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 in Microbiology
Montana State University

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 <u>LISTERIA MONOCYTOGENES</u>
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

in

Microbiology

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Bozeman, Montana

September, 1979

ACKNOWLEDGMENT

My sincere thanks and gratitude are extended to Dr. Larry Jackson, Dr. Dave Ward, and Dr. John Jutila for their expert advice and support throughout this project.

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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;

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$\pm$ 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 cm^2 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 10^6 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. Tenfold 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).

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.

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]

Mouse group	Ration	Number in group	Number in group surviving test period (10 days)
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 syptoms 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)

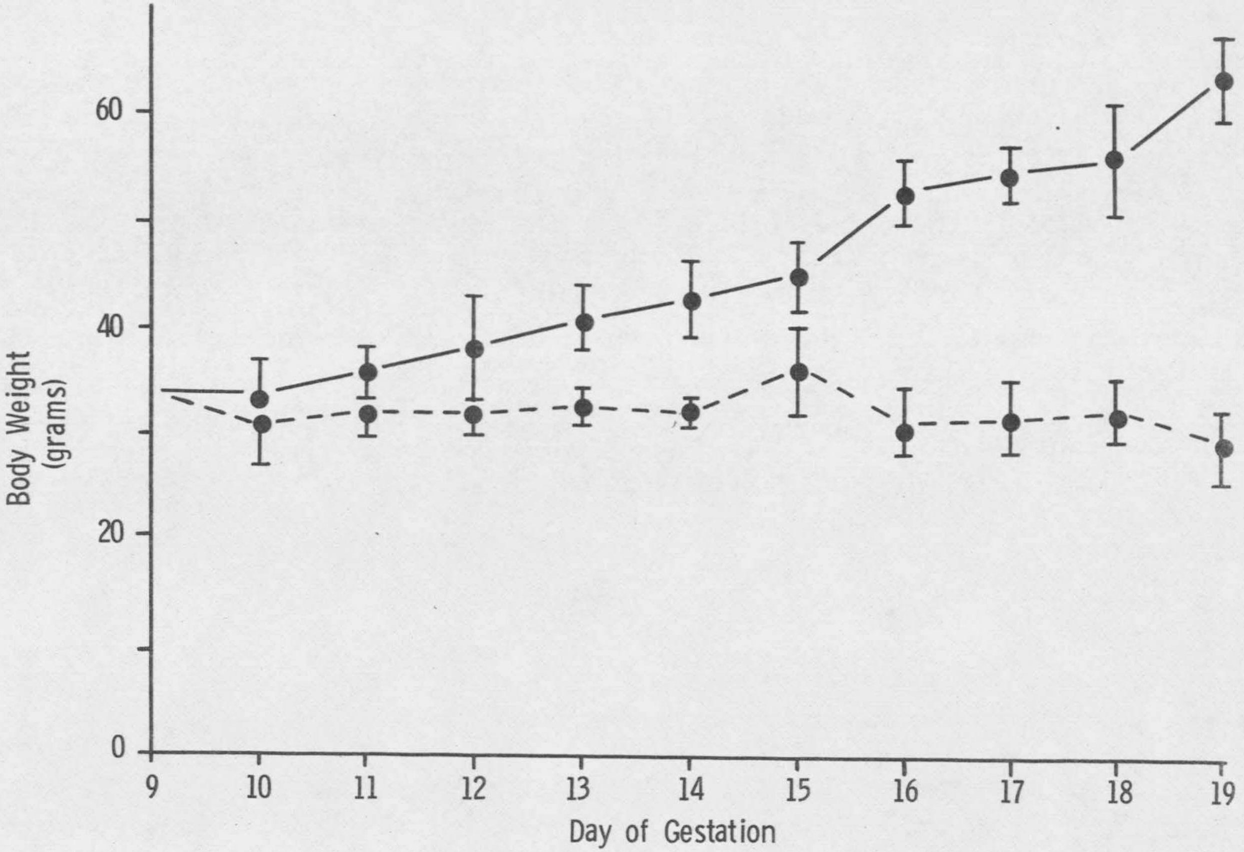
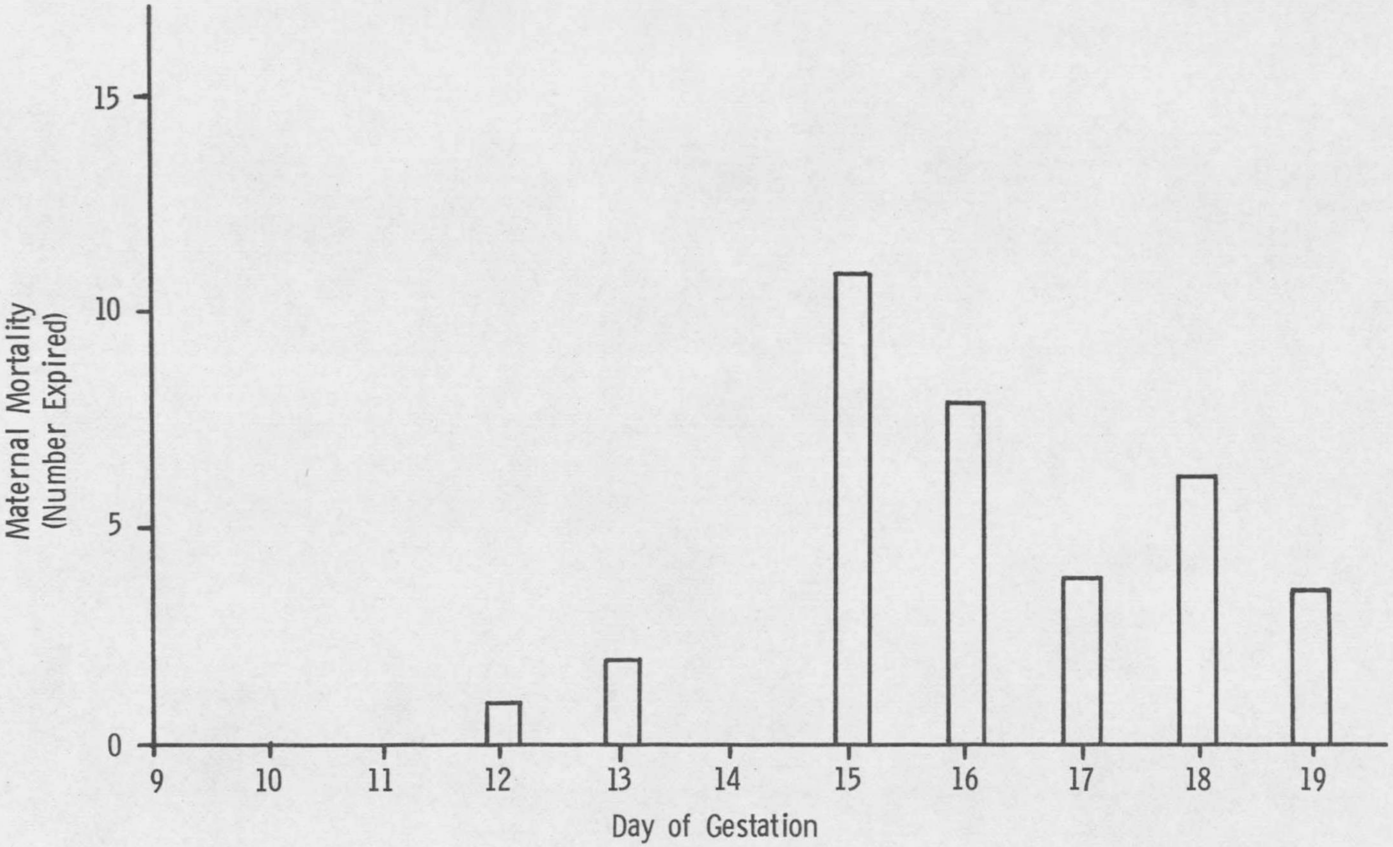

Body Weight
(grams)
60
40
20
0
9
10
11
12
13
14
15
16
17
18
19
Day of Gestation

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


Maternal Mortality
(Number Expired)
15
10
5
0
9
10
11
12
13
14
15
16
17
18
19
Day of Gestation

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 pregancy. 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.

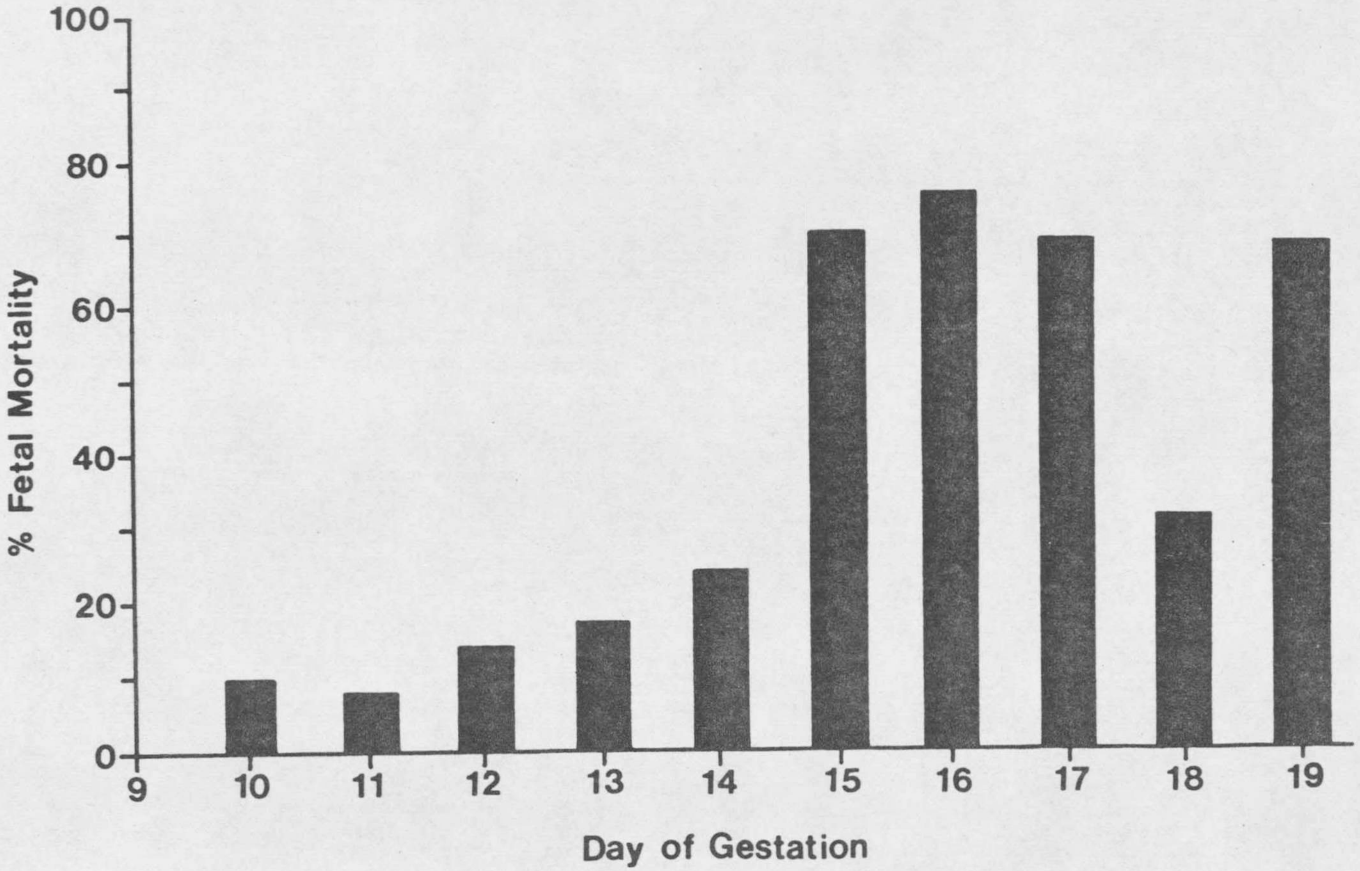

% Fetal Mortality
0
20
40
60
80
100
9
10
11
12
13
14
15
16
17
18
19
Day of Gestation

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)

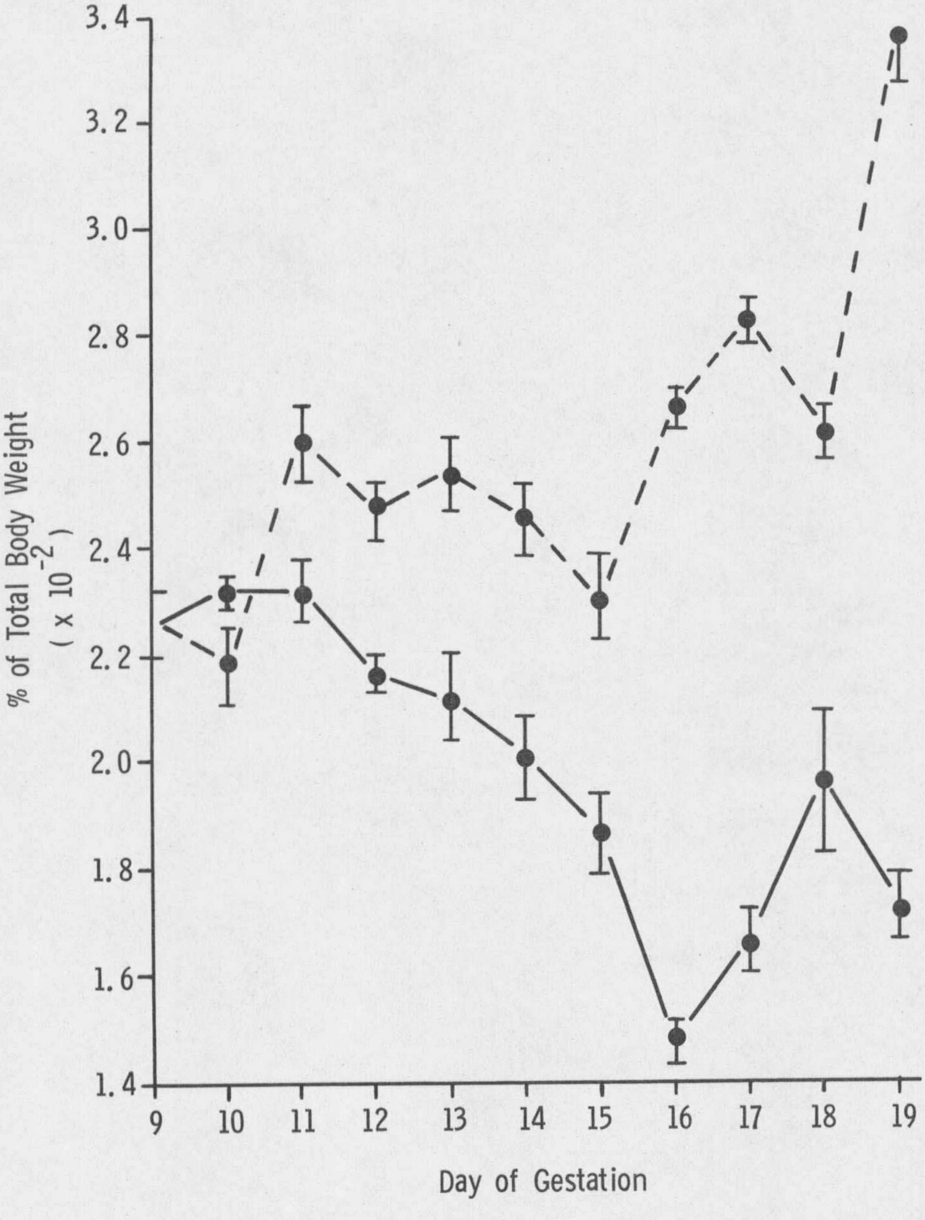

% of Total Body Weight
(x 10^{-2})
3.4
3.2
3.0
2.8
2.6
2.4
2.2
2.0
1.8
1.6
1.4
9
10
11
12
13
14
15
16
17
18
19
Day of Gestation

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)

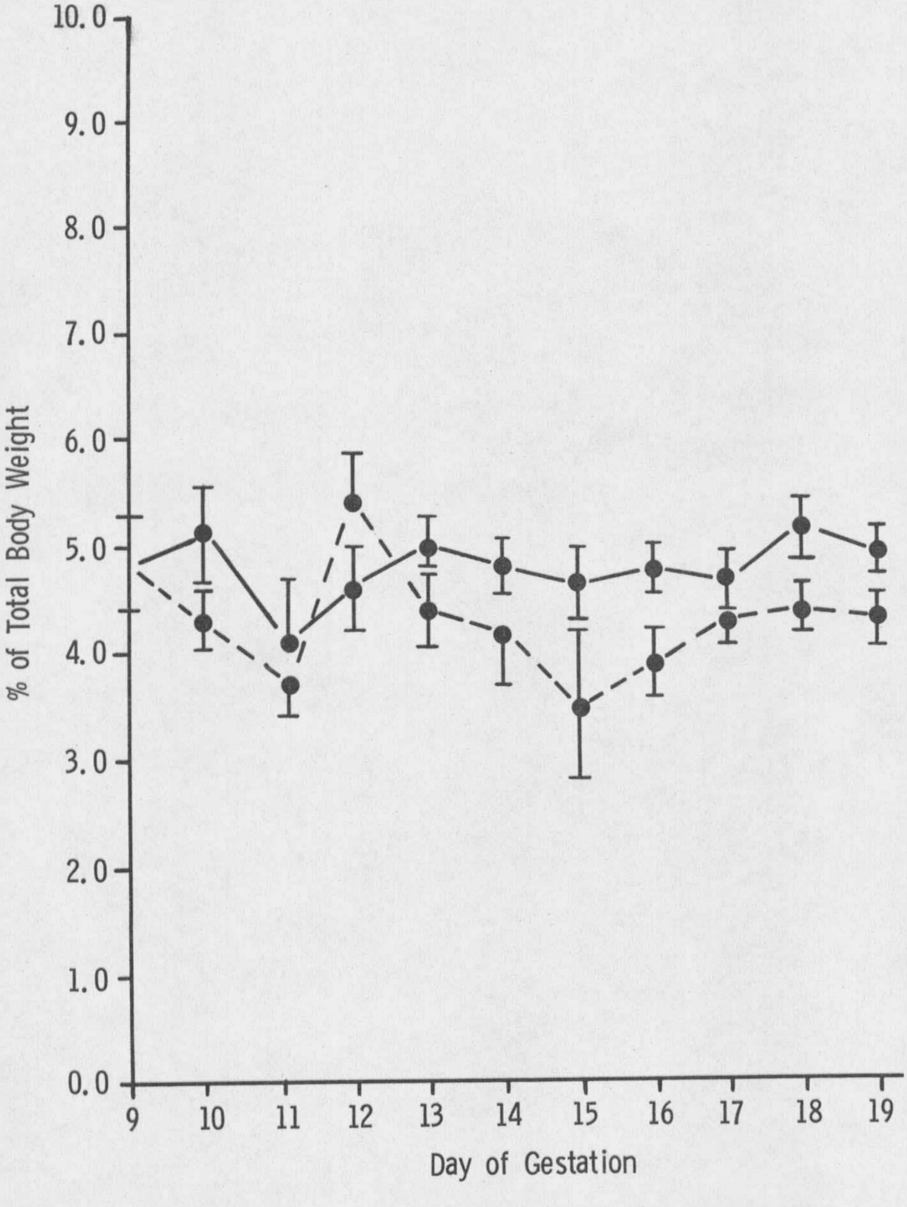

10.0
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
% of Total Body Weight
9
10
11
12
13
14
15
16
17
18
19
Day of Gestation

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)

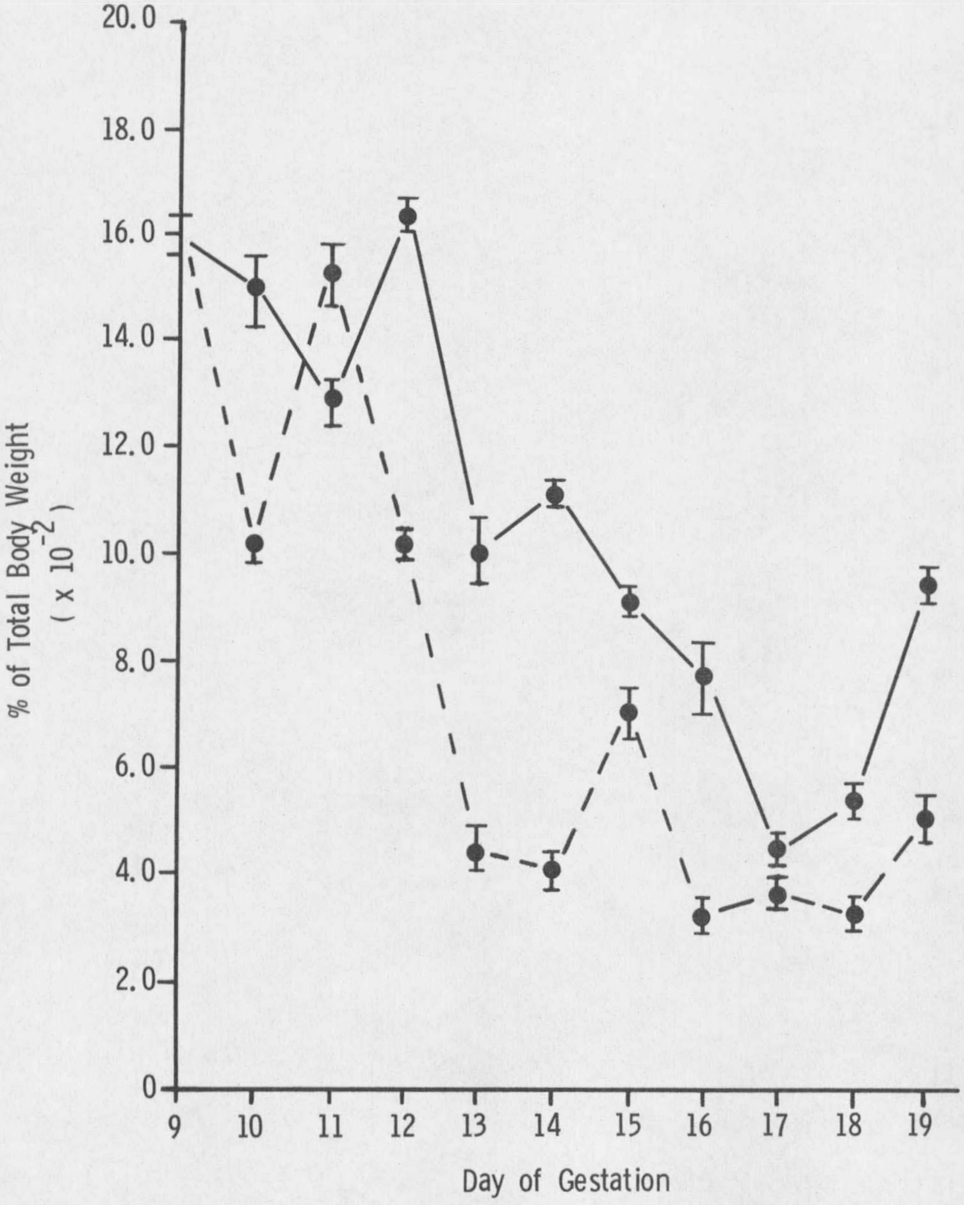
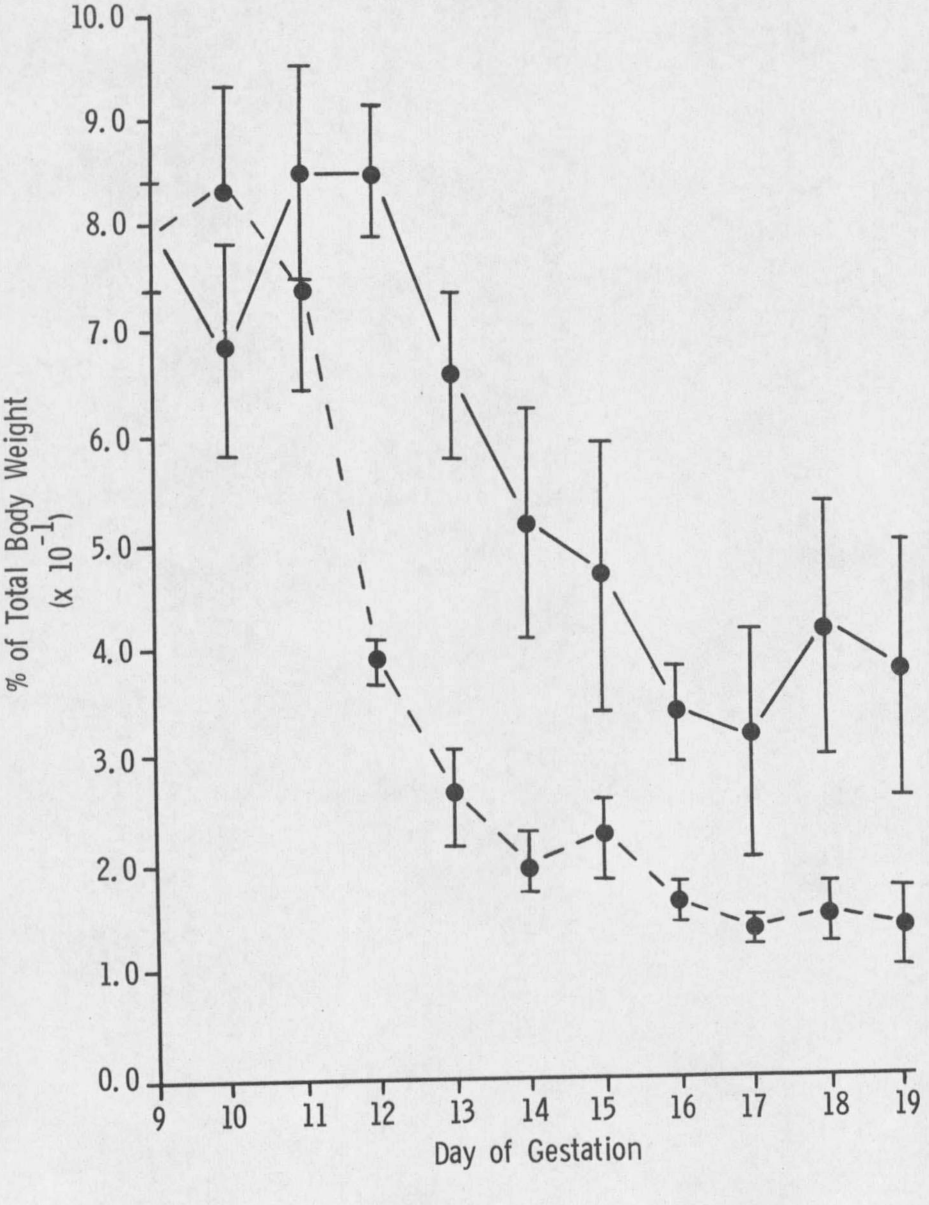

20.0
18.0
16.0
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0
% of Total Body Weight
(x 10^{-2})
9
10
11
12
13
14
15
16
17
18
19
Day of Gestation

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)


10.0
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
% of Total Body Weight
(x 10^{-1})
9
10
11
12
13
14
15
16
17
18
19
Day of Gestation

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-Posistive 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

[a]The numerator indicates number of mice positive for presence of the gram-positive cocci.

[b]The denominator indicates the total number of mice in the study.

[c]One broth culture from a control mouse had a contaminating form growing which was subsequently identified as a strain of Staphylococcus.

[d]Because of the large population normally present in the cecum, it was impossible to definitely determine the presence of the Gram-positive coccobacilli.

[e]Not 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)