



The development of infections with *Listeria monocytogenes* following the ingestion of ponderosa pine needles
by Charlotte Jutila Adams

A thesis submitted in partial fulfillment of the requirements for the degree of MASTER OF SCIENCE
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Abstract:

An infectious microorganism, identified as *Listeria monocytogenes*, has been isolated from the tissues and fluids of mice fed a diet containing *Pinus ponderosa* needle. The ability for this organism to proliferate appears to be more pronounced in pregnant and immature mice, in contrast to adult, non-pregnant female and male mice. Pathologic changes in pine needle-fed mice similar to those observed in cattle and other mammals undergoing abortions and/or toxemia attributed to the ingestion of ponderosa pine needles suggests that *L. monocytogenes* may be part of the etiology of "pine needle abortion" in other species. A medium that appears to enhance the selective proliferation of *Listeria* was also developed which may increase the incidence of isolation of *Listeria* from natural sources.

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THE DEVELOPMENT OF INFECTIONS WITH LISTERIA MONOCYTOGENES
FOLLOWING THE INGESTION OF PONDEROSA PINE NEEDLES

by

CHARLOTTE JUTILA ADAMS

A thesis submitted in partial fulfillment
of the requirements for the degree

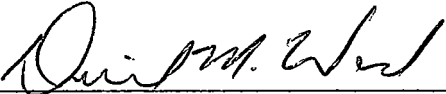
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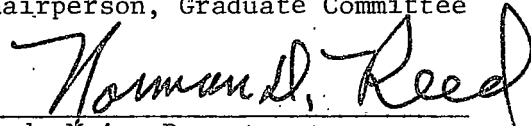
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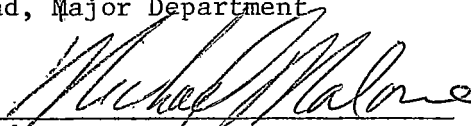
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ABSTRACT

An infectious microorganism, identified as Listeria monocytogenes, has been isolated from the tissues and fluids of mice fed a diet containing Pinus ponderosa needle. The ability for this organism to proliferate appears to be more pronounced in pregnant and immature mice, in contrast to adult, non-pregnant female and male mice. Pathologic changes in pine needle-fed mice similar to those observed in cattle and other mammals undergoing abortions and/or toxemia attributed to the ingestion of ponderosa pine needles suggests that L. monocytogenes may be part of the etiology of "pine needle abortion" in other species. A medium that appears to enhance the selective proliferation of Listeria was also developed which may increase the incidence of isolation of Listeria from natural sources.

CHAPTER I

INTRODUCTION

Prior to the 1900's, ranchers in Canada and the northwestern United States claimed that pine needles and buds were the causative agents of abortions within their herds (MacDonald, 1952; Cogswell, 1974). These reports were not considered seriously because for years the abortifacient activity had been attributed to brucellosis, phosphorus deficiency, and vitamin A depletion. In 1950 MacDonald undertook the first formal investigation that determined that Pinus ponderosa needles caused abortions in cattle (MacDonald, 1952).

The effects of the needles when ingested were probably known decades before the turn of the century. Indian folklore in the western United States reports that Indian women made an aqueous extract of ponderosa pine needles to induce abortion (Tucker, 1961; Chow et al., 1972).

Clinical characterizations of the pine needle abortion include the following: 1) Pine needles appear to be palatable to the cows; they are seen grazing on the pine needles even when there is abundant feed and grain (Smiley, 1975; Fraser, 1977). 2) The abortion can occur in as little as 24 hours after ingestion. 3) The placenta is usually retained;

2.

uterine hemorrhage is common and septic metritis followed by peritonitis are consistently observed (Stevenson et al., 1972). 4) The animal appears well, although weak parturition contractions are usually present (Stevenson et al., 1972; Jacobsen, 1969; James et al., 1977). 5) Ranchers report that the induced reproductive dysfunction occurs year-round as long as pine needles are available (Tucker, 1961; Fraser, 1977).

It is estimated that the economic loss to the cattle industry as a result of the lowered calf yield and maternal mortality ranges in millions of dollars (Cogswell, 1974). Because of the increasing economic impact this loss has on the ranchers several studies have been undertaken to delineate the pine needle induced abortion phenomenon in some detail.

The pine needle-induced reproductive dysfunction has been successfully reproduced in mice and rats (Cogswell, 1974; Allen and Kitts, 1961; Cook and Kitts, 1964; Chow et al., 1974; Anderson and Lozano, 1977) and these experimental animals are now used extensively in studying the pine needle-induced abortion problem.

Although many investigators have attempted to define the etiological agent in pine needles, the problem has not yet

been resolved (Chow, et al., 1972; James et al., 1977). Many studies suggest that the agent is in the water extracts of the pine needles and is heat-labile (Allen and Kitts, 1961; Chow et al., 1974; Anderson and Lozano, 1977). In addition, it has been proposed that there is a heat stable, water-insoluble compound involved in some way to the toxic signs observed (Anderson and Lozano, 1977).

Some extracts of the pine needles have been shown to contain estrogenic and/or antiestrogenic activity (Cook and Kitts, 1964; Chow et al., 1972). One of the most active areas of study is the investigation of anti-estrogenic or estrogenic activity of phenolic compounds and terpenes (Emmens, 1970; Rosen and Millman, 1955) that are known to be constituents of the Pinus ponderosa needles (Tagahashi, 1960; Zavarin et al., 1971).

Microorganisms were first implicated as the etiological agent responsible for the pine needle abortion by Tagahashi et al (1974). Chow et al. (1972) reported that an aqueous fraction extracted from the needles of Pinus ponderosa could disrupt fetal development of mice. However, the aqueous fractions of pine needles harvested in the following two years induced little reproductive dysfunction in mice. A study was undertaken to determine whether metabolites

produced by fungi which had been observed on the pine needles could be the cause of the reproductive failure. Results indicated that the mycotoxins contained abortifacient activity (Chow et al., 1974); however, Anderson and Lozano (1977) suggested that toxins produced by fungi were of secondary importance.

Clinical aspects of the problem support the notion that microorganisms or their products (toxins) may be involved. Stevenson et al. (1972) observed cattle experiencing "pine needle abortion" and reported excessive uterine hemorrhaging, a characteristic nauseating odor, septic metritis and peritonitis as consistent characteristics.

Although most infectious processes are not known to affect the fetus or alter the course of pregnancy, there are a number of examples, such as brucellosis and listeriosis (Jubb, 1963). Such infections can be placed into two categories: those involving the fetus and placenta, usually resulting in stillbirth or abortion, and infections restricted to the lumen of the maternal reproductive tract (Parr, 1970). Through indirect infection of the fetus (from systemic dissemination in the maternal reproductive tract) or as a result of toxic effects by maternal organisms some maternal infections may (1) disrupt the pregnancy process

leading to abortion (2) cause fetal death in utero or (3) have a teratogenic effect insufficient to kill the fetus (Eichenwald and Shinefield, 1962). There are two lines of evidence supporting the hypothesis that, in pine needle-induced abortions, the toxic effects on the fetus originate in the maternal system. James et al. (1977), in their investigation of pine needle-induced aborted feti, found tissues that were consistently autolytic indicating fetal death in utero. The other line of evidence involves compounds contained within the needles; several phenolic and steroidal constituents of Pinus ponderosa needles are considered to be teratogenic in nature (Keeler, 1975).

Direct fetal infection may result from (1) amniotic infection with vaginal organisms that entered the amniotic sac, usually after rupture of the membranes, or (2) hematogenous dissemination of microorganisms carried to the placenta (Blanc, 1961). Certain infections are reported to induce abortion (Jubb, 1963). Probably the most widely known abortifacient organism is Brucella abortus which characteristically infects the mother first and then is attracted to the placental tissues by a chemotactic substance in the placenta, erythritol (Smith and Fitzgeorge, 1964; Keppie et al., 1969). Other infectious agents that

characteristically cause abortion are certain Vibrio species, Listeria monocytogenes, several species of Salmonella, Klebsiella pneumoniae, E. coli, and several viruses such as the rhinopneumonitis virus (Jubb, 1963; Blanc, 1961; Corner, 1963). These disease agents generally cause abortions during the late stages of gestation with resulting maternal septicemia and lesions of vital organs in the mother such as the liver and intestines.

Abortifacient agents can be transmitted via several mechanisms. The most common ways are direct contact between animals within a herd or transmission of the disease by handling of contaminated feed (Sorenson et al., 1959), coprophagy (McBee, 1970), and crowding (Davis et al., 1973).

One obscure mechanism of inducement of abortifacient disease in animals is thought to involve certain plants (Keeler, 1975). One example is that of the plant, Gutierrezia microcephala (broomweed or turpentine weed). Animals ingesting this plant show similar signs as those noted in pine needle abortion including loss of weight, vaginal discharges, listlessness, organ lesions, septicemia, and abortions. It is not known whether the abortifacient activity is induced from the septicemia or the action of the identified toxic element, a steroidal saponin

(Dollahile et al., 1972). Other plants containing steroidal components induce similar pathological signs (Keeler, 1975) The consumption of silage is another example of abortifacient activity induced by the consumption of plant material. Pregnant animals ingesting large amounts of silage may develop a Listeria monocytogenes septicemia with subsequent reproductive failure (abortion) (Gray and Killinger, 1966; Kampelmader and Van Noorle Jensen, 1969).

The objective of this research was to isolate, identify, and characterize a possible microbial etiological agent involved in the phenomenon of "pine needle abortion" using the mouse system as a model.

In this study, whole ponderosa pine needles were used as opposed to extracts employed in previous studies for two reasons: (1) results obtained from the use of pine needle extracts were variable from study to study and often irreproducible, and (2) the consumption of the whole pine needles by mice in our study would represent a closer situation to what the cow experiences when consuming pine needles on the range.

CHAPTER 2

MATERIALS AND METHODS

Experimental Animals

Dub/ICR mice were originally obtained from Flow Labs in Bar Harbor, Maine, second inbred in the local colony. Descriptions of the Dub/ICR mouse groups are as follows:

1. Immature mice--Newly-weaned mice weighing 10 ± 2 grams.
2. Mature females-- Adult mice weighing 28.0 grams or more and having a normal estrous cycle as determined by vaginal smears taken daily.
3. Pregnant mice--Females to be bred for experimental purposes were maintained until they reached a weight of 28.0 g. prior to mating them to a male. Pregnancy was determined by examination of vaginal smears and detection of copulatory plugs. Two groups of pregnant mice were employed: (a) pregnant mice on day 4 of pregnancy in order to study the effects of pine needles on early gestation; and (b) pregnant mice on day 9 of pregnancy in order to study the effects of the needles on the latter trimester.
4. Mature males--Mature male mice weighing 28.0 g. or more. Balb/c and C3H female mice from the local colony and at day 9 of gestation were used to compare with the mice in group 3b above.

Collection of Pine Needles and Preparation of Pine Needle Chow Collection and Storage

Ponderosa pine needles were obtained in October 1977, from the William Fraser ranch, 5 miles east of Greycliff, Montana, because of the area's high incidence of "pine needle abortion." The needles were from branches or small trees less than six feet from the ground. The branches or needles pulled from the branches were stored at -14C. Periodically, samples of the needles were ground in a coffee grinder and air dried.

Preparation of Pine Needle Chow

The dried pine needles were reground and mixed with ground Wayne Lab-Blox in a 3:2 ratio by dry weight. Five hundred grams of this mixture was combined with 400 ml of unsulfured molasses and mixed thoroughly. The mixture was allowed to dry and cut into small pellets which were stored at 4C in a closed container until used.

Feeding

Control groups of mice were fed Wayne Lab-Blox ad lib. and experimental groups of mice were fed a diet of pine needle chow ad lib. for varying time periods as designated. Water was available ad lib.

Pathologic Observations

The mice were fed the pine needle chow or the control chow for up to ten days at which time they were sacrificed by cervical dislocation, dissected, and examined for morphologic change in organs and tissues. Pregnant mice were examined for evidence of reproductive dysfunction in addition to other morphologic change. Reproductive dysfunction (fetal death) was represented by barren uteri, resorptions or yellowish feti that did not respond to painful stimuli. The adrenals, spleens, livers and thymuses were carefully removed, weighed to the nearest mg and examined for gross morphology in both the experimental and control groups.

Isolation, Characterization and Identification of the Microorganisms Proliferating in Mice Fluids and Tissues after Consumption of Pine Needle Chow

Bacterial Enumeration in Body Fluids

Uterine fluid (0.2 ml) was aseptically aspirated from the uterine sac (via a 27 gauge needle attached to a 1 ml Tuberculin syringe) and was used to inoculate (0.1 ml) into 10 ml of tryptic soy (TSY) (Difco) broth. Fluid samples were also streaked on tryptic soy agar (TSY) (Difco) plates and blood agar (BA) (Difco) plates. The

inoculated material was incubated in a candle jar at 37C and examined daily for evidence of growth.

Blood samples were obtained from all mice in the study and examined for evidence of septicemia. The blood samples were obtained aseptically by exposing the heart and suctioning 0.1 ml of blood directly from the right atrium via puncture with a 27 gauge needle attached to a 1 ml Tuberculin syringe. Samples were inoculated into TSY broth and incubated at 37C in a candle jar and examined daily for growth.

Cecal fluid specimens were obtained by suctioning 0.2 ml of fluid via an 18 gauge needle on a 5 ml syringe that had been flushed with nitrogen. Samples were inoculated into TSY broth, and into prereduced TSY broth using nitrogen as the gas phase, as described by Hungate (1969). The inoculated broths were incubated at 37C and examined for growth after 48 hours.

0.1 ml samples were spread evenly over a 1 cm² area of a microscopic slide defined by a cover slip and were directly counted using phase contrast microscopy or bright field microscopy if samples were Gram stained. A minimum of 30 cells were counted for statistical purposes. Samples that had more than 10⁶ organisms / ml were recounted using the Petroff - Hauser counting technique for

the purposes of ensuring accuracy. The student's t-test was used for test of significance.

Bacterial Enumeration from Liver, Spleen and Fetal Meconium

The methods employed for enumeration of viable bacteria in liver and spleen were as described by Nickol and Bonventure (1977). The spleens and livers of the pine needle-fed pregnant mice were excised and homogenized in 2.0 ml of sterile Brain Heart Infusion (BHI) (Difco) broth. Ten-fold dilutions were plated in BHI agar, incubated at 37C and colonies were enumerated 48, 72, and 168 hours after inoculation.

From detectable surviving feti, meconium samples were obtained by aseptically dissecting the feti, opening the intestines, and procuring a small sample of meconium. These samples were inoculated into TSY broth and incubated in a candle jar at 37C. Broth tubes were examined daily for 14 days for growth.

Identification of the Predominant Microorganism Isolated

The microbial species found in the pregnant and immature mice fed pine needle chow was identified through the biochemical and physical test described in Buchanan and Gibbons (1974) and Gray and Killinger (1966).

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The tests included determination of:

1. morphological characteristics of the organism by Gram stains of broth and agar cultures;
2. motility at 22C and 37C;
3. hemolytic properties on blood agar;
4. fermentation of the sugars, dextrose, lactose, maltose, sucrose, mannitol, and dulcitol;
5. catalase production;
6. ability to liquefy gelatin and reduce nitrate;
7. colonial morphology when colonies grown on nutrient sugar were observed with an obliquely-transmitted light;
8. the results of instillation of the organism into the conjunctiva of eyes of rabbits;
9. Agglutination tests were performed as described by Seeliger (1961). Antiserum to the most common O antigen combinations found on isolated strains of Listeria were obtained from the Veterinary Research Lab at Montana State University, Bozeman, Montana and included antisera that defined the 1a, 1b, 3a, 4a, and 4b serotypes of Listeria.

Preparation and Use of Ponderosa Pine Needle--TSY Media

Ponderosa pine needles were incorporated into TSY agar in the following manner.

Air-dried ponderosa pine needles were ground, sieved, and ground twice more until a fine powder was obtained. Needles were added to 10 ml of previously-prepared molten TSY agar in 0.05, 0.10, and 1.0 g portions to make 0.5%, 1%, and 10% solutions by wt/vol, respectively. Solutions were poured into petri dishes, autoclaved, cooled, and then stored at 4C until used. TSY agar was used as the control medium.

Anaerobic pine needle agar and TSY were prepared in a similar manner as above except the Hungate roll tube method (Hungate, 1969) was employed, using nitrogen as the gas phase.

Blood and uterine fluid samples obtained as described previously were plated on the pine needle agar. Cecum samples were obtained anaerobically by the use of nitrogen-flushed 5ml syringes attached to 18 gauge needles. 0.1 ml samples were diluted with anoxic (Hungate, 1969) normal saline to 1:10, 1:100, 1:1000 and 1:10000 using sterile and anaerobic transfer methods. Dilutions were plated in duplicate on the aerobic and anaerobic TSY and pine needle agar.

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The roll tubes and plates were then incubated at 37C and examined daily for growth.

CHAPTER 3

RESULTS

Characterization of Morphologic Change Induced by the Consumption of Ponderosa Pine Needles

Susceptibility of Mice to the Effects Induced by the Consumption of Pine Needles

The groups of mice varied in their response to a ten-day diet of pine needle chow (See Table 1). In all cases, mice that were fed the pine needle chow starting on day 9 of pregnancy showed low survival and the greatest pathological change including decidual hemorrhaging (abortions), fetal death and resorptions, blood-filled intestines, speckled livers and kidneys, and reddened adrenals. In addition, a severe loss of coordination, a substantial weight loss and a purulent exudate from the vaginal orifice was noted in the animals in this group. Upon exposure of the viscera, a characteristic pungent odor was detectable. Seven mice in this group expired within six days of diet consumption. Mice fed pine needle chow beginning on day 4 of pregnancy differed in their response in that the toxic effects were observed only the first two days of feeding the experimental rations. This group displayed some loss

TABLE 1

Susceptibility of Various Groups of Mice to the Effects of Diet Containing Ponderosa Pine Needles.^a

<u>Mouse group</u>	<u>Ration</u>	<u>Number in group</u>	<u>Number in group surviving test period (10 days)</u>
Immature mice	Pine Needle	10	0
Immature mice	Control	5	5
Mature females	Pine needle	10	10
Mature females	Control	5	5
Pregnant females -			
Day 4 of gestation	Pine needle	10	10
Day 4 of gestation	Control	5	5
Day 9 of gestation	Pine needle	10	3
Day 9 of gestation	Control	5	5
Mature males	Pine needle	10	10
Mature males	Control	5	5

(a) For description of mouse groups, refer to Experimental animals in the Materials and Methods.

of coordination, lethargy, and weight loss; however, these signs subsided by the time of sacrifice at day 14 of gestation. There was a total loss of feti in all animals started on the pine needle chow at day 4 of pregnancy.

Adult males and non-pregnant females also showed lethargy and loss of coordination during the first two days on the chow, but the signs subsided during the remaining days on the pine needle chow. These mice showed no gross pathological changes upon dissection.

Immature mice showed greater susceptibility than older mice to the effects of the ponderosa pine needles. All immature mice died within three days of feeding. Prior to death, they showed loss of coordination, extreme lethargy, starvation-like symptoms and were cold to the touch. Necropsy revealed hemorrhagic intestines and ulcerated stomachs.

All control mice remained healthy.

Pathologic Observations at Daily Intervals

This study was designed to determine when morphological change occurred in mice fed a continuous diet of ponderosa pine needles. Studies were confined to pregnant mice at day 9 of gestation for two reasons:

1. This group of mice exhibited a marked susceptibility

to the pathologic change induced by the consumption of the pine needle chow.

2. The original premise of this thesis was to determine the etiological agent(s) responsible for the abortifacient activity resulting from the intake of ponderosa pine needles in late pregnancy.

Seventy-three pregnant mice at day 9 of gestation were fed the pine needle chow and 65 were fed a diet of Wayne Lab-Blox as controls. Control mice showed a continuous increase in weight throughout the ten days of study (See Figure 1). In contrast, pregnant mice fed the pine needle chow exhibited little weight gain with an actual weight loss in the latter days of pregnancy.

In the experimental mice, maternal death began to occur at day 12 of gestation after three days of diet consumption (Figure 2); mortality reached a peak after six days of feeding the pine needle chow (day 15 of pregnancy) with the numbers of mice expiring declining after that point. Four to six mice from each group (control and experimental) were sacrificed for pathological analysis at daily intervals. All mice succumbing to the effects of the pine needle diet (24 in number) were cannibalized; thus no data, other than mortality figures were obtained. Control animals remained

Figure 1. Body weight of Pregnant Mice starting at Day 9 of Pregnancy on Pine Needle Chow.

———— Body weight of mice fed Wayne Lab-Blox for controls (with standard deviation)

- - - - - Body weight of mice fed pine needle chow (with standard deviation).

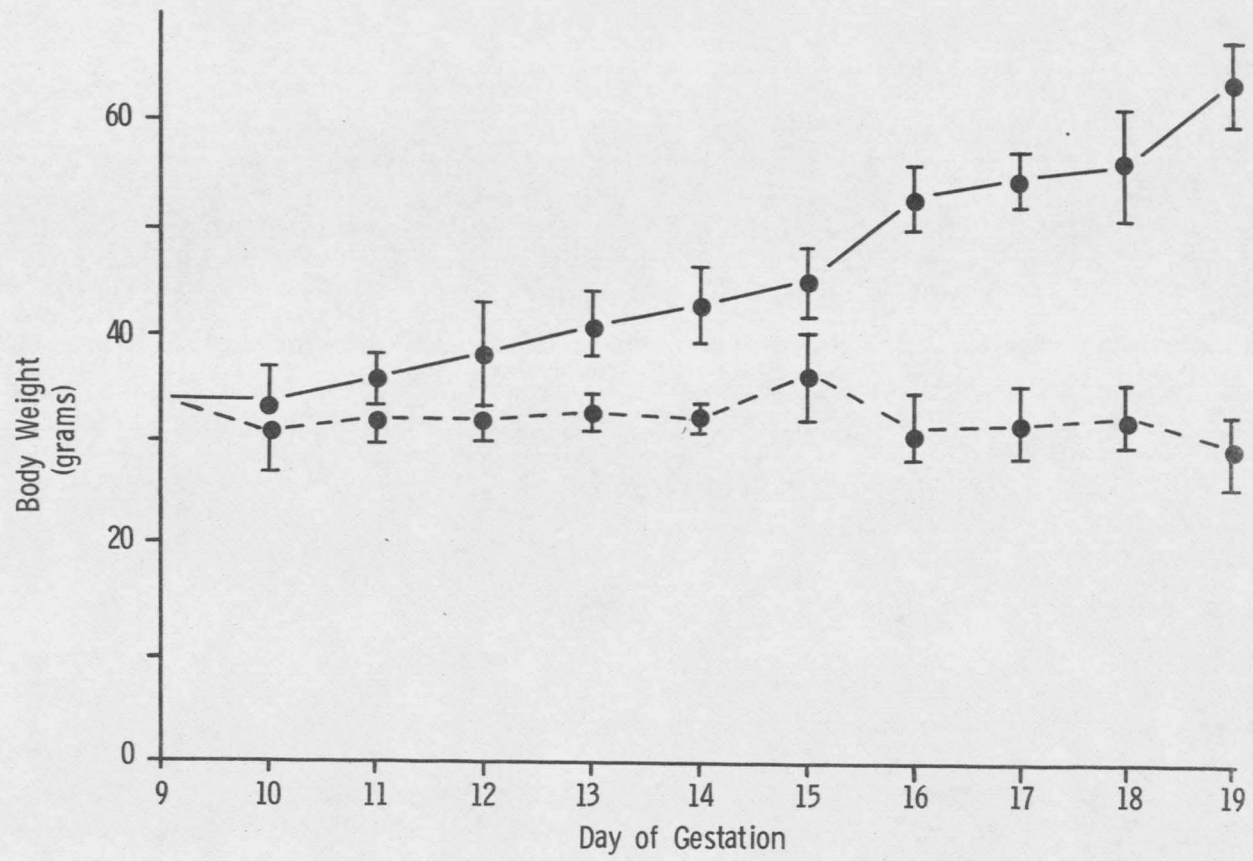
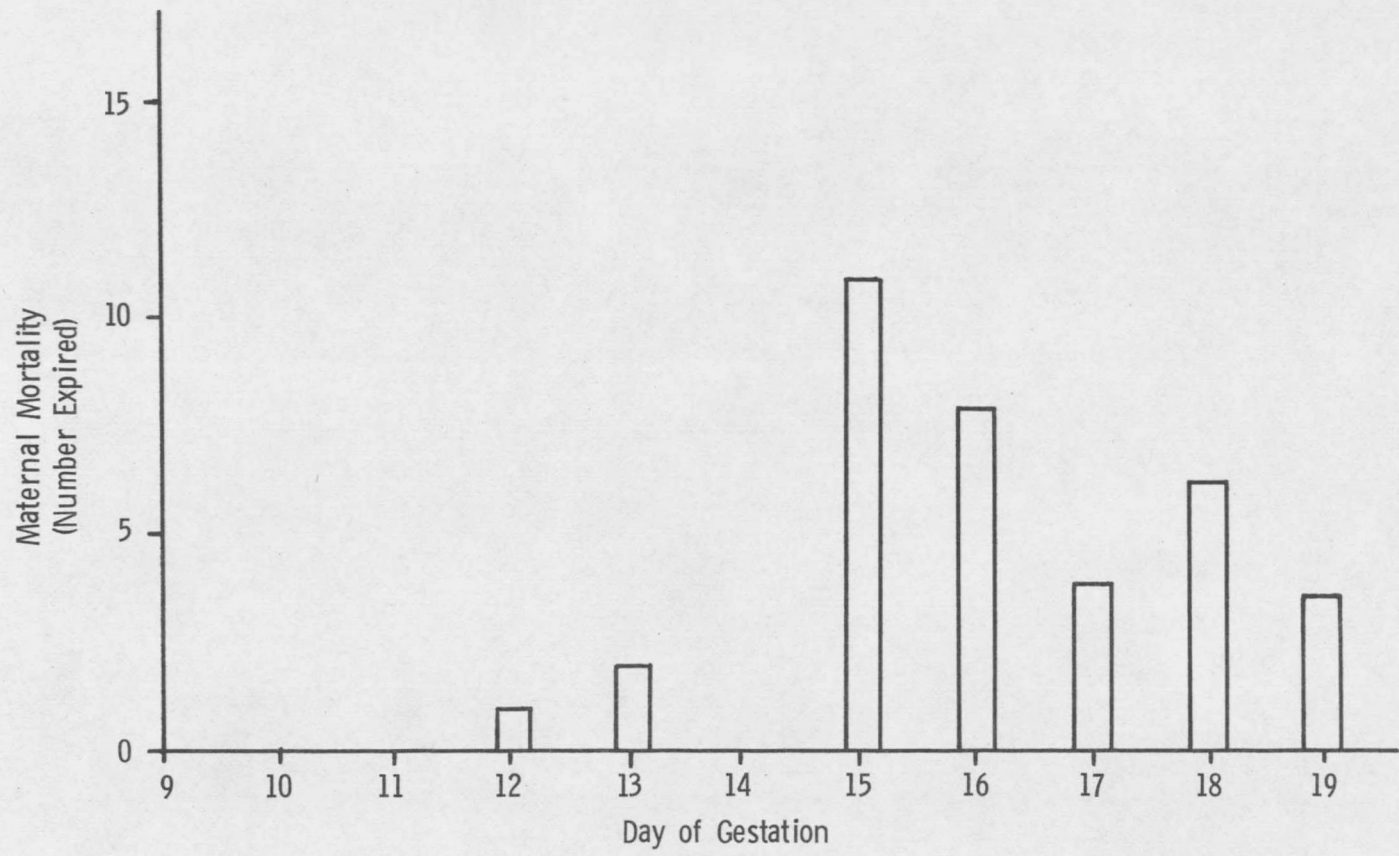


Figure 2. Mortality of Pregnant Mice during Consumption of Pine Needle Chow Starting at Day 9 of Pregnancy.



healthy throughout the study; no pathological change was noted, and feti appeared viable with a 10-15% resorption rate. Normal delivery occurred at day 19 of pregnancy. At day 14 of pregnancy (after five days of diet consumption), fetal death in the experimental mice became apparent with the most dramatic loss during the sixth day of study (day 16 of pregnancy) (Figure 3). After that point, there appeared to be an increase in fetal survival; however maternal death was high at this point and the data were based on fetal survival in live mothers.

Adrenal, liver, thymus, and spleen weights from sacrificed control and experimental mice appear in Figures 4 through 7.

Between days 15 and 19 of gestation, adrenals from the experimental group were visibly enlarged and reddened when compared to those from the control group. At day 16 of gestation, pine needle-fed mice showed a dramatic increase in adrenal weight relative to body weight as opposed to the control group which exhibits a significant decrease (Figure 4). The greatest difference between experimental and control groups is at day 19 when the adrenal weight to body weight of the control group has dropped.

Figure 3. Fetal Mortality in Surviving Mothers During Feeding of Ponderosa Pine Needles to Pregnant Mice Beginning the 9th Day of Pregnancy.

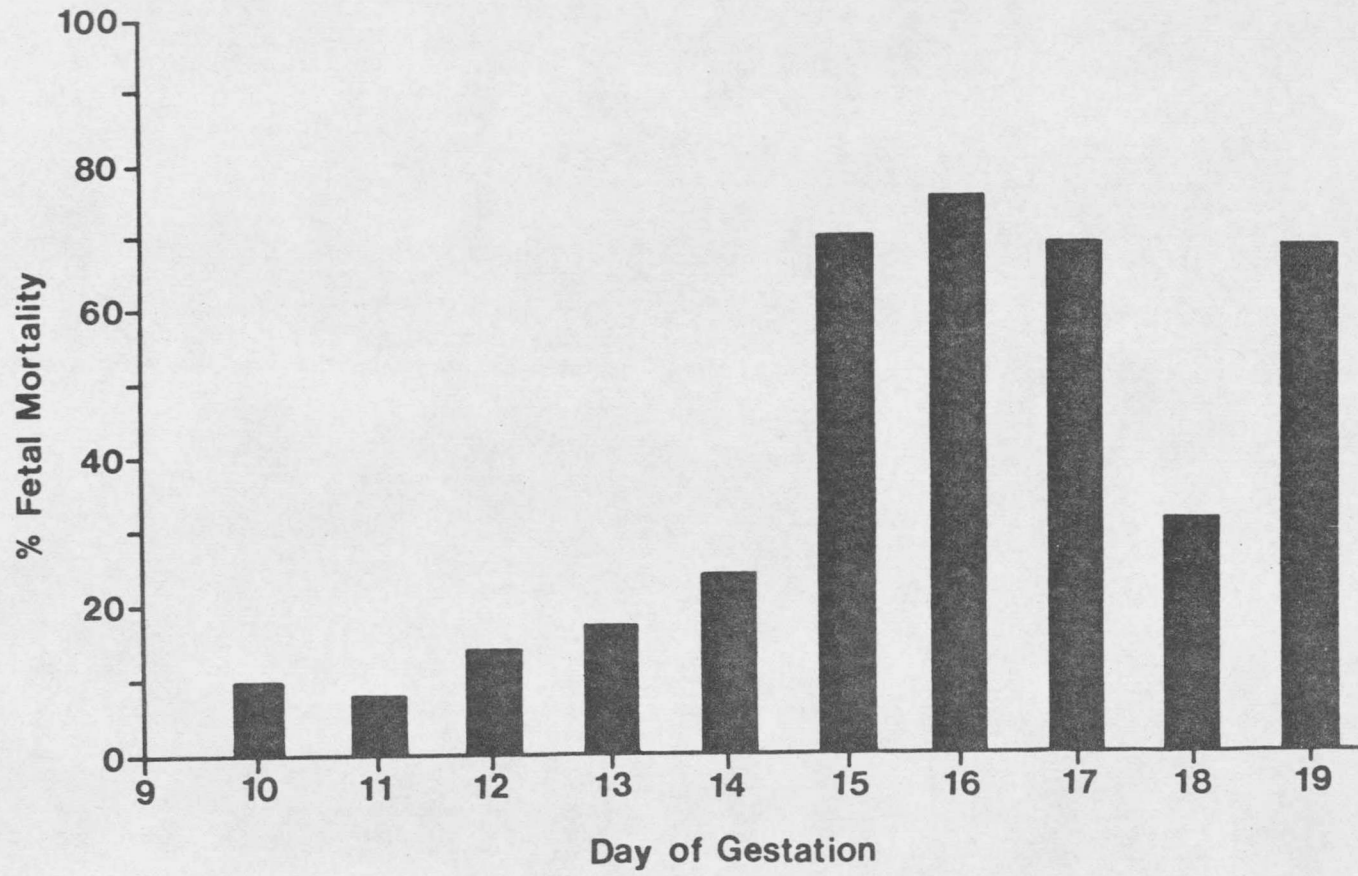


Figure 4. Average Adrenal Weights as Percent of Body Weight of Pregnant Mice fed Pine Needle Chow from Day 9 of Pregnancy.

- Average adrenal weights of pregnant mice fed Wayne Lab-Blox as controls (with standard deviation)
- - - - - Average adrenal weights of pregnant mice fed pine needle chow (with standard deviation)

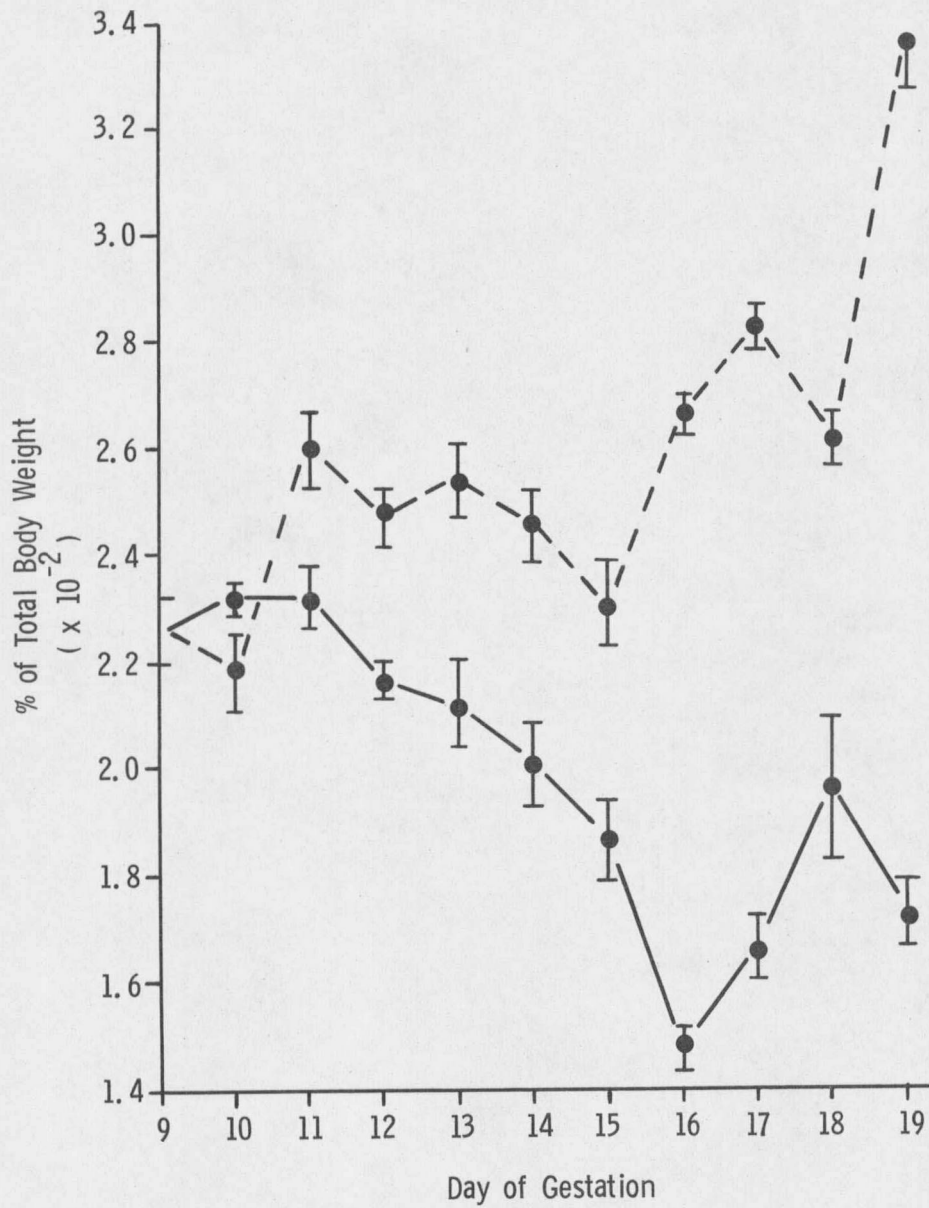


Figure 5. Average Liver Weights as Percent of Body Weight of Pregnant Mice Fed Pine Needle Chow from Day 9 of Pregnancy.

———— Average liver weights of pregnant mice fed Wayne Lab-Blox as controls (with standard deviation)

- - - - - Average Liver weights of pregnant mice fed pine needle chow (with standard deviation)

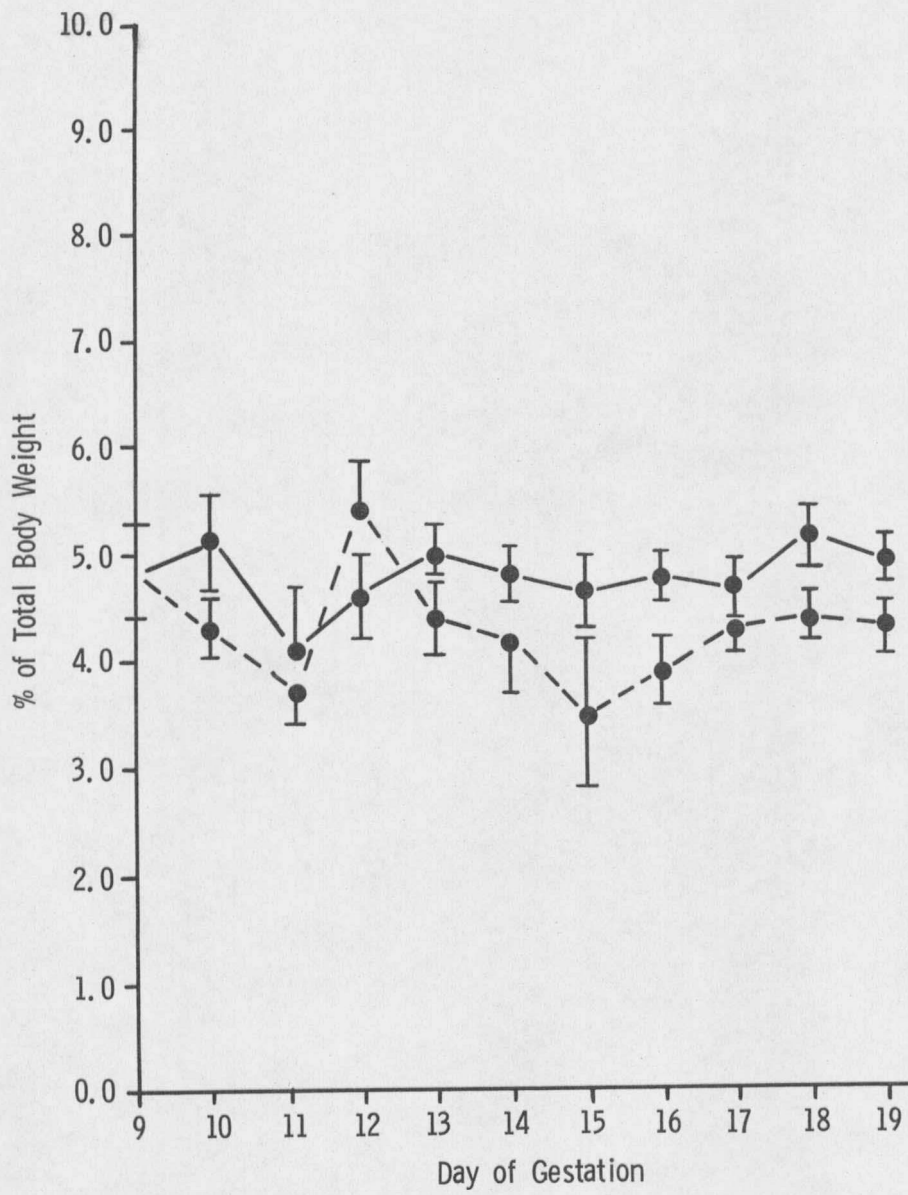


Figure 6. Average Thymus weights as Percent of Body Weight in Pregnant Mice Fed Pine Needle Chow from Day 9 of Pregnancy.

- Average thymus weights of pregnant mice fed Wayne Lab-Blox as controls (with standard deviation)
- - - - Average thymus weights of pregnant mice fed pine needle chow (with standard deviation)

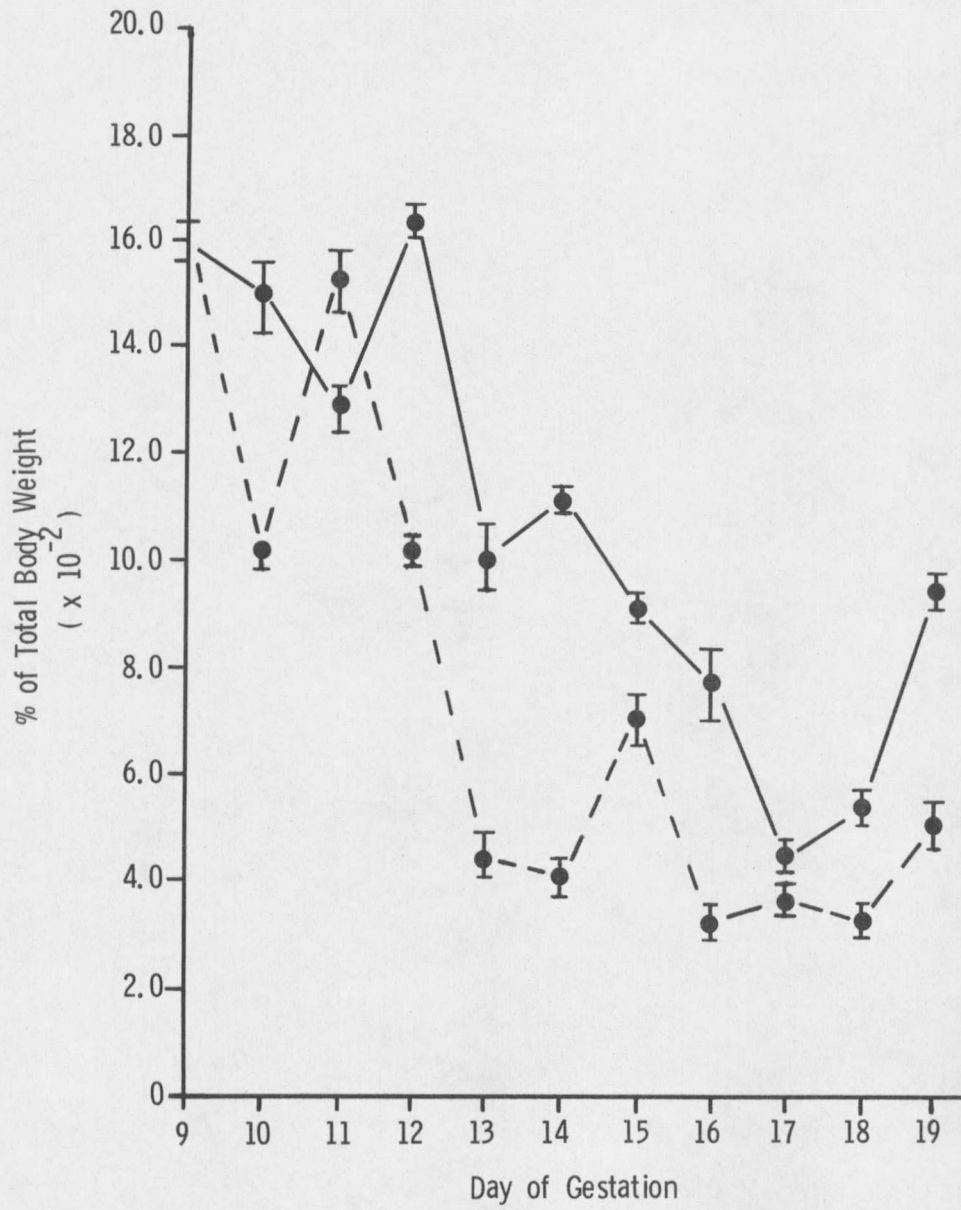
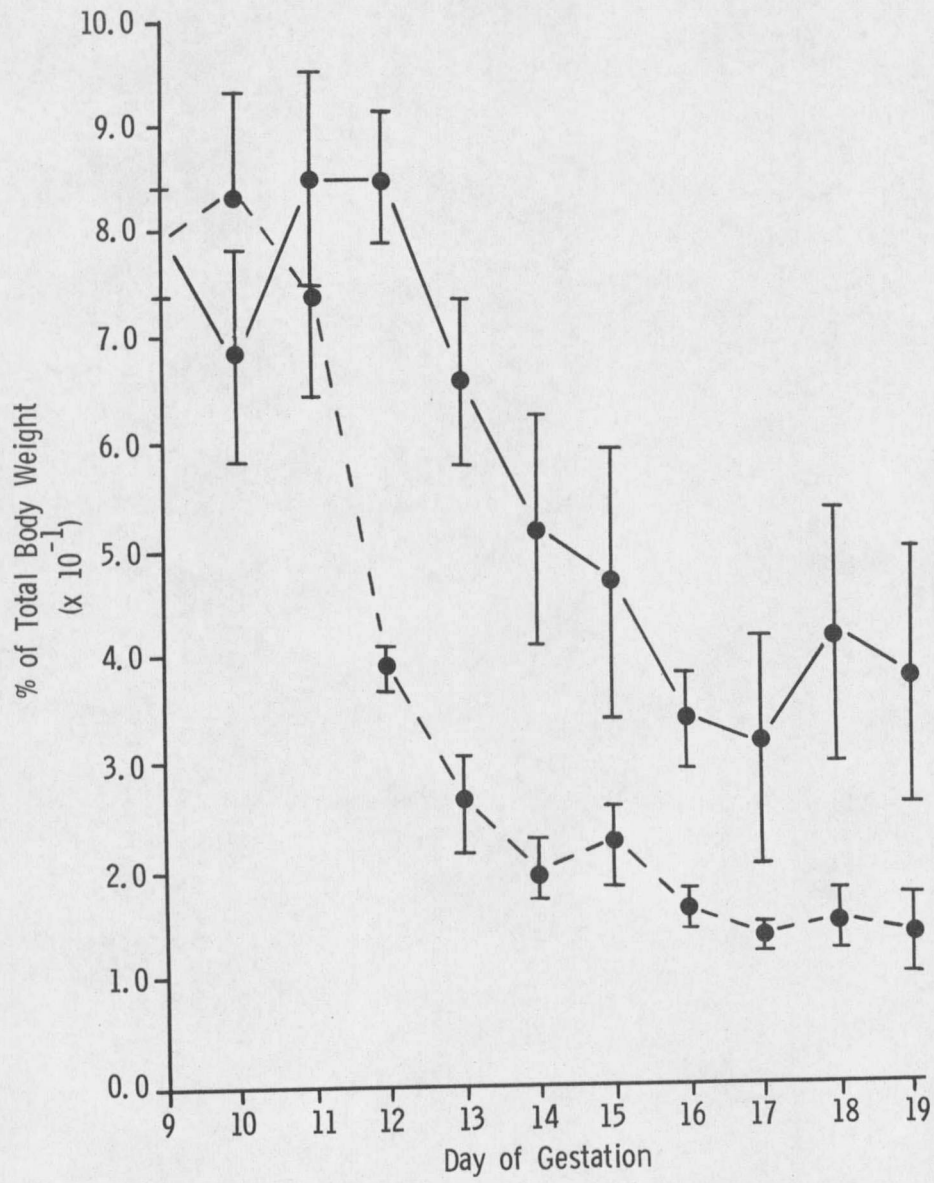


Figure 7. Average Spleen Weights as Percent of Body Weight of Pregnant Mice Fed Pine Needle Chow from Day 9 of Pregnancy.

- Average spleen weights of pregnant mice fed Wayne Lab-Blox as controls (with standard deviation)
- - - - - Average spleen weights of pregnant mice fed pine needle chow (with standard deviation)



Although liver weights do not appear to vary significantly between the groups of mice (Figure 5), livers from pine needle-fed mice were visibly traumatized with a characteristic mottled appearance from day 15 of pregnancy to the termination of the experiment.

Thymuses from the experimental group were smaller than those from the control group (Figure 6).

Figure 7 illustrates average spleen weights as percent of body weights of the control and experimental group. Note the smaller relative weights of spleens in the pine needle-fed group throughout the test period. Spleens from this group were visibly decreased in size and were apparently atrophied by the 14th day of gestation (five days on the pine needle chow).

Confirmation of Pine Needle-Induced Morphologic Change in Other Strains of Mice

Five mice from each strain were placed into control group and ten were put into the experimental group.

Pregnant Balb/c and C3H mice fed pine needle chow from day 9 of pregnancy exhibited similar pathological signs as did Dub/ICR mice. Although no mice died, there was a total loss of feti in both strains of mice by day 19 of gestation

accompanied by hemorrhagic intestines, spleen atrophy, mottled livers, and weight loss.

All control mice remained healthy and delivered normally at day 20 of gestation.

Observation, Isolation, and Identification of Microorganisms
Found in Mouse Fluids and Tissues after Consumption
Of Pine Needle Chow

Seventeen pregnant mice at day 9 of gestation were put into the experimental group; ten were used as controls. Six mice from the experimental group died during the course of study. Those surviving the ten-day test period were sacrificed and dissected at day 19 of pregnancy.

Gram stains of uterine fluid from all pine needle-fed pregnant mice revealed large numbers of microorganisms in concentrations ranging between 10^5 to 10^7 cells/ml as determined by the previously described counting method. (See Materials and Methods). When Gram stained, these organisms were found to be Gram-positive coccobacilli. In contrast, the uterine fluids from control mice exhibited few organisms. Of the organisms detected in the control group, the majority were Gram-negative rods.

Uterine fluid from control animals inoculated into TSY broths showed growth in only one case after 1 week of incubation. Gram stains of the turbid broth of the control case revealed Gram-negative rods. Samples streaked on TSY agar plates resulted in growth in two cases after 48 hours; colonies were mixed Gram-negative rods and mycelia. No growth was obtained on the other TSY plates or on blood agar after incubation for one week.

Samples from pine needle-fed mice that were inoculated into TSY broth showed no growth until after one week of incubation when Gram-positive coccobacilli were detected in large numbers. Scant growth was obtained on TSY plates in the same time period; small, mucoid colonies consisting of Gram-positive cocci were predominant. No growth was obtained on blood agar from these direct uterine fluid samples; however, TSY broth samples containing Gram-positive coccobacilli were streaked on blood agar and colonies resulted within 48 hours that were morphologically similar to those found on the TSY agar. These colonies were also found to be microscopically- β - hemolytic.

Gram stains of blood samples from pine needle-fed mice showed Gram-positive coccobacilli in large concentrations with leukocytosis in all blood specimens obtained from experimental mice (Table 2). Positive cultures resulted from four of the samples inoculated into TSY broth after five days of incubation at 37C; Gram stains of the broths revealed large numbers of Gram-positive coccobacilli. In contrast, no organisms were found in Gram stains of blood smears from control animals. A turbid broth was obtained in one case from a control animal; Gram stains revealed Gram-positive cocci that were subsequently identified as a strain of Staphylococcus.

Because of the hemorrhagic activity seen in the cecum of the intestine, cecal samples were obtained and examined for possible microflora disturbances. Gram stains showed alterations in the normal microflora when samples taken from experimental and control mice were compared; however, samples were difficult to definitely quantitate for specific Gram-positive coccobacilli because of the large numbers of organisms present in the cecum normally and the lack of a selective medium for these organisms. Generally, in the pregnant mice on the control chow, Gram-negative rods predominated. In the experimental mice, the Gram-negative

Table 2. Incidence of Gram-Positive Coccobacilli in Selected Fluids and Tissues of the Pregnant Animal after 10 Days of Diet Consumption

Tissue or Fluid	Experimental Mice		Control Mice	
	Positive Gram Stain	Positive Broth Culture	Positive Gram Stain	Positive Broth Culture
Uterus	4/5 ^a	3/5	0/5	0/5
Blood	5/5 ^b	4/5	0/5	1/5 ^c
Cecum	4/5	mixed culture ^d	0/5	mixed culture
Spleen	4/5	NA ^e	0/5	NA
Liver	0/5	NA	0/5	NA

^aThe numerator indicates number of mice positive for presence of the gram-positive cocci.

^bThe denominator indicates the total number of mice in the study.

^cOne broth culture from a control mouse had a contaminating form growing which was subsequently identified as a strain of Staphylococcus.

^dBecause of the large population normally present in the cecum, it was impossible to definitely determine the presence of the Gram-positive coccobacilli.

^eNot available

organisms were replaced with Gram-positive coccobacilli. Inoculations of cecal samples from both control and experimental mice into TSY broth resulted in abundant growth of Gram-positive and Gram negative rods and cocci within 48 hours of incubation.

Pathologic changes had previously been noted in the spleens and livers of pregnant mice on the pine needle chow; thus these reticuloendothelial organs were studied for evidence of microbial infection. The presence of viable Gram-positive coccobacilli in the spleens and livers of the mice was determined by enrichment in BHI broth and plating dilutions on BHI agar. An average of 4.3×10^4 cells/ml were seen after one week of incubation in 4 out of 5 spleens taken from pregnant mice fed the experimental chow. Gram-positive coccobacilli predominated in the enrichment medium. No Gram-positive coccobacilli were seen in any liver samples taken from the pine needle-fed mice. No bacteria were seen in any liver or spleen samples obtained from control mice.

No organisms were isolated from any meconium sample taken from feti of control and experimental mice.

Time Course for Viable Gram-Positive Coccobacilli in the Cecum, Uterus, and Bloodstream in Pregnant Mice

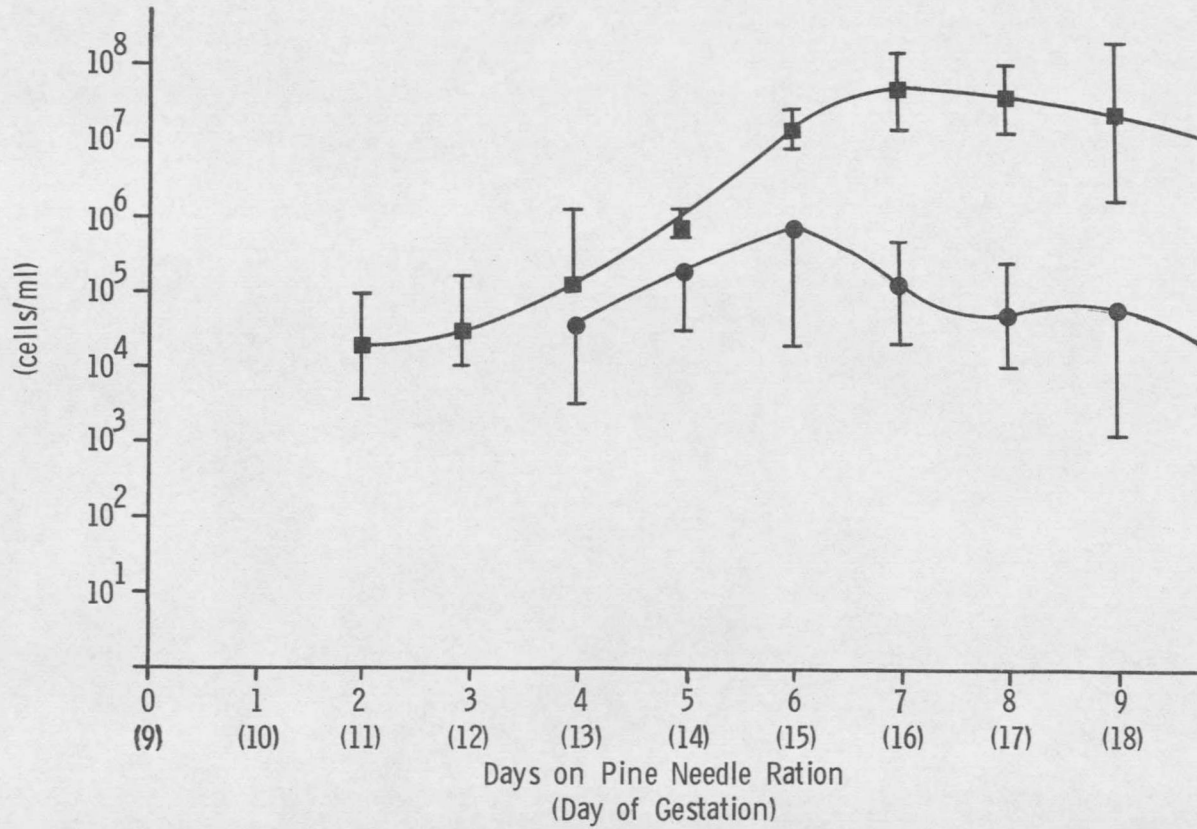
On Day 9 of Pregnancy

On day 9 of pregnancy, 89 mice were placed into the experimental group and 33 were continued on Wayne Lab-Blox as controls. Thirty mice succumbed to the effects of the pine needle chow. Three mice from the control group and 5 - 8 from the experimental group were sacrificed at daily intervals. Blood, uterine, and cecal samples were obtained from the sacrificed control and pine needle-fed mice. The results of the direct quantitation of organisms in the blood and uterus of pregnant mice fed the diet consisting of ponderosa pine needles are illustrated in Figure 8. The figure reveals that Gram-positive coccobacilli were first detected in the blood after two days of diet consumption (Day 11 of pregnancy); approximately two days later the organisms were seen in the uterus (Day 13 of pregnancy). The organism reached a peak concentration in the blood at 7 days of diet consumption and then declined in numbers.

In the control mice, the blood contained no organisms in any case when examined by Gram staining. This was essentially the case in the uterus, although some Gram-negative

Figure 8. Incidence of Microorganisms in the Blood and Uterus in Pregnant Mice Fed a Diet of Ponderosa Pine Needles

- represent concentrations of Gram-positive coccobacilli per ml of blood (with standard deviation).
- represent concentrations of Gram-positive coccobacilli per ml of uterine fluid (with standard deviation)



rods were seen.

The results from quantitation of the cecal samples showed a definite alteration in cecal microflora which was difficult to quantify. In the pregnant mice on the control chow, Gram-negative rods predominated, a condition that persisted throughout pregnancy. In the experimental mice, the Gram-negative flora was gradually replaced by Gram-positive organisms. This alteration appeared to begin at 1 -2 days of diet consumption and would sometimes reach such an extent that only Gram-positive coccobacilli were seen at day 19 of pregnancy. At this point in the study, the observed Gram-positive coccobacilli could not be isolated from the cecal samples. (See below).

All animals again showed the characteristic signs of the disease as described before. After two days of pine needle diet consumption, speckled livers, vaginal exudates, and pale kidneys were noted. Fetal death was not observed until day 3 of diet consumption with most of the feti dead by day 6. A correlation appears to exist between fetal death shown in Figure 3 (p. 25) and the abundance of Gram-positive coccobacilli in the uterus (Figure 8). The lowest

fetal survival percentages occurred when the Gram-positive coccobacilli peaked in concentration in the uterus of pregnant mice fed the pine needle chow. Pathological signs increased with the length of time on the pine needle diet and included the before-mentioned changes and reddened adrenals, enlarged gall bladders, and spleen atrophy. As stated before, accompanying these signs was maternal death with the highest mortality occurring after 6 days of diet consumption (day 15 of pregnancy). The highest maternal mortality (Figure 2) occurred when the concentration curve of Gram-positive coccobacilli in the blood reached its plateau (and highest) region (Figure 8).

Enumeration of bacteria in livers and spleens of pine needle-fed mice revealed infiltration of bacteria into the spleen averaging 2.3×10^4 cells/ml of spleen homogenate from day 12 of gestation throughout the remaining time period. No bacteria were seen in the liver at any time. The livers and spleen of control mice were negative for bacteria.

Influence of Age, Sex, and Pregnancy on the
Proliferation of Gram-Positive Coccobacilli in
Blood Samples

The results from quantitation of the previously-described groups of mice studied are presented in Table 3. From the results, it appears that the pregnant mice in latter gestation and immature mice allow for the most substantial proliferation of the Gram-positive coccobacilli organism although the presence of Gram-positive coccobacilli in the blood of all animal groups indicates that oral consumption of pine needles may result in septicemia in all animals regardless of age, sex, or pregnancy status.

Enumeration of Gram-Positive Coccobacilli in the
Bloodstream of Immature Mice Fed Pine Needle Chow

Thirty mice were fed the pine needle chow; 20 mice were employed as controls. Three mice from the control group and 4 -6 mice from the experimental group were sacrificed daily. The quantitative result of organisms found in the blood of immature mice fed the pine needle chow is presented in Figure 9. No organisms were observed in the blood from the control group. Fifteen pine needle-fed mice expired during the course of study; all were dead within four days

Table 3. Incidence of Gram-Positive Microorganism in Blood Samples Taken from Mice after 10 Days of Diet Consumption^a

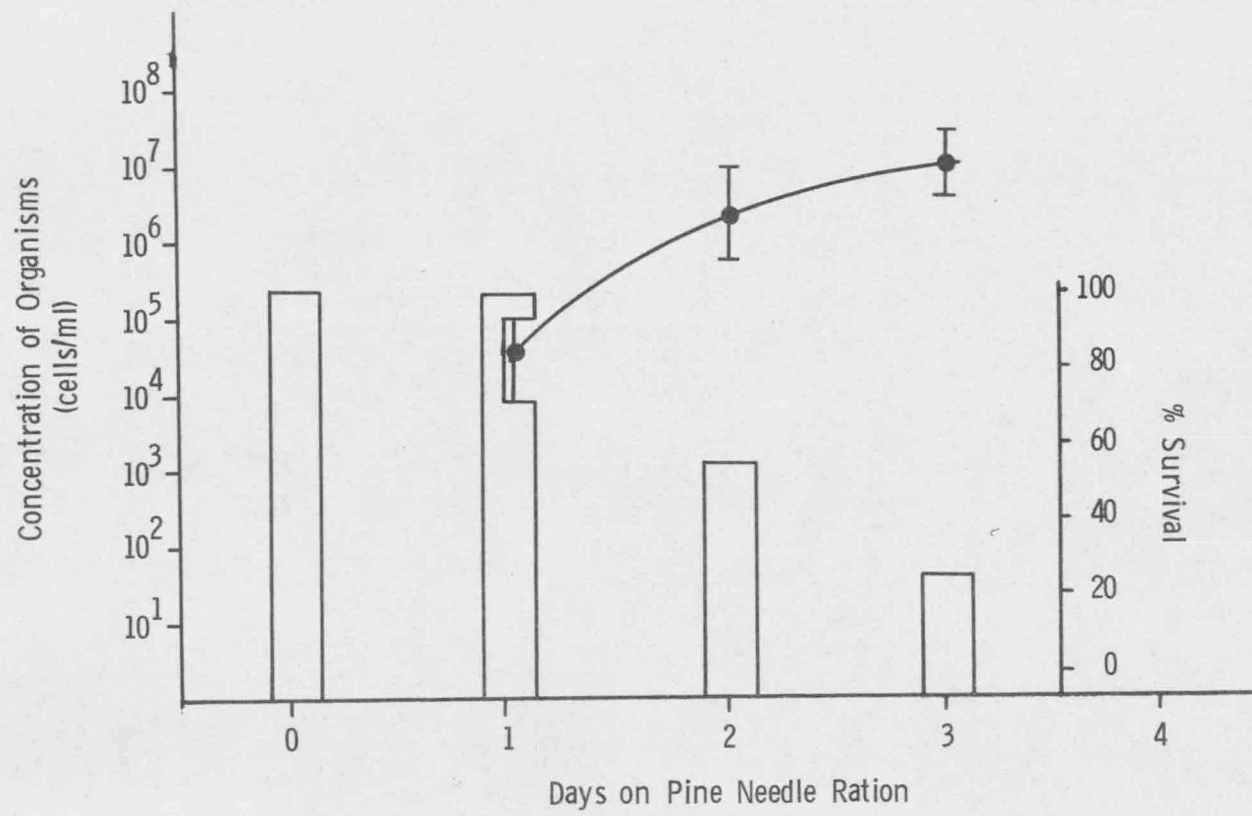
Group	Ration	Number of Mice	Percent Mice Yielding Positive Cultures	Estimated ^b Numbers of Organisms/ml
Pregnant females Day 9	Pine needle	10	90.0	2.3×10^6
Pregnant females Day 9	Control	3	0.0	-----
Pregnant females Day 4	Pine needle	10	80.0	1.1×10^5
Pregnant females Day 4	Control	3	0.0	-----
Adult cycling females	Pine needle	10	60.0	4.0×10^3
Adult cycling females	Control	3	0.0	-----
Adult males	Pine needle	10	100.00	5.7×10^3
Adult males	Control	3	0.0	-----
Immature mice	Pine needle	10	100.00	1.5×10^7
Immature mice	Control	3	0.0	-----

^aExcept in the case of immature mice (See Results)

^bEstimates based on direct counts of microorganisms in 0.1 ml samples of blood

48.

Figure 9. Comparison of the Survival of Immature Mice Fed a Diet of Ponderosa Pine Needles with Concentration of Gram -Positive Coccobacilli Found in Their Blood.



of diet consumption as illustrated in Figure 9. When percent survival of immature mice is related to the concentration curves of Gram-positive coccobacilli found in the bloodstream of these mice, an inverse relation is suggested.

Prior to death, the immature mice showed loss of coordination, extreme lethargy, starvation-like symptoms, and tendencies to huddle with other mice in the group. Necropsy revealed hemorrhagic intestines and ulcerated stomachs.

All control mice remained healthy and showed no ill effects.

Identification of Isolated Microorganisms

The microbial species found in the blood stream of mice fed the pine needle chow and isolated via TSY agar plates was identified as a strain of Listeria monocytogenes. The microbe was a Gram-positive coccobacillus, was motile at 22C and non-motile at 37C and was found to exist intracellularly. It produced acid but not gas from dextrose, lactose, maltose, and sucrose; it did not liquefy gelatin, reduce nitrate or produce acid or gas from mannitol and dulcitol. The isolated organism was catalase positive and reduced

litmus milk. Colonies were a characteristic black color when grown on potassium tellurite agar; colonies exhibited blue-green hue when grown on TSY agar and illuminated with an obliquely transmitted light source. The injection of the organism into the conjunctiva of rabbits resulted in a positive "Anton reaction." These observations are consistent with characteristics exhibited by Listeria monocytogenes. (Buchanan and Gibbons, 1974).

The serovar of the isolate was type 4b, although this typing was not consistent and showed considerable cross-reactivity with other serovars. The Center for Disease Control, Atlanta, also confirmed the serovar of the isolate as type 4b.

Evaluation of the Pine Needles for Microorganism

Gram stains of the pine needles often revealed a mycelium on the surface of the needles. No other morphological form was observed. Inoculation of the pine needles into TSY broth yielded an overwhelming abundance of mycelial growths sometimes with a few Gram-negative rods.

Characterization of Growth Potential of Isolated Gram-Positive Coccobacilli on Ponderosa Pine Needle-TSY Agar

Because of the nonselectivity of the TSY agar for Listeria and the difficulties encountered with growing the organism on TSY agar, an attempt to develop a more selective and enriching medium was made (see Methods). Cecal samples from both pine needle-fed and control mice that were streaked on aerobic and anaerobic TSY agar yielded numerous colonies exhibiting many types of morphology, i.e., Gram-positive and Gram-negative cocci and rods, in addition to mycelia. All dilutions (1/10, 1/100, 1/1000, and 1/10000 resulted in substantial growth.

No growth was obtained on the 0.5% pine needle agar, either aerobically or anaerobically. Data obtained for average numbers of colonies on the aerobic and anaerobic 1% and 10% pine needle agar are shown in Tables 4 and 5. Only samples taken from pine needle-fed pregnant mice yielded colonies on the pine needle agar; no growth was obtained on the agar from samples taken from control mice.

The colonies observed on the pine needle agar were white and mucoid and were morphologically composed of Gram-positive coccobacilli. These colonies were transferred to

Table 4. Number of Colonies found on 1% and 10% Aerobic Pine Needle Agar When Streaked with Cecal Samples from Pine Needle-Fed Pregnant Mice at Day 15 of Gestation

Time (Days)	1/10000	1/1000	1/100	1/10
1% Pine Needle Agar Dilutions				
1	---	---	---	---
2	---	---	---	---
3	---	---	---	---
4	---	---	---	---
5	---	---	---	---
6	---	---	---	---
7	---	---	---	---
14	---	---	---	---
10% Pine Needle Agar Dilutions				
1	---	---	---	---
2	---	---	---	---
3	---	---	---	---
4	---	---	---	---
5	---	---	---	96
6	---	9	23	97
7	---	9	29	103
14	---	9	30	100

TSY plates. After 48 hours at 37C, the resulting colonies were then transferred and maintained in TSY broth.

The organisms from both the anaerobic and aerobic pine needle agar were subsequently identified as Listeria monocytogenes by the methods previously described.

The Listeria found in the bloodstream and uterus of pregnant mice was plated on the 1% and 10% pine needle agar in order to substantiate the agar's potential to support the growth of Listeria. The organism was found to survive and propagate in substantial numbers (50-100 colonies per plate) at both pine needle concentrations. No growth was obtained on .5% pine needle agar.

Table 5. Number of Colonies Found on 1% and 10% Anaerobic Pine Needle Agar when Streaked with Cecal Samples from Pine Needle-Fed Pregnant Mice at Day 15 of Gestation.

Time (days)	1/10000	1/1000	1/100	1/10
1% Pine Needle Agar Dilutions				
1	---	---	---	---
2	---	---	---	---
3	---	---	---	---
4	---	---	---	---
5	---	---	6	11
6	---	---	6	11
7	---	---	6	11
14	---	---	6	11
10% Pine Needle Agar Dilutions				
1	---	---	---	---
2	---	---	---	---
3	---	---	---	---
4	---	---	---	---
5	---	---	10	17
6	---	---	10	17
7	---	---	10	16
14	---	---	10	16

CHAPTER 4

DISCUSSION

Listeria monocytogenes is apparently a ubiquitous bacterium and is found commonly in small numbers in many healthy specimens including several mammals, fowl, fish (Killinger, 1974). L. monocytogenes also exists as a saprophytic organism in soil and on plants (Weis and Seeliger, 1975). It is characterized by its diphtheroid-like forms, Gram-positive reaction, motility at room temperature, and tendency to occur in pairs. It is generally considered to be a facultative anaerobe and difficult to cultivate from natural sources; however, a number of selective media have been reported for its isolation (Weis and Seeliger, 1975, and references cited therein.)

The occurrence of Listeria infections is so sporadic as to lead one to believe that its distribution in nature is restricted in some unknown fashion and that the bacterium acquires pathogenic properties only under specific conditions or that the susceptibility of the host varies over time. Factors that cause and perpetuate listeriosis have not been fully delineated and it is not known why the organism can cause such a variety of disease forms including encephalitis,

meningitis, septicemia, abortions, liver diseases, and papular skin lesions (Gray and Killinger, 1966).

Infections by L. monocytogenes in ruminants are considered to produce (1) encephalitis and "circling" in adults and (2) septicemia in fetuses and neonates (Anonymous, 1976). Listeria - induced abortions in cows are a very serious and potentially fatal malady. Most abortions occur in the last trimester of pregnancy. Some calves are born alive but weak. Cows having late abortion frequently have pyrexia, depression, retained placentas, and purulent genital exudates. Abortions in a herd may be sporadic or multiple.

Stevenson et al. (1972) reported that pine needle-induced abortions in cows are likely to occur within 48 hours to two weeks after consumption of pine needles and have a number of similarities to Listeria - induced abortions. The abortion is characterized by weak contractions, incomplete dilation of the cervix and a nauseating odor. If the cow is near enough to term at the time of pine needle consumption, the calf usually is very weak at birth. A persistent retained placenta, atonic uterus filled with uterine fluid, placental debris and blood, and septic metritis which may be followed by peritonitis were constant findings.

In pregnant animals, L. monocytogenes appears to have a predilection for fetoplacental tissues, especially during the last trimester of pregnancy (Anonymous, 1976). Fetuses infected with L. monocytogenes may die and be retained for 24 to 72 hours before being expelled. Those affected near term may be aborted soon after death or may be born alive but weak.

Gray and Killinger (1966) report that similar outcomes have been produced in mice by injecting L. monocytogenes. With few exceptions, infections have been characterized by a septicemia. If the mice are pregnant, as in the ruminants, they may abort. The pathology noted, including the liver speckling, adrenal color change and enlargement, hemorrhagic intestines, abortifacient activity, and loss of coordination in pine needle-fed animals, is consistent with pathological alterations induced by L. monocytogenes (Bojsen-Moller and Jesson, 1966; Gray and Killinger, 1966). Differences in responses to the effects of the pine needles between our test groups are significant in that pregnant and immature mice showed a considerable increased susceptibility to the effects produced by the consumption of the chow which included extensive pathological changes and mortality. Adult males and non-pregnant females exhibited some signs of an adverse

response to the experimental chow which eventually subsided and yielded no gross damage to tissues. This is consistent with findings by Miller and Burns (1970), who reported finding liver lesions in all mice tested on the seventh day of oral feeding of L. monocytogenes. On the fourteenth day, there was little evidence in male and non-pregnant female mice of any lesions or other pathological changes; however, in pregnant mice the lesions were larger and more numerous. Although our observations appear to be similar, the course of the disease seemed to be accelerated in these studies.

Noteworthy are studies done in conjunction with this research, dealing with diet consumption. Mice fed the pine needle chow consume less food by weight than control mice; however, it was shown that the pathological signs one observes in pine needle-fed mice could not be reproduced by limiting the intake of Wayne Lab-Blox by control mice to the amount ingested by pine needle-fed mice (Neff, 1979). In these limited chow studies, pregnancy was maintained and no toxic signs were exhibited except for a minimal loss of weight.

Significant in the studies designed to characterize the isolated Listeria was the determination that the antigenic structure of the isolated organism was suggestive of Type 4b.

Gray and Killinger (1966) report that serovars of Type 4b in ruminants are commonly found to be virulent.

A point of consideration: What makes immature and pregnant mice more susceptible to the infection by L. monocytogenes? A link between these two groups of mice is their immune systems; in the immature mice, the immune mechanisms are not fully developed (Boraker and Hildemann, 1965; Green, 1966) and in pregnant mice, immune alterations have occurred during the gestational period particularly in regard to the T-cells (Maroni and deSousa, 1973). Perhaps the underdeveloped and/or altered immune systems of these classes of mice allow for the proliferation of Listeria. This would seem likely, particularly in the case of the pregnant mouse. Resistance and susceptibility to Listeria monocytogenes in mice has been found to be related to (1) the innate ability of the non-immune macrophages to kill or inhibit the growth of the organism during the first 24 to 48 hours after infection and (2) the onset of acquired cell-mediated resistance (Cheers et al., 1978) that is possibly modulated by the I region of the major histocompatibility (H-2) complex in mice (Cheers and McKenzie, 1978). Additionally, it appears that acquired immunity to listeriosis rests on macrophage activation by stimulated T lymphocytes

(Cheers et al., 1978). If T-cells are depressed in some manner, such as in the pregnant mouse, the ability of Listeria to survive and proliferate is enhanced (Youdim, 1977). Further evidence that the immune response may be involved in pine needle-induced changes comes from Baker and Campbell's (1978) report that factors from the cell wall of L. monocytogenes can reduce host resistance to listerial infection. Patocka et al. (1974) have described an "endotoxin-like" ether-extractable component of Listeria that had some resistance-decreasing properties. In addition, Silverman et al. (1969) have found a heat-stable filtrate of ultrasonically treated L. monocytogenes that had similar properties. It is suggested that purified Listeria cell walls must be present when and where the Listeria infection first occurs and that the effect is an early step in the infectious process (Baker and Campbell, 1978). These investigators found that mice pretreated with Listeria cell walls could not control even early growth of intraperitoneally-injected Listeria, suggesting that some early event is inhibited by the cell walls, although the mechanism is unknown. The above can perhaps be extrapolated to the pine needle-induced morphologic change. Possibly a compound of ponderosa pine needles, when ingested, stimulates Listeria which may

release endotoxin to impair the immune system. It has been reported that L. monocytogenes can often be isolated from mice that have been severely debilitated from another disease process or other stress factors that result in a stressed immune system (Gray and Killinger, 1966). These observations suggest the hypotheses that (1) Listeria resides ubiquitously in healthy carriers and (2) that an altered or stressed immune system may allow for the proliferation of Listeria.

Another possibility for the susceptibility of the immature and pregnant mice to the infection is in regard to their gut microflora. In the immature mouse, the balance between the various species of gut microflora is not established until the third to fifth week of age (Savage, 1970); in pregnancy, the gut microflora is altered, resulting in an increase in Gram-negative rods (Goldsmith, 1965). Perhaps these conditions allow for the proliferation of Listeria, either by the lack of competitive species or by the presence of strains of organisms which enhance the growth of Listeria.

It is not clear where the Listeria infection has its origins. However, it is possible that an initial enrichment of Listeria may first occur in the cecum of the pine needle-

fed mice. Alterations in this region in the form of proliferation of Gram-positive coccobacilli can be seen within 48 hours of diet consumption. Gram stains and culturing of the ponderosa pine needles in TSY broth showed no forms compatible with Listeria residing on the needles. Thus, the needles may contain an agent(s) that when ingested induces the proliferation of Listeria and thus the resulting pathological trauma. Further evidence supporting this idea comes from the observation that Listeria septicemia can be induced by the consumption of silage even though positive identification of Listeria on the material was not possible (Maurothalassits, 1977). The above implies that L. monocytogenes must reside within the healthy animal system. Although Listeria was never isolated from healthy specimens in this study, several investigators have isolated Listeria from fecal material of apparently healthy carriers including mice and humans who had no prior exposure to Listeria (Gray and Killinger, 1966; Kampelmader and Noorle Jensen, 1969; Seeliger, 1961).

L. monocytogenes reportedly can cause focal necrosis in the intestinal wall (Killinger, 1974); thus, the organism may enter the vascular system by causing breakdown of capillary walls and entering the portal system as evidenced by the

hemorrhagic intestines one observes when pregnant or immature mice have been fed the pine needle chow. In the present study Listeria was detected simultaneously in the blood and spleen of pine needle-fed mice at day 12 of pregnancy. At the same time, spleen and thymus weights dropped significantly and maternal death began. It is not known why Listeria could be isolated from the spleen and bloodstream of pine needle-fed mice but not the liver. It is interesting to speculate that perhaps the portal system is altered pathologically in these mice. If Listeria was entering the circulation as previously hypothesized, constriction or obstruction of the portal vein, possibly as a result of products produced by Listeria (i.e. toxin), could account for the absence of Listeria in the liver and its presence in the spleen. One of the side effects of portal system shutdown, in addition to ascites and esophageal varices, is a backup of blood to the spleen via the splenic vein as a collateral supply; perhaps this would allow deposition of microorganisms in the splenic tissue. Additionally, blockage of the portal system can give rise to small areas of infarcts in the liver resulting in a mottled appearance (Robbins, 1974) such as was observed in pine needle-fed mice.

It has been reported that L. monocytogenes in the bloodstream of pregnant mice have a predilection for placental tissue. Listeria is attracted to the uterine region of cattle and mice causing septic metritis, and fetal death (Gray and Killinger, 1976). The actual cause of fetal death is unknown. No Listeria have been isolated from the bodies of the dead feti, i.e., by culturing meconium from feti in mothers at day 15 of pregnancy. Therefore, the possibility that Listeria is directly responsible for the death of the feti is reduced in likelihood. Possibly the vascular system is disrupted resulting in lack of nourishment and oxygen and a buildup of toxic wastes normally metabolized by the mother's system. Toxins known to be produced by Listeria (see above) might cause severe pathological trauma, including death (Baker and Campbell, 1978; Patocka et al., 1974). Another possibility is that the presence of abnormal estrogen surges detected by Neff (1979) in pregnant mice fed the pine needle chow may disrupt the pregnancy since a normal gestational period is strongly influenced by a balance between hormonal levels.

Listeria was first detected in the uterine fluid of pine needle-fed pregnant mice at day 14 of pregnancy; two days after the appearance of Listeria in the blood.

Twenty-four hours later, significant death ensued followed by a maximum in the numbers of Listeria in the uterus when fetal death was at its maximum. After this point (day 16 of pregnancy), the percentage of surviving feti appeared to increase; however, these figures represent the survival percentages of the feti contained within mothers that survived to that time. Maternal death was more pronounced at day 15 of pregnancy coincident with maximum numbers of Gram-positive coccobacilli in the blood. The mothers surviving beyond day 15 may have the capacity to overcome the effects of the needles and therefore would give inaccurate indications as to the fetal survival rate during the last days of the gestational period.

Daily weights were obtained of organs that were observed to be involved in the pathological trauma noted in an attempt to quantitate the extent of morphologic change. Although the liver sometimes developed focal lesions, there was no loss or gain of weight of this organ throughout the test period in the pregnant mice fed the pine needle chow. This again lends credence to the hypothesis that portal vein obstruction could be a factor since obstruction of this system leads to an infarcted liver that does not enlarge or atrophy.

In contrast to the liver, it appears that relative weights do increase by day 16 of pregnancy in the pine needle-fed mouse. It has been reported that adrenal glands enlarge and become reddened under stress conditions (Seyle, 1950). One of the most notable stress situations that cause the increase in adrenal weight is that of certain microbial infectious processes such as the infection by Corynebacterium diphtheriae, a microbe with similar characteristics to L. monocytogenes (Buchanan and Gibbons, 1974). This enlarging and reddening of the adrenals due to stress conditions such as infections perhaps can be associated with the thymus atrophy observed in pregnant mice fed the pine needle chow since various stress elements such as infections, starvation, etc., can also cause "accidental thymic involution".

Listeria infections appear to be modulated primarily by T cells (Cheers et al., 1978). The involution of the lymphoid organs examined (the thymus and spleen) perhaps can be associated with the fact the thymus is responsible for primary processing of T cells and the spleen mainly houses the mature lymphocytes (Fudenberg et al., 1976). A dramatic depletion of lymphocytes in an attempt to overcome the Listeria infection could possibly result in organ atrophy such as what is observed in these studies.

The decrease in spleen weight in the pine needle-fed mice is puzzling. Spleens are not reported to decrease in weight during bacterial infections. Age, chronic hemolytic anemias, cancer, radiation of the spleen, and chronic starvation do cause spleen atrophy (Anderson and Kissare, 1976; Gordon and Katsh, 1949). However, none of these apply to this study. Support that the atrophy is a T-cell depletion resides in the observation that control pregnant females also show a loss of relative spleen weight during the gestation period study; significant in this observation is that pregnancy is characterized by a T cell deficiency. Perhaps the pine needle-fed mice represent an overdepletion of T cells because of the infection resulting in increased spleen atrophy when compared to the control animals. Reportedly, corticosteroids can cause spleen atrophy by causing a sudden and dramatic decline in the number of lymphocytes found in the spleen (Reed and Jutila, 1966). However, this would contradict stress involvement since stress situations influence the increased secretion of epinephrine and norepinephrine which in turn decreases steroid response by the adrenal gland. The corticosteroid involvement, however, becomes more of a reality when one considers that cortisone injections cause not only spleen atrophy but also thymic involution (Reed,

1966), and adrenal enlargement. In addition, cortisone injections reduce the level of L. monocytogenes when administered intraperitoneally prior to inoculation with a Listeria culture.

Obtaining adequate growth of Listeria on TSY plates proved to be difficult. Although scant growth of Listeria colonies could be achieved on TSY plates incubated in a CO₂ environment, it was not until the development of the pine needle agar that Listeria could be isolated from the cecum of the pine needle-fed mice. The capability of this agar to selectively allow the growth of Listeria even in the presence of a wide variety of other cecal microorganisms is evidence for the agar as a useful tool for research involving Listeria. Listeria was never isolated from the cecal material obtained from control mice, leading to speculation that either the Listeria resides in the cecum of control mice in numbers too low to be isolated or that the components of ponderosa pine needles are required for the organism to proliferate into the cecal region from some other part of the mouse system. As indicated before, Listeria could not be isolated from the pine needles, thus the organism did not enter the animal system by this method.

The purpose of this thesis was, by using a model system, to characterize possible microbial etiological agents of pine needle abortion in cattle. Although this work has not yet been repeated in cattle, the confirmation that similar pathological trauma can be induced in various strains of mice by the consumption of the pine needle leads to speculation that this animal model will be extended to a wide variety of mammals, including cattle.

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