sampled biweekly. Within 7 hours, and usually sooner, samples were dried in a forced air oven at 55°C for 24 hours and then ground (Wiley Mill) to pass a 0.5-mm screen. Samples were immediately analyzed by reverse-phase high performance liquid chromatography for ergovaline and ergovalinine (9). Ergot alkaloid concentrations were adjusted up 30% due to losses that occur with oven drying. Lolines (N-acetylloline [NAL], N-formylloline [NFL], and N-acetylnorloline [NANL]) were analyzed using gas chromatography.

Where appropriate, data were analyzed using the Proc GLM procedure of SAS. Due to sampling procedures and different management practices employed by the various farms, fitting the data from all farms to a statistical model was difficult. Therefore, a sub-sample of six farms was analyzed for differences in sample type on ergovaline and total loline concentrations. Data were analyzed as a completely randomized design with a one-way treatment structure.

Results

Concentrations, averaged across all sampling data, of ergovaline (Figure 1) and total lolines (Figure 2) in pure tall fescue clipped samples (TF) differed ($P < 0.01$; $n = 6$) from those taken inside (IN) and outside (OUT) a cherry tree drip line, as well as samples that were a forage/weed composite (COMP). Ergovaline concentrations in TF (0.29 $\mu g \cdot g^{-1}$) samples were more than two-fold higher than the average of those in the COMP, IN, and OUT (0.11 $\mu g \cdot g^{-1}$) treatment groups. Loline levels in TF (559 $\mu g \cdot g^{-1}$) were 7.5 times greater than the average of samples in the COMP, IN, and OUT (73 $\mu g \cdot g^{-1}$) treatments. Because clipped samples from IN and OUT treatments did not differ from COMP, only COMP and TF samples will be discussed for all other results.

At initiation (February) of the monitoring program, both ergovaline (Figure 3) and total loline (Figure 4) concentrations were below detectable levels. However, by March, average concentrations of ergovaline and total lolines steadily increased in both the TF and COMP treatments. The greatest increase in ergovaline levels was observed in TF samples, peaking at 0.6 $\mu g \cdot g^{-1}$during the month of May, whereas the maximum level of ergovaline in COMP samples was 0.2 $\mu g \cdot g^{-1}$. Average loline levels were consistently higher for TF samples as compared with COMP; however, changes in concentration throughout the monitoring program were relatively small.

Ergovaline and total loline levels in both TF and COMP samples, for all farms from April 15 to May 31, are given in Figures 5 and 6. A majority of early fetal losses (EFL) and late fetal losses (LFL) occurred during the month of May. Although results from this monitoring program cannot determine if endophyte-infected tall fescue is directly associated with MRLS, examining alkaloid concentrations

Figure 1. Ergovaline concentrations in pastures monitored for MRLS.

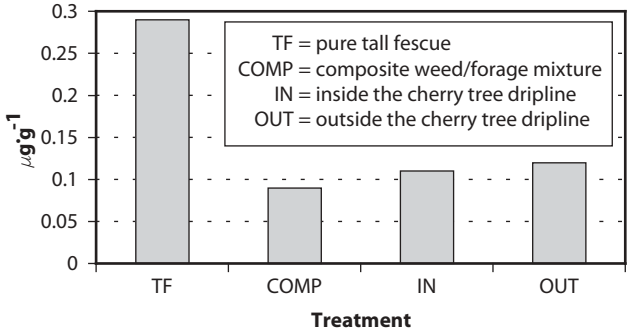


Figure 2. Loline concentrations in pastures monitored for MRLS.

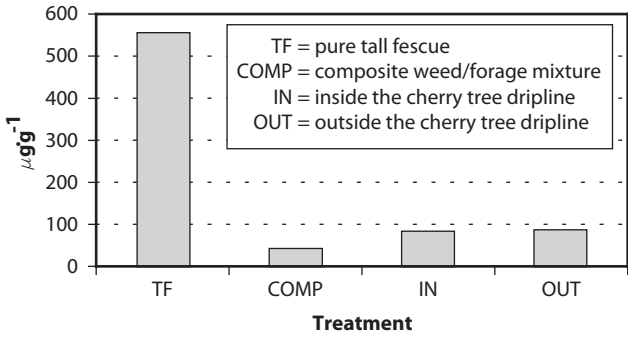


Figure 3. Bimonthly changes in ergovaline concentration.

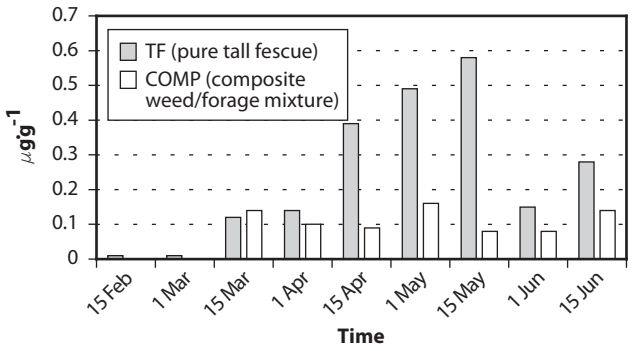
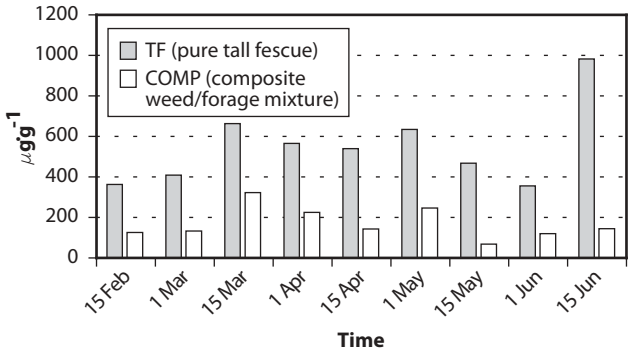


Figure 4. Bimonthly changes in total loline concentrations.



during the time of maximum foal losses is important. Alkaloid concentrations (ergovaline and total lolines) were consistently higher for TF samples as compared with COMP. Ergovaline in TF was between 0.25 and 0.7 $\mu g \cdot g^{-1}$ across all farms, whereas levels in COMP ranged between 0 and 0.23 $\mu g \cdot g^{-1}$. Average concentrations of ergovaline were 0.5 and 0.2 $\mu g \cdot g^{-1}$ for TF and COMP, respectively. Total loline concentrations were also consistently greater for TF versus COMP. Concentrations ranged from 250 to 720 $\mu g \cdot g^{-1}$ for TF and 7 to 225 $\mu g \cdot g^{-1}$ for COMP samples. Average loline levels were 543 versus 169 $\mu g \cdot g^{-1}$ for TF and COMP, respectively.

Discussion

Pure tall fescue clipped samples contained consistently higher levels of both ergovaline and total lolines than found in COMP samples. Minimal research is available on the effects of purified lolines or ergovaline on large animal performance. However, the negative endophyte-infected effects of tall fescue on performance and reproduction of horses are well documented (5,6,7,10). Ergovaline levels found in TF samples on all farms approached or were greater than the suggested 0.3 $\mu g \cdot g^{-1}$ concentration found in literature that results in decreased performance. In fact, greater than 50% (8 of 13) of all farms had ergovaline concentrations above 0.3 $\mu g \cdot g^{-1}$ during the month of May when fetal losses were greatest. However, it cannot be determined from these results if tall fescue was directly involved with any of the losses that occurred during that time. Also, concentrations of ergovaline in COMP samples were numerically lower than that found in TF. Composite samples were a forage/weed mixture with such grasses as tall fescue, timothy, orchardgrass, and bluegrass making up some of the forage species of the mixture. Horses are selective grazers, and the intake of tall fescue relative to total pasture ingested on a day-to-day basis was not recorded, making it difficult to estimate alkaloid intake.

Conclusion

With the type of monitoring program implemented, it is difficult to determine if endophyte-infected tall fescue played a role in MRLS. Further research is needed to properly answer that question. However, most importantly, one cannot discard the fact that high concentrations of ergovaline in horse pastures during late-term pregnancy can result in high foal mortality, decreased udder development and milk production, dystocia, and prolonged gestation. Monitoring mares during this time and removal from infected pastures at 300 days of gestation and subsequently after they have foaled and been rebred through 40 days of gestation have been suggested as a means of preventing the above reproductive complications (11).

Figure 5. Ergovaline concentrations for COMP and TF samples monitored for MRLS from April 15 to May 31.

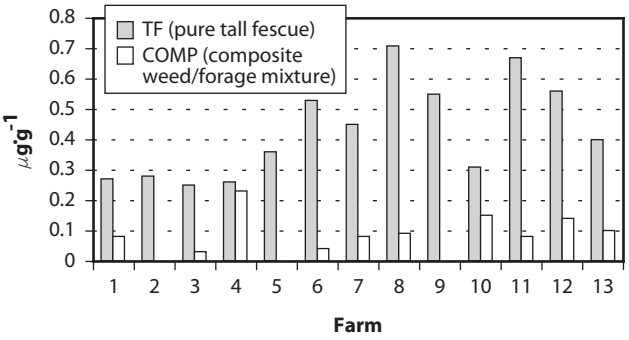
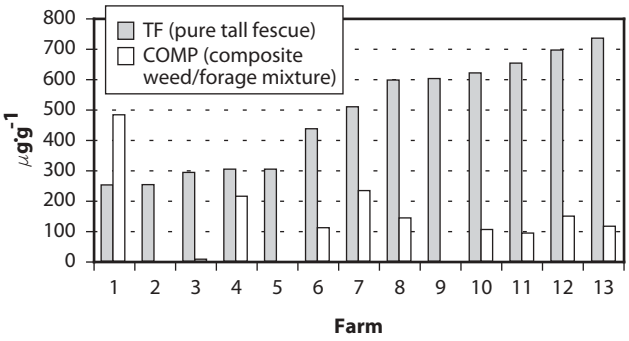


Figure 6. Total loline concentrations for COMP and TF samples monitored for MRLS from April 15 to May 31.



References

1. Sleper, D. A. and Buckner, R. C. Fescues. In: Barnes, R. B.; Miller, D. A.; Nelsen, C. J., and Heath, M. E., editors. Forages. v. 1. An introduction to grassland agriculture. 5th ed. Ames: Iowa State University Press; 1995; pp. 345-356.
2. Livestock on farms, by class and value January 1, 1999 and 2000. Kentucky Agricultural Statistics. 1999/2000;59.
3. Bacon, C. W.; Porter, J. K.; Robbins, J. D., and Luttrell, E. S. *Epichloe typhina* from toxic tall fescue grasses. Applied and Environmental Microbiology. 1977; 34(5):576-581.
4. Paterson, J.; Forcherio, C.; Larson, B.; Samford, M., and Kerley, M. The effects of fescue toxicosis on beef cattle productivity. Journal of Animal Science. 1995; 73(3):889-898.
5. Porter, J. K. and Thompson, F. N. Effects of fescue toxicosis on reproduction in livestock. Journal of Animal Science. 1992; 70(5):1594-1603.
6. Arns, M. J.; Pruitt, J. A., and Sharp, C. Influence of endophyte-infected tall fescue seed consumption on the establishment and maintenance of pregnancy in mares. Professional Animal Scientists. 1997; 13(3):118-123.

7. Brendemuehl, J. P.; Boosinger, T. R., and Shelby, R. A. Influence of endophyte-infected tall fescue on cyclicity, pregnancy rate and early embryonic loss in the mare. Theriogenology. 1994; 42(3):489-500.
8. Putnam, M. R.; Bransby, D. I.; Schumacher, J.; Boosinger, T. R.; Bush, L.; Shelby, R. A.; Vaughan, J. T.; Ball, D., and Brendemuehl, J. P. Effects of the fungal endophyte *Acremonium coenophialum* in fescue on pregnant mares and foal viability. American Journal of Veterinary Research. 1991; 52(12):2071-2074.
9. Siegel, M. R.; Latch G. C. M.; Bush, L. P.; Fannin, F. F.; Rowan, D. D.; Tapper, B. A.; Bacon, C. W., and Johnson, M. C. Fungal endophyte-infected grasses: alkaloid accumulation and aphid response. Journal of Chemical Ecology. 1990; 16(12):3301-3315.
10. Taylor, M. C.; Loch, W. E., and Ellersieck, M. Toxicity in pregnant pony mares grazing Kentucky-31 fescue pastures. Nutrition Reports International. 1985; 31(4):787-795.
11. Harper, F. and Green, E. Broodmares and tall fescue toxicosis. Horse Express. 1995; 14(2).

Phytoestrogens and Estrogenic Activity in White Clover Samples from No-Loss and High-Loss Fields during Mare Reproductive Loss Syndrome, 2001

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MARE REPRODUCTIVE LOSS SYNDROME (MRLS) WAS IDENTIfied in April of 2001 and thereafter on horse farms in Central Kentucky. It consisted of an excessive number of early fetal losses (EFL) and late fetal losses (LFL). During the spring of 2001, many samples were obtained from horse pastures and hay from Central Kentucky. Samples analyzed for phytoestrogens and estrogenic activity are the focus of this report.

Phytoestrogens are substances found in many plants that can interact with the estrogen receptor and elicit estrogenic effects (1-3). Common plants such as clovers, alfalfa, and soybeans contain phytoestrogens. Phytoestrogens in soybeans are reported to have sufficient estrogenic activity to relieve some of the symptoms associated with menopause (3). Estrogenic substances, such as estrogenic mycotoxins and phytoestrogens, have long been known to be deleterious to reproduction in swine and ruminants. They can increase interovulatory intervals in swine and decrease interovulatory intervals as well as cause embryonic and fetal abortions in ruminants (4-7).

In the 1940s, subterranean red clover caused abortions and other reproductive problems in sheep in Australia (8). The causative agents in the clover were the phytoestrogens biochanin A and formononetin.

While phytoestrogens are not known to cause reproductive problems in horses, the unique conditions in late spring of 2001 may have triggered an unusual combination of phytoestrogens to which the mares were exposed and possibly caused or contributed to MRLS. White clovers are the pasture plants most likely to contain phytoestrogens in horse pastures in Central Kentucky. Therefore, white clover in pasture and hay samples collected from fields where mares sustained high versus low incidences of fetal loss were analyzed for phytoestrogen content and estrogenic activity.

Materials and Methods

Between May 6 and June 6, 2001, 25 samples of white clover from pastures and hay were obtained from 10 different horse farms. The farms reported the degree of fetal loss for each field. Portions of all samples were sent to Dr. Patricia Murphy, Food Science and Human Nutrition Department, Iowa State University, Ames, Iowa, where they were analyzed for a panel of phytoestrogens. Additionally, portions of eight of the samples, representing three farms, were also sent to Dr. George Clark, Xenobiotic Detection Systems Inc., Durham, North Carolina, where they were analyzed for estrogenic activity. The analysis for estrogenic activity requires that samples be preserved by freezing immediately upon collection. Only the eight samples, representing three farms, were frozen upon collection and were thus deemed suitable for the analysis.

Results

More than 300 separate tests were performed for estrogenic activity and/or phytoestrogen content. Total estrogenic activity ranged from less than 1 ng/g sample to ap-

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proximately 10 ng/g (tested against the standard estradiol-17ß) (Table 1). There were no consistent differences among samples obtained from fields with mares reported to have high incidences of fetal loss versus those that reported little or no fetal losses.

The phytoestrogens included in the analyses were daidzin, genistin, glycitin, malonyl daidzin, malonyl genistin, malonyl glycitin, acetyl daidzin, acetyl genistin, acetyl glycitin, daidzein, genistein, glycitein, coumestrol, formononatin, and biochanin A (Table 2). The individual isomers of genistein, daidzein, and glycitein were not detectable in any of the samples. Formononetin was the only phytoestrogen that was consistently detectable, with levels on all but two samples ranging from 100 to 300 µg/g sample. The other two samples had the highest levels detected, at 1,037 and 3,295 µg/g sample. Those same two samples were the only samples with detectable levels of biochanin A, at 286 and 2,386 µg/g sample.

Table 1. Estrogenic activity of clover samples taken from high-loss and low-loss fields.

Farm ID	Reported Incidence of Fetal Loss for Field Sampled	Mean of Duplicate Estimates (ng/g)
A	severe	1.28
A	none	3.05
B	no report	5.32
A	severe	0.83
A	none	3.71
B	high	3.56
B	none	10.25
C	severe	10.72

Discussion

Formononetin and biochanin A were the only phytoestrogens that were consistently detectable in the samples of white clover obtained from selected horse farms in June of 2001. These are also the phytoestrogens found in subterranean red clover that caused the Australian sheep infertility problems of the 1940s. However, those concentrations were substantially higher, at approximately 10,400 and 19,000 µg/g sample, respectively. The concentrations found in our samples were consistently lower, regardless of degree of fetal loss associated with those fields. Additionally, they were below the concentrations in some reports for alfalfa sprouts sold for human foods (9,10).

Reported signs associated with MRLS include EFL and LFL as well as increased incidences of pericarditis and uveitis. Signs that one would associate with hyperestrogenism, such as increased interovulatory interval in nonpregnant mares, inappropriate estrous behavior, and inappropriate uterine edema were not reported as part of MRLS. Estrogens can be luteolytic in cattle and sheep and luteostatic in swine (4-7;11-13), but estrogens are not luteolytic or luteostatic in horses (14-16).

We cannot exclude the possibility that phytoestrogen levels might have been higher in horse pastures in the middle of April of 2001 when fetal losses were first detected than during May and June when our samples were collected. Neither can we exclude the possibility that phytoestrogens other than these 15 common ones were present in the samples that we tested. However, based on the low to nondetectable concentrations found in the samples reported here and that those samples represented fields from which mares sustained severely high to no fetal losses, it is unlikely that phytoestrogens as measured in these sample are responsible for MRLS.

Acknowledgments

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References

1. Arnold, S. F.; Klotz, D. M.; Collins, B. M.; Vonier, P. M.; Guillette, L. J., and McLachlan, J. A. Synergistic activation of estrogen receptor with combinations of environmental chemicals. Science. 1996; 272(5267):1489-1492.
2. Safe, S. H. Environmental and dietary estrogens and human health: is there a problem? Environmental Health Perspectives. 1995; 103(4):346-351.
3. Barrett, J. Phytoestrogens, friends or foes? Environmental Health Perspectives. 1996; 104(5):478-482.
4. Ford, S. P.; Weems, C. W.; Pitts, R. E.; Pexton, J. E.; Butcher, R. L., and Inskeep, E. K. Effects of estradiol-17beta and progesterone on prostaglandins F in sheep uteri and uterine venous plasma. Journal of Animal Science. 1975; 41(5):1407-1409.
5. Eley, R. M.; Thatcher, W. W., and Bazer, F. W. Luteolytic effect of oestrone sulphate on cyclic beef heifers. Journal of Reproduction and Fertility. 1979; 55(1):191-193.
6. Thatcher, W. W.; Terqui, M.; Thimonier, J., and Mauleon, P. Effect of estradiol-17beta on peripheral plasma concentration of 15-keto-13,14-diydro PGF2alpha and luteolysis in cyclic cattle. Prostaglandins. 1986; 31(4):745-756.
7. Frank, M.; Bazer, F. W.; Thatcher, W. W., and Wilcox, C. J. A study of prostaglandin F2alpha as the luteolysin in swine. III. Effects of estradiol valerate on prostaglandin F, progestins, estrone and estradiol concentrations in the utero-ovarian vein of non-pregnant gilts. Prostaglandins. 1977; 14(6):1183-1196.

Table 2. Phytoestrogen content of clover samples taken from high-loss and low-loss fields.

Farm ID	Reported Incidence of Fetal Loss for Field Sampled	Total Dein[1]	Total Gein[2]	Total Gly[3]	Coumestrol –g/g sample	Formononetin –g/g sample	Biochanin –g/g sample
Assayed for estrogenic activity and phytoestrogen content							
A	severe	ND	ND	ND	175	145	ND
A	none	ND	ND	ND	ND	376	ND
B	no report	ND	ND	ND	ND	174	ND
A	severe	ND	ND	ND	ND	156	ND
A	none	ND	ND	ND	ND	102	ND
B	high	ND	ND	ND	ND	96	ND
B	none	ND	1	ND	ND	174	ND
C	severe	ND	ND	ND	ND	172	ND
Assayed for phytoestrogen content only							
D	severe	ND	ND	ND	ND	250	ND
E	moderate	ND	ND	ND	ND	3295	2386
E	moderate	ND	ND	ND	ND	198	ND
F	severe	ND	ND	ND	ND	246	ND
G	severe	ND	ND	ND	ND	282	ND
H	none	ND	ND	ND	ND	223	ND
I	light	ND	ND	ND	521	388	ND
A	severe	ND	ND	ND	ND	240	ND
A	severe	ND	ND	ND	ND	276	ND
A	none	ND	ND	ND	367	225	ND
A	none	ND	ND	ND	ND	153	46
J	moderate	ND	ND	ND	ND	308	ND
hay	not applicable	ND	ND	ND	ND	222	ND
hay	not applicable	ND	ND	ND	ND	204	ND
hay	not applicable	ND	ND	ND	ND	1037	286
hay	not applicable	ND	ND	ND	ND	161	ND
hay	not applicable	ND	ND	ND	ND	260	ND

[1] Total Dein is the sum of the individual isomers of daidzein and includes daidzin, malonyl daidzin, acetyl daidzin, and daidzein.

[2] Total Gein is the sum of the individual isomers of genistein and includes genistin, malonyl genistin, acetyl genistin, and genistein.

[3] Total Gly is the sum of the individual isomers of glycitein and includes glycitin, malonyl glycitin, acetyl glycitin, and glycitein.

8. Bennets, H. W.; Underwood, E. J., and Shier, F. L. A specific breeding problem of sheep on subterranean clover pastures in western Australia. Australian Veterinary Journal. 1946; 22(1):2-12.
9. Murphy, P. A.; Song, T. T.; Buseman, G.; Barua, K.; Beecher, G. R.; Trainer, D., and Holden, J. Isoflavones in retail and institutional soy foods. Journal of Agricultural and Food Chemistry. 1999; 47(7):2697-2704.
10. Murphy, P. Documentation for the USDA-Iowa State University isoflavones database [Web Page]. 2002 Mar 15. Available at: <http://www.nal.usda.gov/fnic/foodcomp/Data/isoflav/isfl_doc.pdf>.
11. Frank, M.; Bazer, F. W.; Thatcher, W. W., and Wilcox, C. J. A study of prostaglandin F2alpha as the luteolysin in swine. IV. An explanation for the luteotrophic effect of estradiol. Prostaglandins. 1978; 15(1):151-160.
12. Rampacek, G. B. and Kraeling, R. R. Effect of estrogen on luteal function in prepuberal gilts. Journal of Animal Science. 1978; 46(2):453-457.
13. Geisert, R. D. and Yelich, J. V. Regulation of conceptus development and attachment in pigs. In: Foxcroft, G. R.; Geisert, R. D., and Doberska, C., editors. Control of Pig Reproduction V: Proceedings of the Fifth International Conference on Pig Reproduction; Kerkrade. Cambridge: Journals of Reproduction and Fertility Ltd.; 1997: 133-149. (Journal of Reproduction and Fertility. Supplement no. 52).
14. Goff, A. K.; Sirois, J., and Pontbriand, D. Effect of oestradiol on oxytocin-stimulated prostaglandin F2alpha release in mares. Journal of Reproduction and Fertility. 1993; 98(1):107-112.
15. Woodley, S. L.; Burns, P. J.; Douglas, R. H., and Oxender, W. D. Prolonged interovulatory interval after oestradiol treatment in mares. In: Rowlands, I. W. and Allen, W. R., editors. Equine Reproduction II: Proceedings of the Second International Symposium on Equine Reproduction; Davis. Cambridge: Journals of Reproduction and Fertility Ltd.; 1979: 205-209. (Journal of Reproduction and Fertility. Supplement no. 27).
16. Ginther, O. J. Reproductive biology of the mare: basic and applied aspects. 2nd ed. Cross Plains: Equiservices; 1992.

Review of Mycotoxins as a Possible Cause of Mare Reproductive Loss Syndrome

K. Newman

THE ROLE OF MYCOTOXINS IN MARE REPRODUCTIVE LOSS Syndrome (MRLS) has been a primary focus of the investigations since early in May of 2001. At that time, a number of forage samples were tested for the mycotoxin zearalenone and found to contain quantities ranging from 200 to 1,500 parts per billion (ppb). Since zearalenone has been demonstrated to cause abortions in a number of animal species, the focus on mycotoxins as being one of the primary suspects in this investigation seemed quite justified. Combine this with the fact that drought and temperature stress can evoke toxin production from certain molds (1,2) and the fact that the spring of 2001 was a very dry, warm period followed by a dramatic drop in temperature leading to frost occurring on April 17 and 18, and it seemed quite cut-and-dried that the cause of MRLS was mycotoxin intoxication. Mycotoxins are not transmissible from animal to animal and do not respond to drug and antimicrobial therapy. In addition, most mycotoxins are toxic but not lethal at environmental levels (example: zearalenone has an LD_{50} greater than 16 g/kg of body weight). Zearalenone has estrogenic properties, although it is not chemically an estrogen. For this reason, females are traditionally more susceptible than males. In swine, a swollen, red vulva, which may lead to rectal and vaginal prolapse, is normally only seen in prepubertal gilts (3). In MRLS, maiden and previously barren mares seemed to be more susceptible to the syndrome. A number of studies in other animal species have demonstrated the effects of zearalenone over extended periods of time; a more probable scenario under field conditions (and in examining MRLS) is dosing of toxin over a short period of time. Studies with 16 gilts given pure zearalenone (108 mg on post-mating days 2 to 6, 7 to 10, or 11 to 15) showed that gilts given zearalenone on post-mating day 7 to 10 had reduced embryonic survival than control or other treatment groups (1 of 4 pregnant in the 7 to 10 day group; 4 of 4 pregnant in control and other treatment groups). FSH and estradiol-17ß concentrations were unaffected by zearalenone consumption. Serum concentrations of prolactin in the 7 to 10 and the 11 to 15 group were lower than control or the 2 to 6 post-mating groups (4).

Similar problems associated with abortions in cattle have also been attributed to zearalenone-contaminated hay (5). Because of the critical effects of zearalenone on stage of pregnancy, it was thought that this toxin may have played a critical role in the events described in the spring of 2001. This may account for the observation of affected mares being 40 to 100 days pregnant or near term.

In addition, North Carolina State University (NCSU) and the University of Guelph last year (personal communication) have presented data demonstrating the presence of a significant amount of different mycotoxins in forage samples. In the study from NCSU, they detected aflatoxin, deoxynivalenol (DON), trichothecene (T-2), and zearalenone (ZEA) in forage (Table 1). It is important to emphasize that, in both the Guelph and NCSU trials, commercial ELISA test kits were used for the analysis.

Table 1. Mycotoxins present in forage samples as detected by commercial ELISA test kits.

	Aflatoxin	DON	Fumonisin	T-2	Zearalenone
Detected	Yes	Yes	No	Yes	Yes

(6)

In the case of MRLS investigations, the early results demonstrating the presence of zearalenone were also determined by ELISA kits. These kits are not validated for the complex matrices of forage, caterpillar, and caterpillar frass samples. ELISA procedures are prone to false positive results on nonvalidated matrices and should be confirmed using high performance liquid chromatography (HPLC) and/or thin layer chromatography (TLC) testing. When confirmation of these positive results was requested, one of the major problems was the availability and quantity of samples from the critical time frame. However, no Fusarium toxins were detected in any of the samples submitted for HPLC analysis. What was once a simple task of validation of what was already suspected turned to a "what do we do now?" situation.

Since the presence of mycotoxins is often very difficult to detect under the best circumstances, the inability to confirm their presence in the few samples available for duplicate analysis by other methods and the failure to detect them in forage samples taken after the critical insult period does not eliminate mycotoxins as a possible cause for MRLS. As with any investigation, the need to follow up on the clues presented at the time was the next step. An important observation came from Dr. Bruce Webb,

Venture Laboratories Inc., Lexington, Kentucky.

an entomologist at the University of Kentucky. From his extensive work on caterpillars, he observed that caterpillar frass was an excellent growth medium for molds. From this observation, a theory was developed that caterpillar frass served as a "fertilizer" for mold growth and mycotoxin production leading to MRLS. Field samples taken during the early summer of 2001 confirmed the observation of frass supporting mold growth, with *Aspergillus*, *Fusarium*, and *Penicillium* sp. being the predominant genera isolated (Table 2).

Table 2. The ability of ETC frass to support the growth of various fungi taken from environmental samples.

Environmental Fungal Isolate	Growth on Frass
Aspergillus flavus	Yes
Aspergillus fumigatus	Yes
Fusarium sp.	Yes
Fusarium graminearum	Yes
Fusarium poae	Yes
Penicillium sp.	Yes

Taking this theory one step further, it was observed that *Penicillium* sp. dominated the mycoflora of cultures of ETC and frass from trials where caterpillars were either mixed in the feed or intubated in pregnant mares and subsequently caused symptoms consistent with MRLS (Table 3). Caterpillar frass samples contained approximately 100 times higher concentrations of penicillium than the caterpillar samples (10^7 CFU/g versus 10^5 CFU/g). These concentrations of fungi supported the theory that frass was an excellent growth medium for fungi; however, with the exception of the first mare trial, frass alone has not been shown to cause mares to abort. *Penicillium* sp. have been isolated from every frass sample (seven) obtained in 2002. Fresh caterpillars (taken from trees less than 30 minutes prior to testing) demonstrate $<10^3$ CFU/g of mold from the gastrointestinal tract contents. However, *Penicillium* sp. have been consistently isolated from microbial examinations of entire caterpillars from spring of 2002, with *Aspergillus* and *Fusarium* sp. making up the vast majority of the mold populations from these samples. No known mycotoxins were identified from any of the caterpillar homogenates.

Substantiating the frass fertilizer theory was a recent publication that showed uric acid enhancing toxin production from penicillium molds (7). Uric acid is a primary component of frass (4 to 6%) and seems to be a good nitrogen source for mold growth and a catalyst for production of certain toxins. Published *in vitro* trials looked at different uric acid concentrations and the ability of uric acid to improve alkaloid production by penicillium mold.

In our own observations, uric acid also stimulated the growth rate of five separate *Penicillium* sp. (5 to 30% increase) and three *Fusarium* sp. (7 to 21% increase). Since a period of very warm weather followed by frost was observed in the spring of 2001, and frost exposure being a catalyst for toxin production of certain molds, an examination of the effects of frost on environmental isolates from predominant mold isolates seemed a logical additional piece of required evidence. As expected, exposure to freeze-thaw situations increases toxin production especially in the case of *F. poae* (30% increase).

Table 3. The concentrations of fungi observed from samples of ETC and ETC frass from environmental sources.

Sample	Mold Concentration	Predominant Mold Genera
Frass from intubation trial	1.2×10^7 CFU/g	Penicillium
Frass from affected farm (A)	1.2×10^9 CFU/g	Penicillium; Aspergillus
Frass from affected farm (B)	9.5×10^7 CFU/g	Penicillium
Frass from environmental samples (n = 4)	$4.0 - 6.6 \times 10^8$ CFU/g	Penicillium, Fusarium
Caterpillar GI tract contents	$<10^3$ CFU/g	Not Applicable
Caterpillars from intubation trial	1.7×10^5 CFU/g	Penicillium; Mucor
Intact, entire caterpillars	1.5×10^5 CFU/g	Penicillium

From a scientific standpoint, the circumstantial evidence was beginning to build for a possible mycotoxin insult being responsible for MRLS. Uric acid was shown to stimulate mold growth and toxin production with frost conditions apparently exacerbating toxin production. Then, two separate trials by B. Webb et al. and B. Bernard et al. (this proceedings) found that caterpillars, not frass, were associated with symptoms consistent with MRLS under controlled conditions. Samples of these caterpillars were tested for mycotoxins using a combination of TLC, ELISA, and HPLC and found to be below detectable limits for the major known fusarium, penicillium, and aspergillus mycotoxins. If the data presented from the trials of the above are valid, then mycotoxins are not responsible for MRLS fetal loss. This is a logical finding considering that it seems highly unlikely that a caterpillar would somehow sequester mycotoxins either in it or on it (no known fusarium, penicillium, or aspergillus toxins were detected from caterpillar homogenates). This does not eliminate other biological toxins from being involved in MRLS fetal loss, pericarditis, or eye problems. A variety of microorganisms have been associated with tent caterpillars. The identity of these organisms and possible toxic agents associated with them warrants further investigation. It is also possible that there may be a role for mycotoxins in pericarditis and/or eye problems that were also observed in a small percentage of the horse population during the same time period. There are data in the literature to support a role for mycotoxins in cases of unilateral blindness and heart problems in a variety of species.

References

1. Northolt, M. D.; Verhulsdonk, C. A. H.; Soentoro, P. S. S., and Paulsch, W. E. Effect of water activity and temperature on aflatoxin production by *Aspergillus parasiticus*. Journal of Milk and Food Technology. 1976; 39(3):170-174.
2. Schindler, A. F. Temperature limits for production of aflatoxin by twenty-five isolates of *Aspergillus flavus* and *Aspergillus parasiticus*. Journal of Food Protection. 1977; 40(1):39-40.
3. D'Mello, J. P. F.; Placinta, C. M., and Macdonald, A. M. C. Fusarium mycotoxins: a review of global implications for animal health, welfare and productivity. Animal Feed Science and Technology. 1999; 80(3-4):183-205.
4. Long, G. G. and Diekman, M. A. Characterization of effects of zearalenone in swine during early pregnancy. American Journal of Veterinary Research. 1986; 47(1):184-187.
5. Kallela, K. and Ettala, E. The oestrogenic fusarium toxin (zearalenone) in hay as a cause of early abortions in the cow. Nordisk Veterinaermedicin. 1984; 36(9-10):305-309.
6. Barnett, D. T.; Mowrey, R. A.; Hagler, W. M.; Bristol, D. G., and Mansmann, R. A. The correlation of selected mycotoxins to the incidence of colic in horses [Web Page]. 1994. Available at: <http://www.cals.ncsu.edu/an_sci/ann_rep94/dtbar78.html>.
7. Helbig, F.; Steighardt, J., and Roos, W. Uric acid is a genuine metabolite of *Penicillium cyclopium* and stimulates the expression of alkaloid biosynthesis in this fungus. Applied and Environmental Microbiology. 2002; 68(4):1524-1533.

Cherry Trees, Plant Cyanogens, Caterpillars, and Mare Reproductive Loss Syndrome: Toxicological Evaluation of a Working Hypothesis

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THE ONSET OF MARE REPRODUCTIVE LOSS SYNDROME (MRLS) in 2001 coincided with an exceptional abundance of eastern tent caterpillars (ETC), *Malacosoma americanum,* in Central Kentucky. Preliminary field studies by Henning and his co-workers also showed a high geographic correlation between ETC, black cherry trees, and MRLS. This correlation was strongly confirmed by a later epidemiological survey by R. Dwyer et al. (1). Further support for this association was provided by a toxicology report of high cyanide concentrations in the hearts of three late fetal loss (LFL) foals (L. Harrison, personal communication).

The MRLS 2001 syndrome was a completely new entity, having never been identified or described previously. It was also extremely transient, appearing and peaking in less than two weeks. As such, for the remainder of 2001, the syndrome was viewed, analyzed, and researched in an increasingly retrospective manner.

It was soon hypothesized that cyanide from black cherry tree leaves was the proximal cause of MRLS. Cyanide or cyanogens were thought to be transferred from the trees to the environment of the horse by ETC voraciously denuding these trees (Figure 1). The precise mechanism of the transfer was unclear. The source(s) of cyanogens could be the caterpillars themselves, caterpillar frass, leaf fragments, mandelonitrile ingestion, water contaminated by caterpillars, combinations of these factors, or by other sources such as pasture clover. The concentrations of cyanide involved were assumed to be sub-lethal for mares since mares did not show clinical signs, and it was assumed that the fetus was more sensitive than the mare to cyanide. If the hypothesis was correct, it should be possible to abort mares by exposing them to sub-clinical concentrations of cyanide.

Since there was virtually a complete absence of published literature about cyanide in the horse, let alone the pregnant mare, the objectives of this study were to define "normal" cyanide concentrations in equine blood in Central Kentucky, determine the "threshold" toxic level for cyanide toxicity in equine blood, and determine fetotoxicity of cyanide in the pregnant mare. The overall goal of this project was to reproduce MRLS in the laboratory.

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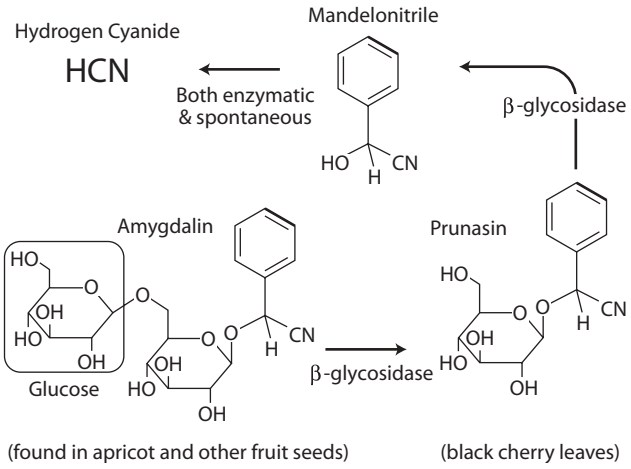
Figure 1. Cyanogenic cascade in black cherry (prunasin) and apricot (amygdalin). Both cascades proceed through prunasin, and mandelonitrile is the proximal cyanide donor.



Materials and Methods

Horses

Mature Thoroughbred mares weighing 428 to 504 kg were used for this study. The animals were maintained on grass hay and feed (12% protein), which was a 50:50 mixture of oats and an alfalfa-based protein pellet. Horses were fed twice a day. The animals were vaccinated annually for tetanus and were de-wormed quarterly with ivermectin (MSD Agvet, Rahway, NJ). A routine clinical examination was performed before each experiment to assure that the animals were healthy and sound. Additionally, cardiac and ophthalmic evaluations were performed to ensure that those organs had no evidence of previous disease. All animals used in these experiments were managed according to the rules and regulations of the University of Kentucky Institutional Animal Care Use Committee, which also approved the experimental protocol.

Cyanide Infusion

An intravenous catheter (Abbocath-T, 14g x 5½", North Chicago, IL) was inserted into the jugular vein and sutured in place. Sodium cyanide (NaCN) solutions were prepared by dissolving NaCN (J.T. Baker, Philipsburg, NJ) in saline and were infused at 3, 6, and 12 mg/minute for 1 hour and 1 mg/kg for 1 hour using an ambulatory withdrawal pump (Dakmed, Buffalo, NY). During infusion, the horses were monitored closely for signs of toxicity, which included restlessness, anxiety, flared nostrils, rapid respiration, sweating, and increased heart rate. Heart rates (HR) were recorded at 1-minute intervals by an onboard heart rate computer (Polar CIC Inc., Port Washington, NY). For the 3, 6, and 12 mg/minute infusions, blood samples were taken before and at various times during and after infusion for complete blood counts, chemistry panels, and blood lactates. For the 1 mg/kg for 1 hour infusion, blood samples were obtained for analyses before infusion (0 hours), during infusion (0.25, 0.5, and 1 hour) and after infusion (0.08, 0.17, 0.5, 1, 2, 4, 6, 8, 12, 24, 48, 72, 96, 120, 144 hours) into Vacutainer® serum tubes (Becton Dickinson, Rutherford, NJ) and Vacutainer® plasma tubes (Becton Dickinson, Franklin Lakes, NJ) and analyzed immediately.

Mandelonitrile Administration

In a second series of experiments, four mature Thoroughbred mares were administered oral mandelonitrile (3 mg/kg). Blood samples for cyanide analysis were obtained before (0 hours) and after dosing at 0.05, 0.08, 0.17, 0.25, 0.5, 1, 2, 4, 6, 8, 12, and 24 hours into Vacutainer serum tubes and Vacutainer plasma tubes and analyzed immediately.

Safety Precautions

Two antidotes were prepared to counter any adverse effects from cyanide infusion. A 3% solution of sodium nitrite was prepared by adding 1.8 g of Na nitrite (J.T. Baker, Philipsburg, NJ) to 60 ml of saline. This mixture was to be administered intravenously at a rate of 10 to 20 ml/minute. A 25% solution of sodium thiosulfate (J.T. Baker, Philipsburg, NJ) was prepared by adding 100 g Na thiosulfate to 400 ml of saline. This mixture was to be administered immediately after the sodium nitrate at a rate of 200 ml/minute.

Analytical Detection of Cyanide

As detailed previously (1), an inexpensive, disposable alternative to the costly Warburg Distillation Flask was developed, which allowed simultaneous running of 100 cyanide analyses. Briefly, a 10-ml plastic cup was suspended by means of Scotch® tape inside a 120-ml plastic cup with a screw lid; 10 ml of 1 molar sulphuric acid (H_2SO_4) was pipetted into the larger cup. Exactly 2.5 ml of 0.25 normal sodium hydroxide (NaOH) was pipetted into the smaller cup. The cyanide-containing sample (e.g., 1 to 2 ml blood) was pipetted into the H_2SO_4, and the cup was immediately sealed with its lid and allowed to sit overnight at room temperature while cyanide as HCN gas was evolved from the acid solution and trapped in the NaOH. The small cup was then removed, and the NaOH solution was decanted into an autoanalyzer sample cup. In the presence of chloramine-T, the cyanide ion was converted to cyanogen chloride, which reacted with pyridine-barbituric acid to form a red-blue color, the intensity of which was measured spectrophotometrically at 578 nm. Use of an autoanalyzer ensured a precise and reproducible interval during which color developed and thus improved the detection limit to as low as 2 ng/ml in a 1-ml sample.

Standard curves were linear in the range of 2 to 300 ng/ml, with a regression coefficient $r^2 > 0.99$.

Toxicokinetic Analysis

The toxicokinetic parameters of cyanide were determined by compartmental analysis. Equations of a two-compartment model with zero-order input rate were fitted to the individual blood concentrations versus time by least squares nonlinear regression analysis using a nonlinear regression program (Winnonlin, version 3.1) (Pharsight Corporation, Cary, NC). The closeness of the fit was evaluated by the Akaike Information Criterion (AIC), residual plots, and visual inspection. The data were weighted as $1/(y_{pred})^2$, where y_{pred} was the model-predicted concentration at the actual time. Area under the curve (AUC) following intravenous administration was measured by use of a linear trapezoidal approximation with extrapolation to infinity, and slope of the terminal portion (ß) of the log plasma drug concentrations versus time curve was determined by the method of least-squares regression (2). The rate constant of distribution α and distribution half-life ($t_{1/2}\alpha$) were determined using the method of residuals (2). Total body clearance (Cl_s) was calculated by use of Equation 1 (3).

$$Cl_S = \text{IV Dose}/AUC_{0\text{-inf}} \qquad \text{(Equation 1)}$$

The volume of distribution in central compartment (Vd_c) and volume of distribution at steady state (Vd_{ss}) were calculated according to Equations 2 and 3, respectively (4).

$$Vd_c = \text{Dose (IV)}/(A+B) \qquad \text{(Equation 2)}$$

$$Vd_{SS} = \text{IV Dose}/AUC_{0\text{-inf}} \times MRT \qquad \text{(Equation 3)}$$

A and B are the Y intercepts associated with distribution and elimination phase, respectively, and AUMC is area under the first moment curve and calculated by the trapezoidal method and extrapolated to infinity.

The mean residence time (MRT) (5) was determined according to Equation 4.

$$MRT = AUMC_{0\text{-inf}}/AUC_{0\text{-inf}} - (\text{Infusion time}/2) \qquad \text{(Equation 4)}$$

The pharmacokinetic variables (elimination half-life, area under the curve) of the cyanide following oral administration of mandelonitrile were determined using a noncompartmental approach (2). The maximum blood concentration of the cyanide (C_{max}) and the time to reach this concentration (T_{max}) were obtained directly from the blood-concentration versus time curves. The absolute bioavailability (F) was calculated from the $AUC_{0\text{-inf}}$ ratio obtained following oral and IV administration according to Equation 5 (4).

$$F = AUC_{0\text{-inf}}(\text{Oral})/AUC_{0\text{-inf}}(\text{IV}) \times \text{IV Dose/Oral Dose} \qquad \text{(Equation 5)}$$

Total oral clearance (Cl_o) was calculated by use of Equation 6.

$$Cl_O = \text{Dose (Oral)}/AUC_{0\text{-inf}} \qquad \text{(Equation 6)}$$

Results and Discussion

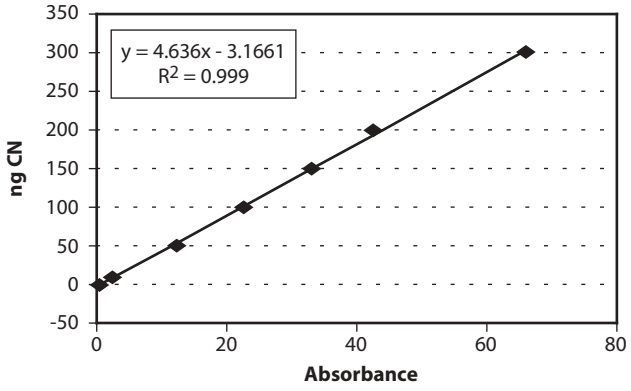
Developing a Highly Sensitive Analytical Method

Most analytical methods for cyanide are used in the forensic detection of cyanide associated with lethal intoxication and have a limit of detection in the mg/ml range. However, it was obvious that if cyanide was involved in MRLS, the concentrations found in mares would be less than those associated with clinical signs of toxicity since no signs of intoxication were noted in any mares. Therefore, an analytical method was needed for cyanide detection in the subtoxic range (ng/ml). Such a method was developed in our laboratory and has been described previously (6). Figure 2 shows the standard curve of that method for the quantification of cyanide in biological fluids.

Defining "Normal" Blood Cyanide in Horses at Pasture in Kentucky

The first step in this project was to define what constitutes a "normal" blood cyanide for a mare at pasture in Central Kentucky. Figure 3 shows that the distribution of blood cyanide concentrations in horses at pasture in Kentucky averaged 8.5 ng/ml in the fall of 2001 (n = 48) and 3.2 ng/ml in the spring of 2002 (n = 100). The blood cyanide concentrations apparently follow log normal distributions, and the concentrations appeared to differ from field to field and also between fall and spring. These blood

Figure 2. Standard curve for the quantification of cyanide in biological fluids.



cyanide concentrations are very low, and it should be kept in mind that cyanide is substantially concentrated in blood. Free blood cyanide and presumably free tissue concentrations of cyanide in horses at pasture in Kentucky are likely about 1/50 of these concentrations (7).

Defining "Toxic" Blood Concentrations of Cyanide in the Horse

The next step was to define the blood concentrations of cyanide likely to be acutely toxic in the horse. To this end, horses were infused with increasing concentrations of cyanide, starting at 1 mg/minute and increasing to 12 mg/minute. Figure 4a shows the blood cyanide concentrations attained following infusion of 12 mg NaCN/minute, the dose that produced the first signs of toxicity. As shown in Figure 4b, there was a sharp increase in heart rate at the point of toxicity during the 12 mg/minute infusion, when the heart rate peaked at 150 beats per minute (bpm). Other signs of toxicity were sweating, rapid breathing, and apparent anxiety.

When clinical signs of toxicity appeared, the infusion was immediately stopped. Thereafter, the heart rate returned to normal, and the horse quickly returned to normal behavior without administration of an antidote. In earlier experiments, cyanide toxicity would sometimes cause the horse to weaken and drop to the ground. These clinical signs were always rapidly reversed following intravenous infusion of 3% sodium nitrite and 25% sodium thiosulfate.

These experiments suggested that if the experimental blood cyanide concentrations were held to less than 2,000 ng/ml, clinical signs of cyanide toxicity in the experimental horses were unlikely. Additionally, these results suggest that there is a large difference between the concentration of blood cyanide in normal horses at pasture and the concentration required for toxicity allowing for considerable experimental latitude to evaluate the fetotoxicity of cyanide in pregnant mares.

Defining the Toxicokinetics of Cyanide in the Horse

Based on the data of Figure 4, we infused four horses with NaCN at 1 mg/kg for 1 hour. Blood cyanide concentrations increased linearly to about 1,000 ng/ml at 1 hour, at which point the infusion was stopped. After infusion was stopped, there was rapid redistribution of cyanide with an alpha half-life of 0.74 hours, followed by a beta or terminal phase of metabolism, which was much slower, with a half-life of 16 hours (Figure 5). The insert shows the logarithmic plot of concentration versus time for the post-administration portion of the data. These data show clearly that the termination of action after a bolus administration of cyanide is by re-distribution and that cyanide is a classic agent for which its acute pharmacological/toxicological effects can be terminated by redistribution.

Figure 3. Distribution of blood cyanide concentrations in horses at pasture in Kentucky a) in the fall of 2001 (n = 48) and b) in the spring of 2002 (n = 100).

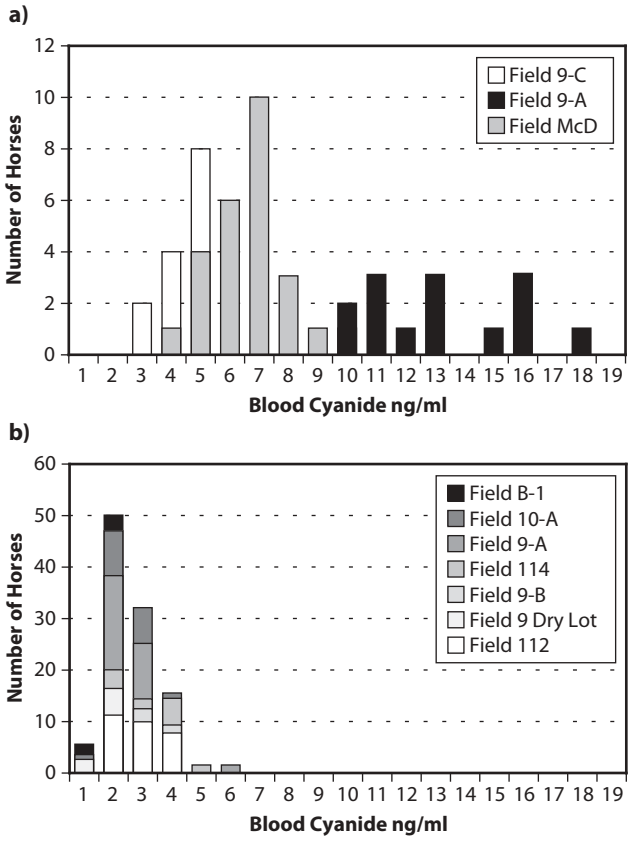


Figure 4. a) Mean blood cyanide concentrations attained following intravenous infusion of 12 mg NaCN/min, the dose that produced the first signs of toxicity; b) Heart rate of infused horse showing the point of toxicity, when the heart rate peaked at 150 bpm.

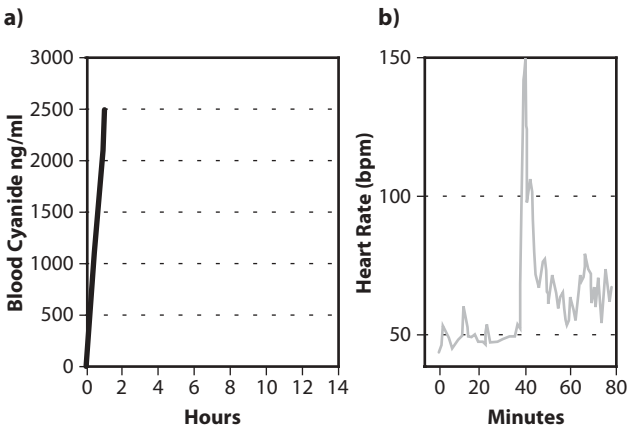
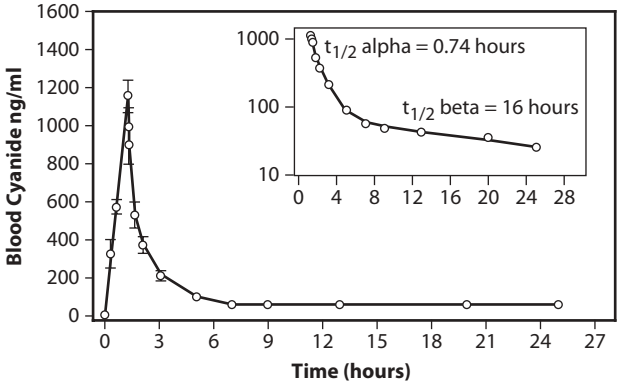


Figure 5. Blood cyanide concentrations following NaCN infusion at 1 mg/kg x 1 hour. Insert shows the logarithmic plot of concentration versus time for the post-administration portion of the data with an alpha half-life of 0.74 hours, followed by a terminal phase, with a half-life of 16 hours.

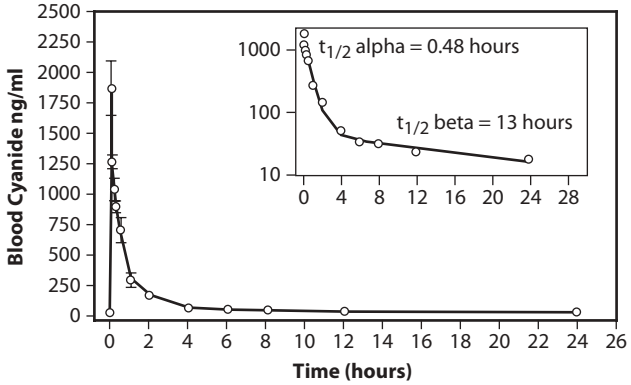


Defining the Toxicokinetics of Mandelonitrile in the Horse

Cyanide is not present as such in black cherry tree leaves but rather as prunasin and mandelonitrile, as set forth in Figure 1. Mandelonitrile is the proximate precursor in the cherry tree system for cyanide release. Therefore, to more closely mimic the possible etiology of MRLS, horses were dosed with oral mandelonitrile, and the resulting blood cyanide concentrations were measured. Figure 6 shows the mean blood cyanide concentrations following dosing with 3 mg/kg mandelonitrile (n = 4). Mandelonitrile is about 20% cyanide; therefore, horses received about 600 mg HCN orally. There was immediate release and absorption of cyanide as indicated by the peak blood cyanide concentration at 3 minutes. In an earlier ranging experiment, in which a dose of 3 g/horse of mandelonitrile was used, the test horse became weak and uncoordinated for about 30 seconds.

The insert of Figure 6 shows a semi-logarithmic plot of cyanide concentration versus time for the post-absorption portion of the data. The apparent bioavailability of cyanide from mandelonitrile was about 57%; absorption was followed by rapid redistribution of cyanide, with an alpha half-life of 0.48 hours. This phase was followed by the beta or elimination phase with a much slower half-life of about 13 hours.

Figure 6. Mean blood cyanide concentrations following bolus oral dose of 3 mg/kg mandelonitrile (n = 4). Insert shows semi-logarithmic plot of cyanide concentration versus time for the post-absorption portion of the data. Absorption was followed by rapid redistribution of cyanide, with an alpha half-life of 0.48 hours and a beta phase half-life of about 13 hours.



Attempted Reproduction of MRLS by Administration of Mandelonitrile to Pregnant Mares

We next attempted to reproduce MRLS in pregnant mares by administration of mandelonitrile. With regard to developing a suitable dosing schedule for mandelonitrile, our pharmacokinetic results showed that it would be difficult to maintain a "steady-state" plasma concentration of cyanide following intermittent oral dosing with mandelonitrile. As a best experimental approach, seven pregnant mares were dosed with mandelonitrile (2 mg/kg twice a day in applesauce) for 14 days. Peak values (taken immediately after dosing) had a mean of 315 ng/ml and varied widely (range: 215 to 594 ng/ml) as indicated by the upper curve. The trough values (taken just before dosing) had a mean of 62 ng/ml and were more consistent than peak values (Figure 7).

Throughout this experiment, no fetal losses were recorded, and no clinical signs suggestive of fetal loss were observed. These results suggested that consistent blood concentrations of cyanide of about 60 ng/ml and/or considerably higher spikes of blood cyanide can occur without associated fetal losses. As such, these results are inconsistent with and do not support the original working hypothesis that cyanide from the black cherry tree is closely associated with or a proximal cause of MRLS.

Other Factors Possibly Influencing Blood Cyanide Concentrations

As the 2002 MRLS season approached, we paid particular attention to the possible impact of pasture clover content and overnight freezing conditions on the cyanide content/bioavailability of clover cyanide and the blood concentrations of cyanide in horses grazing such pastures. Because damage from freezing temperatures has been reported to increase the cyanide content and/or its bioavailability from some clovers, James Crutchfield and his colleagues studied the cyanide content/yield of clover and the effects of morning temperature. As shown in Figure 8, morning temperature had little effect on Kentucky clover cyanide content/yield, in contrast with the reported effects of low temperatures on cyanide content/yield from

Figure 7. Peak and trough blood cyanide concentrations following oral dosing of mandelonitrile (2 mg/kg, twice a day, in applesauce) for 14 days.

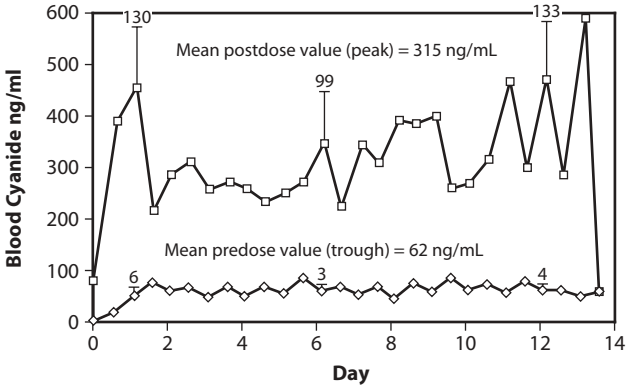
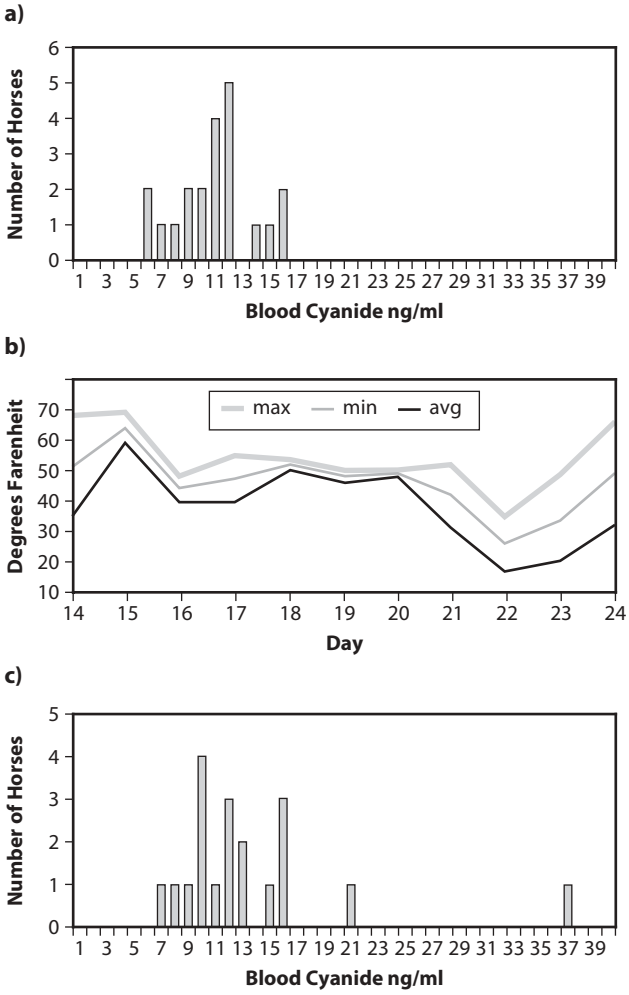


Figure 8. a) Blood cyanide concentrations in horses at pasture before a "freeze" (March 21); b) minimum, maximum, and average temperatures during mid-March; c) blood cyanide concentrations of same horses on March 22, after hard freeze.



clovers in other geographic locations. There was no significant difference between the blood cyanide concentrations before and after freezing.

Consistent with these findings, blood cyanide concentrations in horses at pasture showed little change following an overnight frost. Figure 8a shows the blood cyanide concentrations in horses at pasture before a "freeze" on March 21, and Figure 8c shows the blood cyanide concentrations of the same horses on March 22 after the hard freeze of the night of March 21. Figure 8b shows the minimum, maximum, and average temperatures during mid-March. Consistent with the results of the Crutchfield group experiments, there was no apparent difference in blood cyanides between each group of horses prior to or after the freezing night of March 21, 2002.

Field and Experimental Results from the 2002 MRLS Season

The interpretation of these experimental results is consistent with field and experimental data developed during the 2002 MRLS season. During the 2002 MRLS season, blood samples were drawn from a band of 20 pregnant mares grazing in proximity to black cherry trees. As set forth in Figure 9, the blood cyanide concentrations of these mares was not significantly different from "normal" mares shown in Figure 3. Furthermore, two of these mares (indicated with asterisks on Figure 9) had early fetal losses (EFL) consistent with MRLS, even though blood cyanide concentrations in these two mares were well within normal limits.

In cooperation with Drs. Webb and McDowell, we also monitored blood cyanide concentrations in mares undergoing ETC-induced EFL. Blood cyanide concentrations of mares after ETC exposure on May 29 were not significantly greater ($p < 0.05$) than blood cyanide concentrations before exposure on May 17 to ETC (Figure 10). Again, the results do not support suggestions that increased blood cyanide concentrations are a factor in ETC-induced fetal losses.

Figure 9. Blood cyanide concentrations of mares grazing in proximity to wild cherry trees from April 28 - May 3, 2002. Asterisks denote blood cyanide concentrations of two mares that suffered EFL during this period.

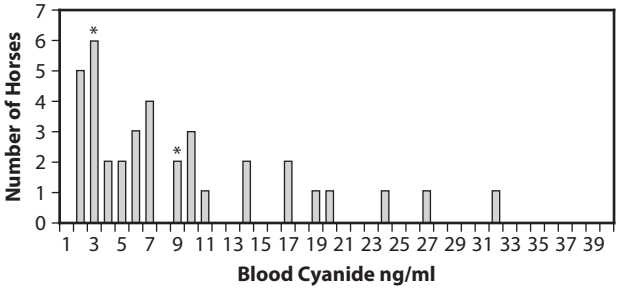
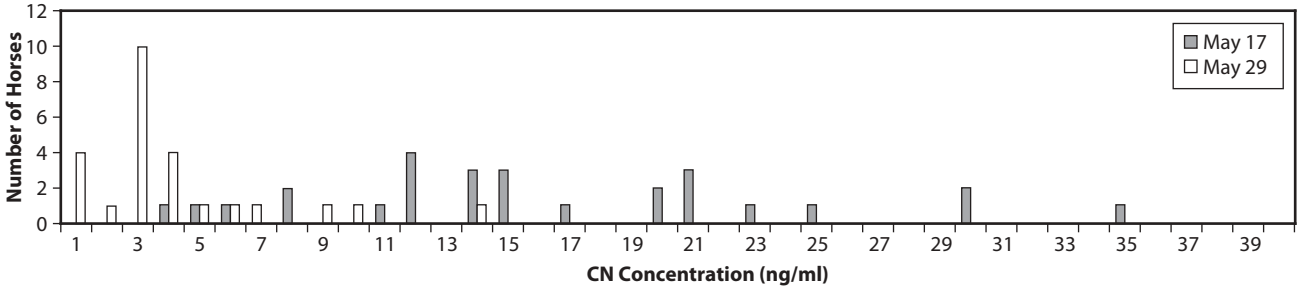


Figure 10. Blood cyanide concentrations of pregnant mares before exposure (May 17) and after exposure (May 29) to ETC.



Conclusions

Field and experimental events reported elsewhere in this workshop from the 2002 MRLS season have provided strong evidence in support of the close involvement of ETC in MRLS. On the other hand, the experimental and field work with cyanogens leading up to the 2002 MRLS season and results obtained during the 2002 MRLS season reported here make the involvement of cyanide in MRLS very much less likely.

Moreover, a study tracing the movement of cyanide from cherry trees to tent caterpillars and into the detritus pool sheds additional doubt on the possible involvement of caterpillar-borne cyanide in MRLS (8).

Simply put, we have been unable to reproduce MRLS by administration of mandelonitrile, a proximal cyanide donor, and no evidence whatsoever has been developed in support of suggestions that exposure to black cherry trees, ETC, or cyanogenic clovers was likely to increase the blood cyanide concentrations of mares to the point that such blood cyanide concentrations could be implicated in MRLS.

In further support of this conclusion, MRLS was seen to occur in mares in which blood cyanide concentrations were no different from or actually less than those seen in "normal" or "control" mares, consistent with suggestions that cyanide from ETC or any other source has not been a critical factor in MRLS as we know it.

Acknowledgments

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References

1. Dwyer, R. M.; Garber, L. P.; Traub-Dargatz, J. L.; Meade, B. J.; Powell, D. G.; Pavlick, M. P., and Kane, A. J. A case-control study of factors associated with excessive proportions of early fetal losses associated with mare reproductive loss syndrome in central Kentucky during 2001. Journal of the American Veterinary Medical Association. 2003; 222(5):613-619.
2. Hughes, C.; Lehner, A. F.; Crutchfield, J.; Woods, W. E.; Harkins, J. D.; Karpiesiuk, W.; Dirikolu, L.; Sebastian, M.; Troppmann, A.; Boyles, J.; Bosken, J.; Harrison, L., and Tobin, T. Analytical detection and "normal" population data of cyanide and thiocyanate in equine blood and serum. Toxicology Mechanisms and Methods. 2003; 13 [In Press].
3. Gibaldi, M. and Perrier, D. Pharmacokinetics. 2nd ed. New York: Marcel Dekker; 1982.
4. Benet, L. Z. and Zia-Amirhosseini, P. Basic principles of pharmacokinetics. Toxicologic Pathology. 1995; 23(2):115-123.
5. Yamaoka, K.; Nakagawa, T., and Uno, T. Statistical moments in pharmacokinetics. Journal of Pharmacokinetics and Biopharmaceutics. 1978; 6(6):547-558.
6. Martinez, M. N. Noncompartmental methods of drug characterization: statistical moment theory. Journal of the American Veterinary Medical Association. 1998; 213(7):974-980.
7. Hughes, C.; Lehner, F.; Dirikolu, L.; Harkins, D.; Boyles, J.; McDowell, K.; Tobin, T.; Crutchfield, J.; Sebastian, M.; Harrison, L., and Baskin, S. A simple and highly sensitive spectrophotometric method for the determination of cyanide in equine blood. Toxicology Mechanisms and Methods. 2003; 13 [In Press].
8. Dirikolu, L.; Sebastian, M. M.; Hughes, C.; Crutchfield, J.; Harkins, D.; Boyles, J.; Bosken, J.; Lehner, F.; Troppmann, A.; McDowell, K.; Harrison, L.; Boskin, S. I.; Tobin, T., and Fitzgerald, T. D. The toxicokinetics of cyanide and mandelonitrile in the horse and their relevance to the mare reproductive loss syndrome. Toxicology Mechanisms and Methods. 2003; 13 [In Press].
9. Fitzgerald, T. D.; Jeffers, P. M., and Mantella, D. Depletion of host-derived cyanide in the gut of the eastern tent caterpillar, *Malacosoma americanum*. Journal of Chemical Ecology. 2002; 28(2):257-268.

Summary

T. Tobin

MARE REPRODUCTIVE LOSS SYNDROME (MRLS) SEEMS TO BE strongly associated with the caterpillars. If you put the caterpillars near horses, or in horses, or, as was described here this morning, if the horses eat the caterpillars—and it seems that young horses may do more so than older horses, which may be associated with a learned response—you get problems.

On the other hand, muzzling seems to be very effective, which has been readily apparent in this 2002 season. This information has come back from the monitoring program, and we heard it again this morning from Dr. Riddle. So the bottom line is: if you can keep the caterpillars out of the mouth/intestinal tract of the horse, then we would seem to have gone a long way toward solving our problem.

When caterpillars do get into the oral cavity, what we pick up throughout the affected horses are bacteria that are apparently mouth commensals, the *Actinobacillus* and the non-hemolytic *Streptococcus* species. These bacteria, normally mouth residents, suddenly start to appear elsewhere in both pregnant and non-pregnant horses. They appear in the early and late fetal losses (EFL/LFL), and they also appear in the pericardial sac; we don't know what appears in the eye because we've not done bacteriology in the eye. Actinobacillus also appears in the brain: Drs. Sebastian and Harrison have reported three cases of Actinobacillus encephalitis occurring in or about the time of MRLS. When the caterpillars appear, something goes through *all* caterpillar-exposed horses, but it is in the pregnant mare that we see by far the most dramatic effects.

So, what happens when the caterpillars get into the horse's mouth? I like Dr. LeBlanc's analogy. Dentists working in my mouth put me on prophylactic antibiotics immediately because I have a heart murmur, and they don't want to risk a bacterial vegetative endocarditis. Likewise, something happens when horses are exposed to caterpillars in that we suddenly have oral commensal bacteria appearing shortly thereafter at multiple locations in the body. (In this regard, Dr. Sebastian has since drawn my attention to an un-referenced citation in an early edition of Blood and Henderson noting that mouth lesions in horses are associated with "hairy caterpillars" [1]).

We have been to some extent overwhelmed by the EFL and LFL—these are what has drawn attention to this whole problem—but there are also related things going on at a much lower rate in all Central Kentucky horses exposed to caterpillars.

Dr. Bernard isn't here, but I understand that he has shown that if you take a caterpillar and separate the exterior from the interior, the fetal losses appear to be associated with the integument, the outside of the caterpillar. This and other considerations drove the first mouse setal experiments that Dr. Sebastian has described to you. The setal hypothesis started with the thought that perhaps there was a toxin associated with the setae. Then we backed up and said that perhaps it's simply the setae themselves facilitating the movement of bacteria into the blood. We wondered about the setae themselves becoming little septic emboli in the body and carrying little quanta of infected material to various locations in the body. The infected material would be contaminated with bacterial commensals from the point at which the setal fragments entered the body of the horse. In MRLS cases occurring in the field, these would be the mouth commensals, the *Actinobacillus* and the non-hemolytic *Streptococcus* species. In experimental MRLS, where we delivered the caterpillars into the stomach by nasogastric tube, the bacterial picture is different, apparently consistent with the different point of entry for the bacteria.

Tissue localization of such septic emboli would not cause significant problems in most areas of the body, where the immune system can handle it, but some areas of the body may be particularly susceptible, such as the fetal membranes, and perhaps the eye, where the results of such effects are easily visible, and also the pericardial sac.

So, let me just simply say: Do we need a toxin? Well, we don't have a candidate toxin. Dr. Whitwell very kindly asked the toxicologists here to nominate a toxin, and one wasn't forthcoming. My sense at this time is that we need to look carefully at the link between the bacterial commensals in the mouth and how the outside of the caterpillars (and those barbed setae) may facilitate distribution of mouth commensals to distant locations in the body. At this point, I am far from persuaded that there's a classic toxicity mechanism involved, and I am a toxicologist, more or less, by training.

Reference

1. Blood, D. C.; Radostits, O. M., and Henderson, J. A. Veterinary medicine: a textbook of the diseases of cattle, sheep, pigs, goats and horses. 6th ed. London: Bailliere Tindall; 1983, p. 780.

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