Session 4

Potential Candidates for the Eastern Tent Caterpillar Mare Reproductive Loss Syndrome-Inducing Agent

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Placental Toxicology: Recognized Placental Toxicants in Veterinary Medicine and Consideration of a Likely Role of a Placental Toxicant in Mare Reproductive Loss Syndrome

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AS THE PHYSICAL AND FUNCTIONAL INTERFACE BETWEEN THE conceptus and its maternal host, the placenta must fulfill many critical tasks associated with transfer and catabolism supporting fetal nutrition, portal or excretion of fetal waste, partner in the endocrine cross talk with maternal systems, and barrier to immunological rejection and invading microorganisms. Given the anatomic and functional complexities of these tissues, there is great potential for disruption by toxicants at many different points. This overview will briefly address some basic aspects of placental toxicology, discuss several toxic conditions of domestic animals recognized to have placental involvement, and examine the evidence that placental toxicity is a component of Mare Reproductive Loss Syndrome (MRLS).

Interest in the toxic effects of chemicals on placental function has been centered in two areas: drug safety testing by pharmaceutical companies and concern about human health and fetotoxicity from exposure to chemicals either through the environment or through the food supply (1-9). In regard to the latter, the most immediate concern relates to the toxic effects of cigarette smoking on the developing fetus. To a lesser degree, human placenta damage in pregnant women who abuse drugs such as cocaine and morphine is also of concern. There is strong evidence of a direct toxic effect from cigarette smoking on the human placenta (1-9). Tobacco smoking retards fetal growth, with babies of smokers being an average of 200 g lighter than normal newborns. Changes in uterine blood flow and placental development occur, and they are associated with abnormal placental vascular growth in women who smoke (1).

Pharmaceutical companies have expanded their drug safety testing programs to include more detailed study of chemicals on different reproductive processes. "Developmental toxicology" has become an important component in drug safety testing with determination of effects on reproductive cyclicity, conception rates, and embryonic and fetal losses. Second and third generations of progeny from animals dosed are also examined for reproductive performance. Until relatively recently, drug effects on placental tissues were not specifically separated from effects on the fetus, thus leaving a void in our basic understanding of both the importance of and mechanisms responsible for placental toxicity. Greater attention is now being given to placental pathology in drug testing.

Toxins and the Placenta

Toxin insult to placental function can potentially take many forms. Key biological components of placental development and function susceptible to toxic insult include altered placental development, direct cytotoxic effects on maternal and fetal placental tissue, inducers of apoptotic cell death, alternation to cell-cell adhesion, endocrine disrupters, vasoactive effects on either the maternal or fetal cardiovascular system, altered placental responsiveness to normal physiologic demands (altered homeostasis), and immune modulators and loss of immune modulation enabling maternal rejection.

Historically, the placenta was thought of as being a protective barrier, preventing passage of noxious substances to the fetus, preventing maternal rejection, and limiting any invading microorganisms from passing to infect the embryo or fetus. More recent medical evidence, however, suggests that most compounds can and do cross the placenta; some do so only at a very low rate, others more quickly. It also appears that most toxic materials that do pass through the placenta do so by simple diffusion (with the exception of some antimetabolites that are actively transported) (2).

The lipid solubility of a compound is an important characteristic with enhanced movement of chemical compounds that are more highly lipid soluble. Another important aspect of placental toxicity is that there are many enzyme systems (P450 systems, etc.) that can act on relatively inert chemical compounds resulting in substrates that are toxic ("biotransformation"). Less commonly, some compounds can directly affect placental tissues. One example of this is the production of frank placental necrosis that occurs in cadmium toxicity in rodents (10).

The toxicity of a chemical compound is dependent on the dose, susceptibility of the animal, presence of placental enzyme systems involved in either biotransformation (chemical alteration that occurs in the animal) or transport across the placenta, and stage of pregnancy. The interested reader is referred to the extensive literature on reproductive toxicology (2-9).

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Toxicities in Veterinary Medicine

There are three plant-associated toxic conditions affecting cattle, sheep, or horses that have been recognized for many years (fescue toxicity, pine needle abortion, and locoweed toxicity). Each of these is associated with pregnancy failure, and, for each, there is evidence that placental function is compromised. Fescue toxicosis in horses is caused by an endophytic fungus infection of tall fescue grasses. Cattle that ingest pine needles may abort or give birth to underdeveloped calves, and cattle and sheep that ingest locoweed (*Astragalus* and *Oxytropis* spp.) are also known to have delayed placentation, hydropys amnion, and abnormal placental development (11). The first two conditions will be discussed in greater detail.

Fescue Toxicosis

Approximately 700,000 horses in the United States are maintained on tall fescue (*Festuca arundinacea*) and therefore at risk to toxins from endophytes that grow on these grasses (12). Tall fescue is planted on approximately 35 million acres in the United States and Canada, and the direct effects on pregnancy are fairly well documented. These include a syndrome including agalactia, prolonged gestation, abortion, thickened placentas, and foal losses (12). In an early study by Garrett et al. (13), they reported relative incidence of 38% prolonged gestation, 18% abortion, and 9% thickened placentas in mares with fescue toxicosis. Fescue toxicosis is associated with an endophytic fungus infection named *Neotyphodium coenophialum* (14). Multiple toxins are produced by the endophyte, and these toxins include peramines, lolines, and ergopeptine alkaloids (12). Of these, it appears that the lolines and ergopeptine alkaloids have the most effect on the mammalian systems. Lolines have been shown to have vasoconstrictive effects on the vascular system in cattle and horses, and the ergopeptine alkaloids are agonists of the dopamine D2 receptors. The relative importance of dopamine in the reproductive aspects is to inhibit prolactin. Decreased prolactin concentrations are a major functional aspect of the pathogenesis of the agalactia and decreased mammary gland development noted in mares.

Another effect of exposure to products of these toxins from this endophyte is on the normal production by the placenta of progesterone that becomes depressed during the last 30 or 40 days of gestation. It is postulated that the reason for this is lower placental production of progesterone caused by ergot alkaloids that inhibit adrenocorticotropic hormone (ACTH) secretion in the fetus. ACTH secretion is important in controlling placental function. Progesterone normally acts in concert with prolactin and is also directly involved in mammary gland growth, development, and lactation. Interference with ACTH release is felt to be partially responsible for the failure of appropriate signaling for parturition. As noted above, prolonged gestation is a feature of fescue toxicosis.

Further effects of fescue toxicity on placental function are thought to be related to vasoconstriction (15-17). This is especially important given the very nature of the fetal and maternal placenta, of being an extensive vascular interface between them. Placental lesions that have been reported in fescue toxicity include edema, fibrosis, and mucoid degeneration of arteries (17). The incidence of retained placentas is 62% in mares consuming endophyte-positive fescue pasture grasses. This is in contrast to 12.5% for mares on fescue pastures that are endophyte negative. Clearly the toxic factors released by the endophyte have dramatic broad effects on placentation, and a study by Loch et al. (18) has shown that the fetus is also affected. Wet weights and dry fat-free weights of ponies fed different amounts of endophyte fescue seeds demonstrated that tissue dry fat-free weights increased with increasing percentages of feed in the diet. Fetal development is not only compromised, but fetal growth is altered.

Many of the clinical signs can be reversed or ameliorated by administration of domperidone, a dopamine receptor antagonist (19). A recent study by Ryan et al. (20) demonstrated that mares experimentally fed fescue contaminated with endophytes also had significant reduction in the circulating levels of relaxin.

Pine Needle Abortions

It has been recognized since the early 1950s that consumption of ponderosa pine needles by pregnant cattle may result in abortion or birth of small, weak calves and placental retention (21). Isocupressic acid isolated from ponderosa pine (*Pinus ponderosa* Laws) needles when administered to pregnant cattle induced abortions (21), and experimental studies have demonstrated that ingestion of these pine needles causes increased tone in caruncular arteries of the uterus with associated reduction in blood flow to the uterus (22).

There is little information in the literature about abortion caused by exposure to insects. Only rarely has pregnancy failure been reported to be associated with exposure to insects, and these are case reports. A pregnant woman in South America who contacted Lonomia caterpillars and went into renal failure also experienced premature labor and delivered a live baby (23). Crude extracts of cantharides are taken as part of Chinese medicine to induce abortion (24). The completion of trials to reproduce the MRLS under experimental conditions has demonstrated that ingestion of eastern tent caterpillars (ETC) leads to abortions. These studies have been critically important first steps. It will now be important to learn if there is a dose-dependent effect and, if so, if feeding of higher doses might be expected to produce more

dramatic fetal or placental lesions that could focus our attention on why and how the MRLS abortions occur.

Examination of Evidence for Placental Toxicity in MRLS

One might seek clues regarding pathogenesis of abortions in MRLS from either clinical observations or lesions found in maternal or fetal tissues. Although the syndrome is slowly becoming more precisely defined, especially now that it has been experimentally reproduced, there are still large gaps in our knowledge. Fetal losses have been reported to occur either early or late in gestation, yet given the short window of exposure and the tight breeding season, the sensitivity of the mid-gestation mare to abortions is not really known. Relatively little pathologic information is available from material from early gestation because these fetuses die so quickly and are absorbed or are autolyzed. Similarly, pathology findings reported by N. M. Williams et al. (this proceedings) are complicated by the co-existence of bacterial infection in many of the cases; more than 85% of these cases are so affected. What is the evidence that damage to the placenta is of importance in the pathogenesis of MRLS abortions?

The very high incidence of bacterial infections and the fact that the bacteria commonly isolated are not common abortifacient pathogens of the mare suggest that the placenta as a functional barrier has been damaged.

Hemorrhage and placental edema are observed in a large number of MRLS cases. The degree varies between cases, and the edema is not as severe as that found in fescue toxicity. One mechanism for the development of edema is alteration in endothelial cell permeability. This can either be the results of endothelial cell damage or cell death (apoptosis or necrosis). Severe edema can lead to placental separation. An important feature was the premature separation of the placenta ("red bag"). The pathogenesis of the placental separation has not been studied, and given the inconsistency of its association with significant edema of the chorioallantois, toxic damage to the trophoblastic cells is a consideration.

Ultrasound findings reported by T. W. Riddle (this proceedings) of echogenic allantois and to a lesser degree amniotic fluid suggests direct damage to the allantoic and amniotic epithelium and possibly the skin of these early fetuses. These changes, along with sudden fetal death, were considered the earliest and most diagnostic features of MRLS abortion in early pregnancy. Further evidence of placental involvement reflected in effects on fetal growth have been alluded to in a paper by Pantaleon et al. (to be published). Fetal femoral growth in fetuses in mares "exposed to MRLS" as measured *in utero* by ultrasound were found to be 2.5 to 7 days delayed when compared to fetuses of unexposed mares. This *in utero* growth retardation might be due, at least in part, to compromised placental function.

The rapidity of fetal demise, presence of hemorrhage, and edema suggest primary placental vascular damage in MRLS. Investigations to date have been limited by the nature of tissue available for investigation. The quality of material available for careful examination needs to be very good to detect such changes, and morphologic evidence may come only after pregnant mares are killed to enable collection of appropriate material.

More precise information about the impact of toxic compounds (from ETC) might require studies using chronically instrumented equine fetuses. Although mouse studies have begun, with some limited success, the standard model to study fetal/placental pathophysiology is the fetal lamb. Given the expense and complexity of conducting trials in pregnant mares, initiation of trials in pregnant ewes may be advised. Although the llama and pig have placentas anatomically more similar to that of the horse, there is significantly less information available about their normal fetal physiology or placental function. There is an extensive body of information available for the ovine fetus. Laboratories that use chronic instrumentation of the pregnant ewes are established in many research settings around the world. It would seem prudent to initiate either feeding or direct injection studies in pregnant sheep to determine if abortions are induced and, if so, then to plan studies using instrumented animals to define the pathogenesis of the abortion process. Disruption of the complex endocrine systems of pregnancy is also another possible pathogenetic mechanism responsible for pregnancy failure in MRLS, and chronic studies of fetuses *in utero* may be needed to characterize such an effect.

Clearly, additional animal studies should be undertaken, perhaps in those species for which there can be more in-depth evaluation of effects of fractionated compounds from ETC on the fetus and placenta.

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An Overview of Fetotoxic Agents and Their Possible Role as Agents in Mare Reproductive Loss Syndrome

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THE OBJECTIVE IS TO PROVIDE A FOCUSED OVERVIEW OF reproductive and fetal toxicology, and that can be done only by painting very broad brush strokes on this subject matter. Reproductive loss due to toxicologic agents is a very complex topic because reproduction is a dynamic and complex process, and there are multiple sites that are susceptible to toxicant modification or damage. Historically, with few notable exceptions, there has been less attention paid to reproductive toxicology than to other target organ toxicology. There is a large volume of information about teratogens, but beyond these toxins that affect fetal development, relatively little is known and what is known is problematic because research must identify the multiple factors that go into a toxic effect. Here we are interested primarily in two specific reproductive toxicants, cyanide and ergopeptides, that have been investigated as possible etiologic agents in Mare Reproductive Loss Syndrome (MRLS).

Over the past several years, there has been increased interest in feto- and reproductive toxicology with concern about environmental endocrine disrupters. Much current investigation is focused on the mechanism of action of toxins that affect the endocrine system. A great amount of valuable information will probably be available in the next five to six years on this subject.

In addition to interest in the relationship between cyanide or ergopeptides and MRLS, it would be useful to examine the scientific literature for potentially toxic compounds in caterpillars. While cyanide has been shown to be present in the eastern tent caterpillar (ETC) feeding on cherry trees (1), other biologically active agents might be present that would be worth consideration. Two caterpillar genera, *Lonomia* spp. and *Lagoa* sp., have toxic substances that affect humans (2,3). However, these two genera apparently do not cause clinical problems in horses.

Reproductive toxicology is defined as the impact or potential impact of a toxicant(s), be they natural or synthetic, at any point in this process of reproduction. The impact of the toxicant can be on the sperm or the egg, the process of fertilization, implantation, embryo development, fetal development, and, after parturition, the neonate. Some effects caused by toxins are not evident until maturity. A good example of delayed effect is the exposure of expectant mothers to diethylstilbesterol (DES) in the 1950s and 1960s. Toxic effects of DES in the offspring were not observed until this population of exposed individuals (females) reached sexual maturity, about 18 to 20 years after birth.

Fetal toxicology is focused more narrowly. The reader will not find a precise definition of fetal toxicology in toxicology textbooks. However, fetal toxicology is the study of the effects of chemicals on embryos after implantation and on fetuses during development, maturation, and parturition. Fetal damage can be direct, or it can be indirect from maternal or placental effects. Direct effects require the passage of the toxicant from the gastrointestinal tract of the mother and through the placenta with subsequent distribution to target sites within the fetus. The placenta is not a significant barrier to the influx of many toxicants that could affect the fetus. The concept of the placenta as a barrier to toxicants is more related to the physical chemical aspects of the toxicant than to any true barrier that is provided by the placenta. Direct effects can be due to the parent toxicant or to a biologically active metabolite.

There are many confounding factors that need to be considered with either direct or indirect fetotoxicity, including maternal susceptibility factors such as maternal age, metabolic state, disease state, ongoing stress, nutritional state, parity, and concurrent exposure to more than one agent. Maternal susceptibility factors can lead to several important and different alterations: anemia, toxemia, endocrine imbalance, nutritional deficits, electrolyte and acid-base disturbances, decreased uterine blood flow, altered organ function, and decreased milk production. Diabetes mellitus is a problem with regard to fetal toxicity, and the physiology of the diabetic mother can have a negative impact on the fetus. Certainly conditions like stress and disease state are important parameters. Maternal hyperthermia can cause very significant teratogenic effects in offspring. Placental toxicity can lead to placental insufficiency: reduced size, reduced blood flow, altered transport, and altered metabolism.

Fetotoxicity can result from a combination of both direct and indirect effects; the ergopeptides are good examples of direct and indirect fetotoxicants. Cyanide might be an example of a direct acting fetotoxicant. However, clinical evidence is lacking.

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There are a number of compounds that are associated with abortions (4). These include plant chemicals such as isocupressic acid (from *Pinus* spp.), swainsonine (*Astragalus* spp., *Oxytropis* spp., *Swainsona* spp.), mimosine (Leucaena leucocephala) and phytoestrogens, mycotoxins such as ergot alkaloids, perloline, peramine, diacetoxyscirpenol (DAS) and ochratoxins, nitrate/nitrites, excess protein/urea, carbon monoxide, glucocorticoids, dioxins, lead, phenothiazine, prostaglandins, oxytocin, organochlorines such as dichloro-diphenyl-trichloro-ethane (DDT), and coumarin. Ingestion of several plants has been associated with abortions, but no toxicant has been identified (*Gutierrezia* spp., *Vicia villosa,* and *Juniperus* spp.).

Additionally, there are a number of other xenobiotics that are known teratogens. However, teratogenity has not been identified in association with MRLS. Therefore, known teratogens are probably not associated with the syndrome.

As mentioned, mycotoxins are associated with abortions in farm animals, and ergot peptide alkaloids of fescue prolong gestation of mares and cause birth of dysmature foals. Abortion associated with mares pastured by fescue has been reported in at least one study. Some of the other mycotoxins like DAS or ochratoxin are not commonly found in North America. It is unlikely that these mycotoxins would be the cause of MRLS in Kentucky.

A rather extensive study of causes of abortion, stillbirths, and neonatal deaths in Kentucky horses was published in the early 1990s (5). While infectious etiologies were the most common causes, a significant percentage (16%) were undiagnosed. Although it is not possible to say with certainty, some of these could be due to toxicants. In addition, other etiologic categories such as placental edema, contracted foal syndrome, and congenital abnormalities could conceivably be due to toxicant exposure.

At least one hypothesis attributes MRLS to exposure to cyanide. Certainly there is interest in this hypothesis because it has as its basis the relationship of ETC to the cyanogenic glycosides in the cherry tree and other various cyanogenic plants. The acute toxicity of cyanide/cyanogens has been investigated for a long time. There are a number of potential sources of exposure to cyanide, such as industrial processes such as electroplating, photographic processing, combustion, cigarette smoke (as a source of low-level cyanide exposure), and plant cyanogenic compounds, for example, found in *Prunus* spp. In addition, it is possible that exposure to chemicals such as acetonitrile and sodium nitroprusside can result in cyanide exposure following metabolism of the parent compound.

Cyanide is rapidly absorbed via multiple routes and is widely distributed to target organs. Cyanide readily crosses the placenta. Elimination is via first-order kinetics with variable half-lives being reported. Cyanide is highly toxic; however, it certainly is not one of the most toxic compounds. Cyanide toxicity depends on the form. For example, the LD_{50} for potassium cyanide (KCN) is 200 mg/kg body weight, which places KCN in the range of a moderately toxic compound.

Cyanide has a very specific mechanism of toxic action. It inhibits a number of enzymes, but clinical toxicosis is due to inhibition of cytochrome oxidase, which is very important for oxidative phosphorylation and energy production by cells. Electron transport is inhibited, oxygen cannot be utilized, and adenosine triphosphate (ATP) cannot be produced; so the cells that are impacted by cyanide become hypoxic. The central nervous system (CNS) is the primary target organ because the cells in the CNS are so energy-dependent. Oxidative stress and enhanced release of excitatory neurotransmitters may also contribute to CNS damage. Dopaminergic neurons in the basal ganglia are apparently the most sensitive cells in the brain and thereby are readily affected by cyanide.

Detoxification is a fairly simple process; it requires sulfur donors. The free cyanide is converted to thiocyanate by an enzyme known as rhodanese. Thiocyanate is produced and eliminated via the urine. This is a very efficient process, and free cyanide is rapidly detoxified unless exposure saturates the detoxification system. Thiocyanate is a good marker of cyanide exposure, and measurement of this metabolite may be an essential assay if further research into MRLS involves experiments with cyanide/cyanogens.

The availability of sulfur donors is a limiting factor in CNS detoxification, and it may be that the fetus has a relative deficiency in the sulfur donors compared to the mother. Therefore, the fetus might be more susceptible to cyanide intoxication. However, one critical question that needs to be asked is whether cyanide can cause fetotoxicity in the absence of toxic effects in the mother.

In looking at the literature, there has been concern about the administration of sodium nitroprusside to pregnant women for essential hypertension. Sodium nitroprusside is a good vasodilator; however, during metabolism, cyanide is released potentially causing fetal hypoxia. This concern has been evaluated using a pregnant sheep model (6,7,8,9). In these studies, the fetus was externalized and catheterized. Maternal and fetal blood cyanide levels were measured. Results suggest that fetotoxicity in the absence of maternal toxicity is highly unlikely.

This argues against cyanide or cyanogenic compounds causing equine fetal death and abortion without clinical signs exhibited by the mares.

Ergot alkaloids can be categorized as endocrine disrupters. Endocrine disrupters are exogenous agents that interfere with the production, release, transport, metabolism, binding, action, or elimination of natural hormones responsible for the maintenance of homeostasis and the regulation of developmental processes. These can be ei-

ther agonists or antagonists. Endocrine disrupters are chemically diverse and include pesticides, phytoestrogens, plasticizers, organometals, and polyaromatic hydrocarbons. Most investigations done with known endocrine disrupters have focused on estrogenic agents, androgenic agents, and compounds that affect the thyroid.

Ergot alkaloids, especially ergovaline, have been associated with reproductive effects in pregnant mares. This alkaloid, along with several other chemicals, is found in tall fescue grass *(Festuca arundinacaea*) and is produced by an endophytic fungus of the grass called *Neotyphodium coenophialum*. Ergopeptides cause both direct and indirect fetotoxicity. Indirect actions include dopamine receptor agonism, causing a decrease in circulating prolactin, and vasoconstriction resulting in placenta insufficiency. Direct actions on the fetus include inhibition of fetal adrenocorticotropic hormone secretion (ACTH) and possibly blockage of corticotrophin-releasing hormone (CRT). There is some thought that implantation of the fertilized ovum in the endometrium can be negatively impacted (10). The effects of the ergot alkaloids on equine reproduction have been studied for a number of years. The MRLS in Kentucky is quite different from that of fescue-associated impairment, suggesting that ergot alkaloids are not responsible for the syndrome.

Several "toxic" caterpillars have been identified throughout the world. Here in the United States, one caterpillar known as the "pus caterpillar" is present in Oklahoma (3). This caterpillar has venomous hairs that cause an acute local reaction, but no primary systemic effects have been reported. A species of caterpillar found in South America, the *Lonomia* species, has a real potpourri of toxic material in them that results in fairly severe chemical reactions in people and causes various coagulopathic effects (2).

There is at least one good example of how some insects utilize plant chemicals for defensive purposes. Monarch butterfly caterpillars feed on milkweed absorbing the cardioactive glycosides that are in milkweed. When birds feed on Monarch butterfly caterpillars, they become sick and regurgitate the insect. The toxic effect of the cardioactive glycoside brings about learned deterrence so that birds stop eating the caterpillars. Since ETC feed on the leaves of *Prunus* species, especially black cherry trees, these insects may contain cyanogenic glycosides. Another compound that is likely to be present in ETC is benzaldehyde. However, this compound has relatively low toxicity and would probably be unlikely to cause a problem. How ETC could cause MRLS is not defined by available information.

Because pericarditis and uveitis, perhaps due to an immunogenic stimulus, are probably components of MRLS, a brief search of cardioactive agents was done. However, none have been shown to be immunogenic. Although cardioactive agents are associated with visual abnormalities in addition to effects on the cardiovascular system, the lack of immunogenic effect would appear to rule out these types of compounds.

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A Laboratory Animal Model of Mare Reproductive Loss Syndrome: Preliminary Evaluation of a Mouse Model

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DURING 2001, CENTRAL KENTUCKY HAD AN EPIDEMIC OF early and late fetal losses (EFL/LFL) that was together called Mare Reproductive Loss Syndrome (MRLS). The LFL began in the last week of April, peaked on May 5, and declined rapidly. EFL was identified on April 26 and had a similar course and ultimately totaled about 1,500 cases (1). The same syndrome was repeated in May and June of 2002 with fewer losses. Concurrent with each epidemic was a local population explosion of eastern tent caterpillars (ETC), *Malacosoma americanum*, with large numbers wandering in pastures. Epidemiological studies, period of occurrence, and experiments conducted during the last year strongly suggest that exposure to ETC plays an important role in this syndrome.

Because of the size, long gestation period, and expense of pregnant mares, a laboratory animal was needed for study of this syndrome. It was hoped that abortions could be induced in mice by exposing them to the products of ETC. A study was undertaken to determine if ETC, frass (droppings of caterpillars), and setae (hairs) administered through different routes could produce abortion and, if so, to study the toxicopathologic effect of ETC.

Materials and Methods

Four challenge experiments with pregnant mice (ICR, Taconic Labs, Germantown, NY) were performed with ETC, frass, and setae administered by various routes. Caterpillars and frass were weighed and mixed in normal saline with a mortar and pestle. The solutions were transferred to a tissue homogenizer and finely homogenized with the volume adjusted to 0.5 ml/mouse. The homogenates were administered by gavage using a ball-tipped needle (Perfekturn, Popper & Sons Inc., New Hyde Park, NY). Setae (15 setae/mouse) were plucked from the skin of caterpillars and homogenized in a tissue homogenizer with normal saline, and the volumes were adjusted to 0.4 ml/mouse. All materials were prepared fresh daily for administration.

In Experiment 1, three groups of mice (12 days pregnant) were administered frass (19 mg; n = 9) that had been frozen, early instar ETC (70 mg; n = 9) that had been frozen, and saline (0.5 ml; n = 8) by oral gavage. The experiment was terminated on day 19 of pregnancy when the mice began to give birth. All mice and pups were euthanized, and complete necropsies were performed.

In Experiment 2, fresh frass and ETC were used. Three groups of mice (12 days pregnant) were administered fresh frass (19 mg; n = 7), late instar ETC (200 mg; n = 7), and saline (0.5 ml; n = 7) by oral gavage. The experiment was terminated on day 18 of pregnancy. All animals were euthanized, and full necropsies were performed.

In Experiment 3, two control groups of mice (5 days pregnant) were administered saline by oral gavage (0.5 ml; n = 4) and saline by intraperitoneal (IP) injection (0.4 ml; n = 4), and three treatment groups (5 days pregnant) were dosed for 14 days with one of the following: fresh frass (19 mg; n = 7) by gavage, late instar ETC (200 mg; n = 8) fed on fresh cherry tree leaves by gavage, or setae plucked from live late instar caterpillars filtered through a bacterial filter (VWR Scientific Products, West Chester, PA) by IP injection (20 setae/mouse; n = 7). The reason for injecting setae was to evaluate the possible role of a soluble setal toxin in MRLS. However, because of a personnel change occurring 4 days into this experiment, all later setal homogenate injections were unfiltered. This experiment can therefore be interpreted only in terms of a setal homogenate effect. The experiment was terminated on the nineteenth day of pregnancy. All animals were euthanized, and full necropsies were done.

In Experiment 4, three groups of mice (5 days pregnant) were treated by IP injection for 14 days. Groups were administered one of the following treatments: saline (0.4 ml; n = 7) to a control group, a filtered homogenate/extract (20 setae/mouse; n = 7), and an unfiltered homogenate/extract (20 setae/mouse; n = 7). Setae were plucked from frozen late instar caterpillars, and the homogenate was filtered through a bacterial filter. The experiment was terminated by euthanasia on the eighteenth day of pregnancy, and full necropsies were performed. Both filtered and unfiltered homogenates were injected to distinguish between a setal toxin (filtered homogenate) and mechanical irritation or bacterial-laden setae disrupting the fetal membranes (unfiltered homogenate).

Results

The gross and histopathological findings of the four experiments are detailed in Tables 1 through 4. In Experiment 1, there were no significant findings in the uteri of

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the mice that died during the experiment. Furthermore, there were no significant findings in the uteri and fetuses of any of the mice in treatment and control groups. The total number of pups born for the treatment and control group mice are shown in Table 1.

In Experiment 2, the uterus of the mouse that reabsorbed one fetus had acute suppurative inflammation at the implantation site. There were no significant findings in the uteri (implantation sites) and fetuses of any mice in the treatment and control groups. The total number of pups in the uteri of treatment and control mice are shown in Table 2.

In Experiment 3, the group administered setae had statistically significant reabsorptions of all fetuses in three mice, and two mice were not pregnant. The uteri of the mice that had reabsorptions had acute suppurative inflammation at the implantation sites. The total number of pups in the uteri of treatment and control mice and the histopathological findings in the uteri of setae-administered mice are shown in Table 3.

In Experiment 4, two fetuses were reabsorbed in the unfiltered setae group, eight fetuses were reabsorbed in the filtered group, and four fetuses were reabsorbed in the control group. The uteri of the mice that had reabsorption showed acute suppurative inflammation at the placental sites. The total number of pups in the uteri of the treatment and control group mice are shown in Table 4. The bacteria isolated from the uteri of mice in Experiment 1, 3, and 4 are detailed in Table 5.

Table 1. Number of pups for each mouse in Experiment 1. Group 1 was administered caterpillar extract (early instar), Group 2 was administered frass, and Group 3 (control) was administered normal saline.

Animal ID	No. of Pups	Comment
Grp1-1	14	DEAD - during experiment
Grp1-2	5	
Grp1-3		No pups born when euthanized - 10 at necropsy
Grp1-4	13	DEAD - during experiment
Grp1-5	11	
Grp1-6	14	
Grp1-7	12	
Grp1-8	14	
Grp1-9	12	one not active
Grp2-1	7	pups not active
Grp2-2	13	DEAD - during experiment
Grp2-3	11	
Grp2-4	12	
Grp2-5	11	DEAD - during experiment
Grp2-6		No pups born when euthanized - 11 at necropsy
Grp2-7	12	
Grp2-8	14	
Grp2-9	11	
Grp3-1	13	
Grp3-2	10	
Grp3-3	13	
Grp3-4	9	
Grp3-5	11	
Grp3-6	14	DEAD - during experiment
Grp3-7	10	
Grp3-8	12	

Discussion

In Experiment 3 (setae from live ETC), three of the five pregnant mice had reabsorption of their fetuses. The same experiment, when repeated with frozen setae (Experiment 4), did not produce reabsorption as observed in Experiment 3. The structure and composition of setae, whether it changes by freezing and subsequent thawing, are not known. Normally 10 to 20% of reabsorption is noticed in the uteri of pregnant mice. Reabsorption in the control groups of Experiments 2, 3, and 4 was not above 10%, and no significant pathological changes were observed. In Experiments 1, 2, and 4, the percentage of reabsorptions was also below 10% in the treatment groups.

It was hoped that the mouse would mimic MRLS seen in horses so that a laboratory animal model could be used to more thoroughly investigate MRLS. Horses aborted following oral dosing with 50 g ETC to a 500-kg horse (0.1 g/kg). The mice did not abort following oral dosing with 200 mg ETC to a 20-g mouse (10 g/kg). So even though mice received a 100-fold increase in ETC per body weight, they were not susceptible to abortion. The reason(s) for the difference(s) in susceptibility of the two animals could be different intestinal enzymes and/or flora, different placentation, or longer gastrointestinal tract in the horse increasing the possibility of intestinal absorption. The intestinal tract appears to be involved because, when it was bypassed with IP injections of setal homogenate/extract from live ETC, mouse reabsorptions did occur in three of the five mice.

There also appears to be a factor associated with setae from live ETC versus frozen ETC. Since Experiment 4 was run after the supply of live ETC had been exhausted, setae were taken from frozen ETC. There were no effects from IP injection of setal extract in that experiment.

Conclusion

Pregnant mice are not as susceptible to ETC-induced abortion as horses. Mice do not produce abortions where the expelled fetuses and placentas can be examined, but rather mice reabsorb the fetuses. The gross and histopathological changes in the experiments conclude that mice (*Mus musculus*) are not a suitable laboratory animal for reproducing MRLS.

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Table 2. Number of pups for each mouse in Experiment 2. Group 1 was administered fresh frass, Group 2 was administered fresh ETC, and Group 3 (control) was administered normal saline.

Animal ID	No. of Pups	Comment
Grp1-1	7	
Grp1-2	12	
Grp1-3	13	
Grp1-4	2	
Grp1-5	13	
Grp1-6	10	
Grp1-7	14	
Grp2-1	12	
Grp2-2	11	
Grp2-3	14	
Grp2-4	15	
Grp2-5	10	
Grp2-6	13	
Grp2-7	12	
Grp3-1	14	
Grp3-2	12	one fetus reabsorbed
Grp3-3	10	
Grp3-4	13	
Grp3-5	13	
Grp3-6	13	
Grp3-7	10	

Table 3. Pups for each mouse in the uteri of setae group of Experiment 3.*

Animal ID	Total No. of Fetuses	Reabsorption	Comment
Control 1	10		
Control 2	4		
Control 3	11	One dead fetus	
Control 4	12		
Setae 1	11		
Setae 2	11		
Setae 3		All fetuses reabsorbed	Acute suppurative inflammation at implantation site
Setae 4			Not pregnant
Setae 5			Not pregnant
Setae 6		All fetuses reabsorbed	Acute suppurative inflammation at implantation site
Setae 7		All fetuses reabsorbed	Acute suppurative inflammation at implantation site
Caterpillar group	No abnormalities noticed in the uterus and fetuses		
Frass group	No abnormalities noticed in the uterus and fetuses		
Control group-2	No abnormalities noticed in the uterus and fetuses		

* The histopathological findings of uteri of mice that had reabsorptions are listed in column 3.

Table 4. Number of pups for each mouse in Experiment 4. Group 1 received unfiltered setae, Group 2 received filtered setae, and Group 3 (control) received normal saline.

Animal ID	Pups Born	Comment
Grp1-1	1	
Grp1-2	2	Left horn thickened
Grp1-3	13	
Grp1-4	Non-pregnant	
Grp1-5	11	Normal
Grp1-6	12	One reabsorbed
Grp1-7	3 dead, 3 live	Had pups before euthanasia
Grp1-8	5 dead	Had pups before euthanasia 1 small fetus
Grp1-9	7 live, 2 dead	Had pups before euthanasia
Grp1-10	9	1 reabsorbed
Grp2-1	15	3 reabsorbed
Grp2-2	12	normal
Grp2-3	Non-pregnant	
Grp2-4	Non-pregnant	
Grp2-5	11	
Grp2-6	10	
Grp2-7	5	4 reabsorbed
Grp2-8	Non-pregnant	
Grp2-9	5 live, 2 dead	Had pups before euthanasia
Grp2-10	12	1 reabsorbed
Grp3-1	7	Had pups before euthanasia
Grp3-2	1	
Grp3-3	Non-pregnant	
Grp3-4	12	
Grp3-5	11	1 reabsorbed
Grp3-6	12	1 reabsorbed
Grp3-7	9	
Grp3-8	12	Had pups before euthanasia
Grp3-9	14	1 reabsorbed
Grp3-10	13	1 reabsorbed

Table 5. Bacteria isolated from Experiments 1, 3, and 4 and from one of the filters used to filter setae.

Experiment 1	Experiment 3	Experiment 4	Filter
No growth	*Serratia marcescens* - setae group	*Serratia marcescens*	*Pseudomonas maltophila*
	Pantoea agglomerans - setae group	*Pseudomonas maltophila*	Unclassified gram negative bacillus

Summary

Noxious Agents and Disease Patterns Relevant to Mare Reproductive Loss Syndrome

D. S. Kronfeld

THE EMPHASIS IN THIS SESSION IS ON POTENTIAL NOXIOUS agents that might cause the Mare Reproductive Loss Syndrome (MRLS). It seeks to identify the direct or proximate cause and implies a simple cause-effect relationship. This limited view of MRLS has not solved the problem in 16 months since April of 2001. In my opinion, we should be trying to describe the whole pattern of the disease complex, and my intention in this perspective is to summarize likely disease patterns and to note where agents identified by the previous speakers may fit. First we need a broader concept of cause than simply A causes B.

Causation

The cause of abortion is the set of events, conditions, and characteristics necessary and sufficient for its occurrence (1). To determine the set, the usual approach is to list all reasonable possibilities, then try to rule each out, as in differential diagnosis. Possibilities usually arise as associations, for example, MRLS with certain climatic conditions, eastern tent caterpillars (ETC), and waterfowl. Each association can be ruled out by inconsistency. Its inclusion in the causal set can be reinforced by demonstration of appropriate timing and mechanism(s).

Disease Patterns

The horse exhibits many patterns of diseases with multifactorial etiologies and multiple etiologies (2). It is unrivaled in regard to disease complexes, such as rhabdomyolysis and laminitis, in which quite different sets of causes converge on apparent constitutionally weak sites. The muscle membranes and the laminae in the foot are two such sites; perhaps the placenta is another. Multiple etiologies can give the impression of exceptions or inconsistencies and intensify caution in rule-outs.

Septic Setae

T. Tobin (this proceedings) challenges the whole idea of a toxic agent with his septic penetrating setal emboli (SPSE) hypothesis. In this disease pattern, penetrating ETC setae pave the way for oral commensal bacteria to proceed via the blood vessels to the placenta where they lodge, proliferate, and cause abortion. The bacteremia may also cause pericarditis and uveitis.

This hypothesis is novel and testable. It appeals as a disease pattern in need of further development for several reasons. ETC setae are soft compared to the spiky setae of other species that are known to damage eyes, according to T. D. Fitzgerald (this proceedings). Dental hygienists spread oral commensals to heart valves but not yet to human placentas. Penetration of bacteria is more common in the large bowel of the horse subjected to luminal acidosis.

Carbohydrate Overloads

Luminal acidosis is caused by rapid fermentation of soluble carbohydrates to lactic acid (2). The most variable fraction of carbohydrates in pastures includes fructans (fructose oligosaccharides) and soluble fibers (pectins, gums, mucins). Common pasture contents of fructans are about 5% (dry matter) but rise to 25% during rapid growth or following frost kills. Early April of 2001 was warm, which would have encouraged fructan production; then came a frost, causing fructan accumulation.

These oligosaccharides are not hydrolyzed in the small intestine but are rapidly fermented in the cecum. When pH falls below about 6.3, the integrity of the cecal epithelium begins to loosen with increasing risk of bacterial penetration. This disease pattern is best described for liver abscesses in feedlot cattle. It could also operate in the pregnant mare and enable cecal anerobes to reach the placenta. It could be an exacerbating factor in SPSE.

Acidic conditions in cecal fluids if sufficiently severe cause bacterial lysis with the release of endotoxins. These lipopolysaccharides have been implicated in one form of laminitis. They may also affect the microcirculation of the placenta and lead to abortion. Pregnant mares have exhibited brief periods of fever before abortion. An endotoxic component has not yet been ruled out for MRLS.

Overloads of hydrolyzable starch and sugar from meals of more than 5 pounds of grain-molasses sweet feeds have the same effects as fructans on cecal fermentation. Thus, nutritional management of pregnant mares during risk periods should emphasize supplements of hay but not sweet feed because overloads of carbohydrates have not been

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ruled out as conditioning factors in MRLS. If sweet feed is used, it should be given as multiple small meals (2 or 3 pounds).

Meals of hydrolyzable carbohydrates raise blood glucose concentration and initiate a feeding-fasting cycle of metabolites and hormones. Studies in progress at Virginia Tech are showing that chronic feeding of starch-and-sugar meals leads to increased insulin resistance, especially in mares during late pregnancy. Studies at the Gluck Center by Dr. B. Fitzgerald are also revealing that insulin resistance in pregnant mares is increased by obesity. A higher incidence of abortion occurs in women who have pregnancy diabetes, according to K. Benirschke (this proceedings). Until type-2 diabetes and insulin resistance are ruled out as conditioning factors in MRLS, prudence suggests the avoidance of large meals of sweet feed and obesity in pregnant mares.

Plant Poisons

Previous speakers have suggested that climatic conditions may also have promoted overgrowth and consumption of pine needles, locoweed, lupins, veratrum, and hemlock, which have been implicated as causes of abortion in cattle and horses. Only the last of these possibilities has been associated with MRLS, and it appears to have been ruled out because it was also widely available to mares on many MRLS-free pastures. Hemlock poisoning might be conditioned by another concurrent factor, however, that has not yet been identified.

Mycotoxins and Zinc

The association of MRLS with highly constrained climatic conditions suggests that a highly favorable microenvironment might favor the production and accumulation of noxious agents. Previous speakers in this session have identified fescue endophyte alkaloids and an endocrine disrupter, zearalenone. About 10 mycotoxins common in feeds have been evaluated in regard to MRLS as reported by K. Newman (this proceedings), but no broad screen likely to detect unusual mycotoxins has been reported. For example, one screening method claims to detect any of 182 mycotoxins (3).

A likely template is facial eczema, in which growth of a fungus explodes on ryegrass only during brief periods of suitable microenvironmental conditions. The fungus, *Pithomyces chartarum*, was isolated from a black line on a mower blade observed by a farm hand; it had eluded scientists working on the disease for half a century. Its toxin, sporidesmin, was similarly unsuspected. Thus, experience with facial eczema suggests that a wide net needs to be cast for any fungus and mycotoxin involved in MRLS.

Sporidesmin damages the liver, which produces a photosensitive pigment. Severity of sunburn is conditioned by marginal dietary zinc. The toxin promotes production of radical, reactive oxygen species. A key enzyme in their detoxification is copper-zinc-superoxide dismutase. If the cause of MRLS involves a similar mycotoxin, then a conditioning factor may be a high dietary copper:zinc ratio. The copper:zinc 1:4 ratio recommended by the National Research Council was challenged by a 3-fold increase in copper allowance, following studies in Ohio and Kentucky on developmental orthopedic disease. Tolerances are about 50-fold for copper but only 10-fold for zinc, so nutritionists are cautious about raising zinc to preserve a 1:4 copper:zinc ratio.

Caterpillar Consumption

The ETC has been associated with MRLS as reported by R. Dwyer (this proceedings). The first question regarding causation concerns voluntary ETC consumption by horses.

My property line in the Appalachian Mountains of southwestern Virginia has a quarter-mile stretch of cherry trees. The number of tents and ETC vary enormously from year to year. Three times in 14 years ETC have crawled everywhere, including the feed buckets of my two equids, a pony and a Percheron. ETC appear to be attracted by the residual slime of molasses, slobber, and feed dust. After a scoop of sweet feed is dumped on a few or a dozen caterpillars, the horses snuffle up the feed but sort out and leave the caterpillars. I have counted these ETC in feed buckets so often that I'm convinced—my horses do not eat caterpillars. Is that a sufficient rule-out? No, but it's a prompt for testing voluntary caterpillarphagia in Thoroughbreds.

Caterpillar Toxicity

Caterpillars could be carriers of plant toxins or be noxious in themselves; for example, their setae could be mechanically damaging, as suggested by T. T. Tobin. Four administration experiments have been alleged to demonstrate ETC toxicity, but the preliminary reports (in horse magazines and this proceedings) have provided no statistical basis for this conclusion. In fact, some of the results are overtly contradictory unless attention is given to the timeline.

On August 27, I presented the results of my application of Fisher's Exact Probability Test to the published data from these four ETC administration experiments. Subsequently, I obtained the help of Dr. Dan Ward, a statistician at Virginia Tech. He applied a generalized linear model to the data with a logit link and a binomial error distribution, with single degree of freedom contrasts used to test hypotheses of interest. In the discussion that follows, my original P values are given in plain text, Dr. Ward's in *italics*.

A convenient starting point is the June experiment of B. Bernard et al. (this proceedings) because the question was simple and the results clear. Nasogastric administration of 50 g crushed fresh ETC in a slurry with 50 ml of water resulted in EFL in 4 of 5 mares, compared to 0 of 5 mares given 50 ml water (control) or 50 ml of water containing 2.5 g of stored frass. The ETC group was different from the combined control and frass groups (P = 0.004, *P = 0.0004*), and from the frass group or the control (P = 0.048, *P = 0.004*). Thus, ETC was toxic, frass not.

The July experiment of M. Sebastian et al. (this proceedings) extended the nasogastric toxicity of fed Michigan ETC to *LFL* (P = 0.002). In addition, clinical and pathological observations indicated placental detachment leading to fetal ischemia and hypoxia. The congruence of this experimental syndrome with natural MRLS needs to be evaluated.

The April experiment of B. Webb et al. (this proceedings) used ETC fencing and permitted but did not demonstrate ETC consumption by horses. Abortion occurred in 7 of 10 mares in the ETC-plus-frass paddock, and 7 of 9 in the frass-only paddock (P = 1.0, *P = 0.70*). The combined frass groups (14 of 19) were different from the control group, which had 3 abortions in 10 mares (P = 0.046, *P = 0.021*). Clearly, the frass was noxious in April. The three abortions in the control group were most likely attributable to caterpillar/frass contamination of the control pens. The ETC body tissues were either nontoxic or not consumed in April.

In the May experiment of B. Webb et al. (this proceedings), the ETC fences were improved, and the ETC were not fed for two or three weeks, so carried little or no frass. Abortions were 1 of 8 in controls, 0 of 8 in the frass group, and 3 of 8 in the starved ETC group. The control and frass groups were not different (P = 1.0). The two statistical approaches gave different results: ETC versus frass (P = 0.20, *P = 0.027*), and ETC versus the two combined frass and control groups, that is, ETC-free groups (P = 0.091, *P = 0.033*). Clearly, frass was no longer toxic in May, but the ETC body tissues now may (logistic regression) or may not (Fisher's Exact) induce abortion.

A timeline interpretation starts with a climate-conditioned, biological noxious agent that was consumed by ETC in April and was present in the frass in sufficient amounts to cause abortion late in April and early in May. The suspected toxin started to accumulate in ETC body tissues but was not yet enough to cause abortion in April. Accumulation of the noxious agent in ETC body tissues was clearly sufficient after two or more months, that is, later in June.

These experiments would be more convincing if voluntary consumption of caterpillars was actually observed (rather than assumed) before or at the time of the spike in MRLS frequency—the last week of April and the first week of May. The timeline should also be applied to the SPSE hypothesis; soft and furry setae in April and early May are unlikely to penetrate, and hard spiky setae later in July would be less relevant to the high time of natural MRLS.

Sentinels

Association is sufficient and causation not necessary for ETC to be useful sentinels in the management of mares at risk. A profusion of tents or larvae would be an indication for reducing exposure of pregnant mares to pasture. This avoidance reduces risks associated with fructans and other plant substances, endotoxins, myriads of mycotoxins, and ETC tissues, frass, and setae—none of which have been ruled out as components in the complex causation of MRLS. The history of public health is replete with effective interventions based on associations before causation was established.

Genetics

My worst fear is that MRLS may be a side-effect of selection for speed. Has the Thoroughbred's placenta become a vulnerable site like its bleeding alveoli, leaky muscle membranes, and fragile laminae? If so, then the coincidence of several quite different environmental triggers (fructans, mycotoxins, ascending infection, and ETC, etc.) might converge on placental separation at the same time and give the appearance of an outbreak of MRLS.

A relevant disease pattern is infertility caused by a round rump and sloping vulva requiring the Caslick procedure. When I was a veterinary student 50 years ago, perhaps two or three fillies in a 100 had the colt-like oval hindquarters and needed a stitched vulva. Most fillies had a triangular rear end, with a vertical drop from the tail insert. When the anus protruded an inch during defecation, the feces fell clear. A panel of three veterinarians and three farm managers at a meeting in Lexington, Kentucky, in 1990, which I attended, agreed that over 35 years they had bred for a colt-like conformation to obtain more speed from fillies. Now, nearly all Thoroughbred mares have rounded rumps and sloping vulvas, so are Caslicked to reduce the risk of infertility associated with soiling and chronic infection.

Two testable hypotheses arise from this deliberate and overt conformational trade of reproductive unsoundeness for speed. First, could perineal conformation be a conditioning factor in MRLS? One could test for associations between sloping vulvas, ascending infections, and MRLS. Second, what if single-minded striving for speed has unintentionally endowed the racing Thoroughbred with a covert, constitutionally vulnerable placenta that is prone to separate at the slightest insult of any kind? This hypothesis could be tested retroactively by comparing the family racing performances of MRLS mares with spared mares.

Conclusion

The MRLS challenge continues. Conclusive rule-outs must be painstaking; testing for 10 common mycotoxins, for example, is hardly a comprehensive screen. Rule-outs are needed for numerous likely conditioning factors: dietary fructans, sweet feed and obesity, the copper:zinc ratio, sloping vulvas, and fast families. These factors can be evaluated retroactively by epidemiologists with the help of nutritionists, theriogenologists, and geneticists. Looking forward, the timeline is crucial in testing prospective causal agents. Are ETC setae hard and penetrating in April or July? Ethologists must watch mares through telescopes and binoculars to see if they voluntarily consume ETC while unperturbed on pastures in late April and early May. Toxicologists should compare the toxicity of frass and parts of the ETC bodies at times relating to a spike in MRLS frequency. They could also compare cyanide affinities of cytochome oxidase of tissues from mares and foals. Bacteriologists should answer two questions. Do the placental bacteria come from the mouth, cecum, or vagina? More difficult is the timeline. Are the placental bacteria primary invaders that start placental separation or later lodgers in already damaged tissues? Corrective interventions should proceed in the usual way from encouraging clinical experiences to rigorous comparative trials. In the end, we will see if MRLS represents a new pattern of disease or a variation on an old theme.

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