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Pine Needle (Pinus ponderosa) Abortive Factor and its Biological Determination

Carolyn A. Cogswell

PINE NEEDLE (*PINUS PONDEROSA*) ABORTIVE FACTOR AND ITS BIOLOGICAL DETERMINATION

BY

CAROLYN A. COGSWELL

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This thesis is approved as a creditable and independent investigation by a candidate for the degree, Doctor of Philosophy, and is acceptable for meeting the thesis requirements for this degree. Acceptance of this thesis does not imply that the conclusions reached by the candidate are necessarily the conclusions of the major department.

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PINE NEEDLE (*PINUS PONDEROSA*) ABORTIVE
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Abstract

CAROLYN A. COGSWELL

Under the supervision of Professor L. D. Kamstra

A study was conducted to develop a reliable method to measure presence and quantity of the toxic factor in ponderosa pine (*Pinus ponderosa*) needles that interferes with normal maintenance of pregnancy in cattle. Special emphasis was given to biological assay sensitivity during embryo implantation and gestation in laboratory animals. The detrimental component was found to be present in both aqueous and acetone fractions prepared from ponderosa pine needles. There was a reduction in average rat litter size from bred rats fed either the aqueous or acetone pine needle extracts throughout pregnancy. Rats fed pine needle extracts from the winter pine needles delivered smaller litters at term than did those fed summer and fall pine needle extracts and the unidentified detrimental component in winter pine needles was more toxic to both dam and fetus.

Reduction in viable embryos was observed 5 to 7 days post coitum from mice fed aqueous or acetone pine needle extracts from any of six collection dates. Adrenal weight obtained during the implantation study reflected a time x treatment x age interaction but failed to provide definite conclusions about ponderosa pine needle toxicity. Gestation study verified results from the implantation experiment as very few mice fed pine needle extracts delivered normal litters. Mice receiving the concentrated aqueous extract suffered diarrhea and inadequate feed intake.

It appeared evident that a biological assay at implantation could be used to estimate injury from feeding ponderosa pine needle extracts to bred laboratory animals throughout gestation.

Both dried needles and branches collected throughout the year were analyzed for moisture, ash, crude protein, ether extract, acid-detergent fiber, acid-detergent lignin and *in vitro* dry matter digestibility. In general, chemical components and *in vitro* dry matter digestibility changed very little on a month to month basis but low protein content and low digestibility would hinder long term maintenance of an animal on ponderosa pine needles and branches.

The use of biological assay in early gestation research should serve as a useful screening tool for eventual isolation of the "active ponderosa pine needle abortive factor."

TABLE OF CONTENTS

LIST OF TABLES

INTRODUCTION

Cattlemen in the western United States and Canada where the ponderosa pine (*Pinus ponderosa*) is the predominant tree are plagued each year by the loss of calves due to pine needle abortion. The calf losses in the Black Hills area of South Dakota and Wyoming number into the hundreds each year according to a 1974 survey conducted by the Black Hills Area Resource and Development Project. Of the 173 ranchers who answered the six county questionnaire, 129 or 75% indicated that they had suffered calf losses suspected to be due to pine needle abortion. It was estimated over 600 calves were lost by the ranchers in a three year period from 1972 to 1974. Keeping bred cows excluded from ponderosa pine trees is currently the best management practice to help alleviate the problem. It is difficult to fence off pastures on many ranches. The pine needle abortion problem has not been solved but avoided for the present time. With the increased economic trend to use all available ranges, research should be conducted to solve the occurrence of ponderosa pine needle abortion.

Ponderosa pine needle abortion was described as early as 1927 (Bruce, 1927). Little research has been devoted to solve the problem. Attempts to isolate the causative factor have failed in previous research due to a number of reasons. (1) Ponderosa pine needle abortion does not occur every year in areas that have once experienced the problem. (2) There are many factors which can lead to abortion, as yet no measurement has been found which will identify pine needle abortion other than the absence of bacterial or viral organisms in suspected pine needle abortion cases. (3) Researchers have not always been able to produce the detrimental effects of ponderosa pine needle consumption at will. (4) It is not certain whether the abortive factor is present in pine needles during all seasons or if it is a periodic toxic component.

The primary objective of this study was to develop a reliable method to measure not only the presence but the intensity of the "pine needle abortive factor." Measures used to test the sensitivity of the biological assay were:

1. To determine if reproductive disturbances would occur in laboratory rats and mice after the feeding of pine needle extracts.

2. If reproductive disturbances did occur, determine the critical periods when the toxic factor present in ponderosa pine needles was at its highest concentration.

3. Determine which of the pine needle extracts was the most toxic to the pregnant rat and mouse.

4. To obtain information concerning the nutritional value of ponderosa pine for the controlled laboratory experiments and also an insight as to why ruminants readily consume ponderosa pine branches and needles.

LITERATURE REVIEW

General

It is estimated that the net calf crop of states within the western region of the United States is slightly less than 80%. Abortions and stillbirths of calves result in a 2 to 3% annual loss (National Academy of Sciences, 1968). Although many abortions are due to bacterial or viral infections and hormonal imbalances, consumption of toxic plants may account for considerable calf losses. A live healthy calf is the goal of all beef cow ranchers. Ponderosa pine (Pinus ponderosa) needle abortion results in the loss of hundreds of calves each year (S. E. Selway, personal communication). As yet, the chemical or biological cause of ponderosa pine needle abortion has escaped detection.

The presence of antifertility substances in plants does not seem impossible because physiologically active materials such as digitalis, quinine and antibiotics have been isolated from plants. De Laszlo and Henshaw (1954) compiled a list of over one hundred plants that contained substances affecting human reproduction. Antifertility components of plants may act as oral contraceptives, interfere with implantation or gestation, or expedite parturition in the female. In the male, antifertility substances may produce sterility by the prevention of spermatogenesis. Primitive people especially the early tribes in Europe and the Western Hemisphere were said to have used many different plants and herbs as antifertility measures.

Yarrow (Achillea millefolium L.) was used by people in western Europe to suppress estrus. A powder was made of the plant and added to make up a 20 to 50% concentration of the normal diet (De Laszlo and Henshaw, 1954). The Navaho Indians of Colorado were known to use a milkweed (Ascepias hallii) infusion as an oral contraceptive after childbirth (Bailey, 1950). Another plant used as an oral contraceptive by the Navaho Indians of Arizona, was the antelope sage (Epiogsium jamesii). The root was boiled for 30 minutes and then consumed by women to prevent conception (De Laszlo and Henshaw, 1954). A superstition among a Mediterranean tribe was that the white poplar (Populus alba) bark taken with a mule kidney caused sterility in females (De Laszlo and Henshaw, 1954). Some of the early beliefs using plants and herbs to control fertility have remained superstition and folklore, however, many plants and herbs are known to be abortive and also poisonous to both man and animals.

"Poisonous plants are those that contain or produce under natural conditions physiologically active or toxic substances in sufficient amounts to cause harmful effects in animals, including man. The quantity of a poisonous plant ordinarily consumed may contain enough of the toxic principle to cause death or only a diseased condition (Muenscher, 1945)." Classification of the poisonous nature of a plant may be by the presence of one or more different kinds of substances.

The first classification occurs when substances present in the plant are themselves poisonous to the animal. An example is poison hemlock (Conium maculatum) found principally in the western states. The animal dies from respiratory paralysis 2 to 3 hours after consuming poison hemlock. The toxic alkaloid present is coniine. A second classification is found when substances present in the plant themselves are harmless, but may decompose to form toxic products either before or soon after they are eaten. An example of this type of poisoning occurs when the nontoxic glucoside, amygdalin produced in the wild chokecherry (Prunus virginiana) hydrolyzes to form the highly toxic hydrocyanic acid. The third classification occurs when toxic substances are formed by the action of microorganisms or fungi on plants or plant products. Fungi under certain conditions produce mold in hay or silage forming toxic decomposition products (Kingsbury, 1964). The classification of the poisonous nature of ponderosa pine needles is still unknown.

When a noxious influence is placed upon a female animal during the first third of pregnancy, it may cause death of the dam, death with or without abortion of the embryo or congenital deformity of the embryo depending upon the severity and nature of the influence. Abortion per se is defined as a fetus which is imperfectly formed or developed and fails to reach complete development (Hafez, 1968). During early pregnancy before implantation and tissue differentiation, the detrimental effect upon the embryo is likely to be embryonic death and resorption. If the injurious effect occurs during the latter two-thirds of pregnancy, injury to the fetus is unlikely unless the damage is great enough to harm the dam. The end result would likely be a stillbirth or abortion due to fetal damage. The embryo as it is called during the first third of pregnancy does not react to toxic conditions in the same way as the fetus since no inflammation or leucocytosis develops (Klopper and Diczfalusy, 1969). The reaction is one of abnormal development and it is often impossible to determine even histologically whether such an abnormality has been caused by infection or by heredity.

Plants and herbs capable of causing abortion are more prevalent with livestock maintained on range conditions. Often poor management practices are an indirect cause of abortion problems because pregnant animals are forced to graze poisonous plants when their normal vegetation is scarce in grazing areas. A great deal of research remains to be done to counteract the toxic principles in plants. This is difficult since many times the toxic component present in a plant has not yet been isolated and identified.

Plants Containing Abortive or Toxic Components

1. Creeping indigo (Indigofera endecaphylla Jacq.)

Creeping indigo is classified as either an annual or perennial herb. The plant was brought from the tropics to Florida in 1925 and later to the Hawaiian Islands (Emmel and Ritchey, 1941). It was used as a soil builder and forage in Hawaii until toxicity problems occurred. Beta-nitropropionic acid was tentatively identified as the toxic component (Cooke, 1955). However, later studies have indicated that the plant is extremely high in nitrate content (Hutton, Windrum and Kratzing, 1958a,b). Toxicity in cattle is primarily expressed in reproductive difficulties. Dead or very weak calves were produced prematurely or near term in animals pastured on Indigofera for 25 days or longer (Yelf, 1959).

2. Lechuguilla (Agave lecheguilla)

A perennial known as lechuguilla grows in the arid climate of Texas and New Mexico. Severe liver and kidney damage is likely to occur in ruminants that have ingested lechuguilla. A saponin from the plant can produce visceral vasodilation, gastroenteritis and often hemorrhage when administered experimentally to rabbits. In small amounts the saponin caused abortion (Dollahite, Shaver and Camp, 1962). Severe outbreaks that occur during drought can cause mortality as high as 30% in range livestock (Mathews, 1938).

3. Tansy (Tanacetum vulgare)

The herb tansy has been known since the Middle Ages to contain both an antihelminthic and an abortive factor. Medicinal use of an extract derived from the herb used to treat human disorders has side effects that cause convulsions and gastritis (Musson, 1906). Livestock rarely graze on tansy but cows can abort after consuming the herb (Gress, 1935).

4. Poison hemlock (Conium maculatum)

Tall, coarse-growing poison hemlock which is prevalent in the northern tier of states and southern Canada is another abortive-causing plant. The alkaloid in hemlock is present in small amounts in the root and concentrates in the stems, leaves and seeds (Marion, 1950). Both coniine and nicotine contain a pyridine nucleus, therefore; it is not surprising that they function alike in the body. They produce an initial stimulatory action followed by severe depression upon the central nervous system. The heart is slowed with eventual paralysis and coma. In the pregnant animal, abortion may occur if the animal survives (Forsyth, 1954).

5. False hellebore (*Veratrum viride*)

False hellebore is widely distributed in the northern regions of the United States. The toxic principles present in the plant were identified in the 1950's as the *Veratrum* alkaloids (Taylor, 1956). Some are glycoalkaloids and others appear as ester alkaloids. The steroid configuration is present in both types of alkaloids. *Veratrum californicum* has been traced as the toxic source that causes the "monkey face" syndrome in lambs. In some years the syndrome accounted for a 1 to 25% lamb loss to ranchers in Idaho (Babbott, Binns and Ingalls, 1962; Binns, Anderson and Sullivan, 1960; Binns *et al.*, 1962; Binns *et al.*, 1959). The lambs are born with a malformation of the face and cranium. In mild cases the face is shortened and the cranium protrudes because the nose and jaw are so poorly developed. In severe cases the eyes and nose are entirely missing with a rudimentary jaw. Lambs carried to term are usually born alive but die shortly afterwards. Ewes that feed on *Veratrum californicum* during the weeks shortly after conception are very likely to have "monkey faced" lambs. The dam may also abort. So far, the compound interfering with normal pregnancy has not been identified. Despite some attention to the pharmacological action to the *Veratrum* alkaloids, little is known about the metabolic processes involved in the interference with normal fetal development.

6. Broomweed (*Xanthocephalum sarothrae*)

Broomweed is known under a variety of names such as turpentine weed, perennial snakeweed and slink weed. The resinous plant is most toxic in the leaf formation stage of development. It is prevalent in the arid climates of Texas, California, Colorado, Idaho and Mexico. Improper grazing conditions are ideal for its growth and probably why animals sometimes consume the plant. A saponin extracted from the plant has been suggested as the probable cause for the toxic effects of the plant (Dollahite *et al.*, 1962). A wide variability in susceptibility to the abortive effect of broomweed has been reported in cattle (Dollahite and Allen, 1959; Dollahite and Anthony, 1957a,b). As little as 20 pounds of fresh broomweed produced abortions in 7 days. In other experiments cattle ate as much as 1,097 pounds and aborted after 117 days on broomweed feeding. The abortifacient properties of broomweed are less readily demonstrated in goats and sheep than swine, cattle and rabbits.

7. Goldenrod (*Solidago* spp.)

Goldenrod is widely distributed in the United States. Early research indicated that a rust (*Coleosporium solidagenis*) was the probable abortive causing factor (Helmer, 1893). Later research by Rusby (1896) suggested that not the rust but ironweed (*Veronia noveboracensis*) was capable of causing abortions in humans as well as cattle. An outbreak of abortion in cattle has been linked to a *Coleosporium* species of fungus found growing on the goldenrod (Freer, 1949).

8. Chewings fescue (*Festuca rubra*)

Chewings fescue is commonly used in lawn and turf seed mixtures. In Oregon, screenings of chewings fescue seed heavily infested with nematode galls (*Anguina agrostis*) have caused fatal poisoning in both ruminants and nonruminants (Haag, 1954; Hardison, 1953). Symptoms in sheep and cattle have been similar whether they were induced naturally or experimentally (Shaw and Muth, 1949). Abortion appeared common in pregnant ewes and was evident in about 6 to 10 days after consumption of 10% or less of an animal's weight in screenings. Lesions in the intestinal tract, heart and gall bladder may or may not be present. A gangrene similar to ergot (Claviceps purpurea) poisoning was demonstrated in the experimental feeding of rats (Haag, 1954). As yet, the suspected alcohol soluble and heat stable toxic component is unknown.

9. Monterey cypress (Cupressus macrocarpa)

Certain seed bearing plants of the Pinale order are also capable of inducing abortion in pregnant animals. The monterey cypress is also known as the macrocarpa. In North America, this tree grows only on the Monterey Peninsula of California. Outbreaks of abortion in cattle have, however, occurred in New Zealand (Macdonald, 1956). Cows aborted after consuming needles anywhere from two weeks to two months before term. Maternal cotyledons were found to be greatly enlarged. Fetal membranes were darkened in color and firmly adhered to the placenta. The animals suffered severe straining with retained placentas and died unless promazine hydrochloride was administered. The drug rapidly reduced cotyledonary swelling and promoted passage of the placental membranes.

10. Juniper (Juniperus virginiana)

The juniper was listed as toxic to livestock in Old World literature. The bitter taste of the volatiles present in the tree is one important reason why the needles are rarely consumed. Digestive disturbances probably occur when the animal is forced to consume the needles because natural vegetation is inadequate. Abortion may occur in pregnant animals (Norris and Valentine, 1954).

11. Ponderosa pine (Pinus ponderosa)

Indian women in the states of Oregon and Washington have been reported to prepare a water extract of ponderosa pine needles to induce abortion (Tucker, 1961). Indians in the western states also stripped the bark from ponderosa pine trees and ate the cambium layer (Collingwood and Brush, 1955). Historically it is known that many of the Indians in the western states did live near or among the ponderosa pine forests.

Cattlemen and veterinarians in certain areas of the western United States and Canada have frequently observed that beef cows abort or deliver weak calves of low vitality if pine needles are consumed in late pregnancy. Retained fetal membranes are often associated with the abortions and weak calves.

Confirmation of Pine Needle Abortion

The first published reference to ponderosa pine needle abortion was made by Bruce in 1927. He based his belief that pine needles caused abortion upon reliable reports of ranchers who had experienced the problem. Gunn (1948) disputed the pine needle abortion theory because there were numerous other nutritional and disease imbalances that could lead to abortion. A vitamin A deficiency, phosphorus deficiency and brucellosis can also cause abortion. Since accurate experimental proof was missing to prove or disprove the ponderosa pine needle abortion theory, the problem remained questionable until the early 1950's.

Muenscher (1945) stated that a number of conifer trees including the pines could be harmful when large amounts of the browse was consumed or when it composed the exclusive diet of the animals. However, because of the resinous taste animals seldom eat the needle browse unless forced to do so. Range cattle may consume pine needles when their normal forage is covered with snow or badly overgrazed (Bracken, 1968).

With the assistance of the British Columbian Beef Growers Association, MacDonald in 1952 undertook a series of studies to determine whether or not ponderosa pine needles caused abortion in range beef cows. Eighteen brucellosis free pregnant cows were divided into three comparable weight groups. In group I, cows were given fresh pine needles and buds at an initial 5 pounds per head per day and intake gradually increased until at the completion of the trial each cow consumed 8 pounds of pine needles daily. Group II cows were provided free access to fresh pine needles and group III cows received no pine needles in their diet. All cows on trial were provided with an adequate wintering ration for pregnant cows according to the allowances recommended by the National Research Council (Guilbert, Gerlough and Madsen, 1945).

MacDonald concluded that pine needles and buds caused abortion and weak calves based upon his trial results. In the first group of cows, three calves were born dead, one calf born alive but died later, one calf was born weak and small and two calves were normal. Group II cows produced one calf born dead, four calves born alive but died later and one normal calf. At the end of the trial there were five cows in the control group and they all produced normal calves. The experiment confirmed that ponderosa pine needles did cause abortions and stillbirths.

Pine needles may be very palatable to cows and they will actually crave needles even on adequate nutritional rations. Schmutz, Freeman and Reed (1968) claim that cows will easily consume 5 to 6 pounds of fresh pine needles daily. Appreciable consumption of pine needles has been especially noticed during the coldest part of the winter when cows are in the last trimester of pregnancy (Doorenbal, 1955). Nicholson (1957) tried to produce abortions in cattle by the feeding of lodgepole pine (Pinus contorta) needles. Although consumption was as high as 10 pounds per day, no abortions occurred. Apparently the abortive factor is not present in lodgepole pine.

Deem, Osborn and Maag (unpublished data reported by Faulkner, 1968) conducted three experiments using bred cows and heifers that were brucellosis and leptospirosis free. Four cows examined as five to six months pregnant were used in the first trial. Three cows received fresh pine needles and the fourth was used as a control. One cow on pine needles aborted 9 days after being placed on the trial. The other two cows on pine needles delivered small weak calves after 12 weeks on the needles. The two calves died soon after they were born. The cow receiving no pine needles delivered a normal calf at term. Vitamin A blood levels were examined to investigate whether pine needles interfered with vitamin A absorption. Results indicated the vitamin A levels remained in the normal range.

In the second trial of Deem et al., four heifers were started on a pine needle - alfalfa pellet ration during their third month of gestation. The pellets were made by grinding fresh pine needles with alfalfa in a ratio of one part needles to three parts by weight of hay. Four other heifers began receiving the pine needle pellet ration after their seventh month of pregnancy. Four control heifers received a diet of alfalfa pellets. Results showed no reproductive disturbances to any of the heifers on the pelleted pine needle - alfalfa ration. All heifers on experiment delivered normal calves at term indicating that pelleting destroyed the toxic effect of pine needles. Work by Chow et al. (1972) further confirmed that the toxic effect in pine needles is destroyed by the pelleting process.

Fresh pine needles were ground with alfalfa hay daily in the proportion of 25% needles and 75% alfalfa for the third trial (Deem et al.). Four mature cows were started on 9.1 kg of the mixture daily during their third month of pregnancy while four others were placed on the pine needle - alfalfa mixture after the seventh month of pregnancy. Three control cows were fed 6.8 kg of alfalfa hay daily during the trial period. Two of the four cows started on pine needles after the third month of gestation aborted 109 and 142 days after placement on the trial. The other two cows in this group delivered normal calves but had retained placentas. One of the four cows that began to consume pine needles after the seventh month of gestation aborted 21 days after placement on the trial. The other three cows in this particular group delivered normal calves at term but all had retained placentas. The control cows delivered normal calves, although one cow had a retained placenta and another delivered twins. Post mortem examination of the abortion cases showed that the fetus had been dead several days before the abortion occurred.

It has not been established whether cows are less susceptible to pine needle abortion during early pregnancy or the cows simply do not graze on the ponderosa pines during the late spring, summer and early fall. One of the prime effects in a Grant County, Oregon field observation (Stevenson, James and Call, 1972) was retained placentas regardless of the stage of pregnancy when pine needle abortion occurred. Manual attempts to remove the placenta were often associated with excessive hemorrhage. The uterus was atonic and filled with uterine fluid, placental debris and blood. Mortality was high in cows that aborted. Toxicity of the fetus was questionable in their study since some calves were born weak while others born near term showed no toxicity signs.

Suggested Modes of Pine Needle Action Upon Pregnancy

Hormonal Imbalance. Reproductive performance can decrease in an animal due to certain hormonal imbalances. Recognition has been made of the fact that infertility in females can result from consumption of certain plants. Uteri of such females may not, under an estrogen stress, be able to respond to progesterone priming in order to maintain the desirable uterine environment for proper implantation and uterine development. Also the uterus may not be responsive to normal estrogen stimulus if a compound present in a plant blocks or inhibits the effects of estrogen during reproduction (Fox et al., 1957; Ostrovsky and Kitts, 1963).

Estrogenic activity may be assayed either chemically or biologically depending on the purpose of experimental investigations. Very high sensitivity preceded by chemical extraction and purification are required for accurate biological assay determination. The best biological indicators are consistently demonstrated under definite physiological conditions and observed in more than one species. Effects of the hormone are attributed to direct or specific actions. They can be observed easily and quantitatively. Some biological assays may be more sensitive than chemical or physical methods. The most sensitive methods cover the range from 5×10^{-6} to 5×10^{-4} micrograms of estradiol or estrone per mouse assay. A great drawback of assays particularly those employing intravaginal cornification response is the large number of experimental animals needed per assay (Emmens, 1950).

All natural estrogens isolated so far have been steroids. They possess a cyclopenta-perhydro-phenanthrene nucleus. Synthetic estrogens and those that occur in forages and plants are not steroids. The most potent natural estrogen is estradiol-17β. It is produced by the ovary with lesser amounts produced by the adrenals, placenta and the testes of the stallion (Williams, 1968). Another natural occurring estrogen found in urine, the adrenals and placenta is estrone. It is generally more potent than estriol but less potent than estradiol-17β. Intravaginal tests have shown estriol to be less potent than estrone (Biggars and Claringbold, 1954).

Dodds et al. (1939) were the first to produce a series of synthetic estrogen like compounds. Diethylstilbesterol, the most well known synthetic hormone has the potency when injected between that of estrone and estradiol. It has a potency equal to estrone when administered the intravaginal route. In contrast to natural estrogens, the synthetic types such as diethylstilbesterol, hexestrol and dienestrol are active when given orally.

Estrogen functions in many ways. Two of the immediate important roles to be considered here are its influence in preparing the uterus for the arrival of the fertilized ovum and its role in the birth of the fetus. Estrogen causes growth of the uterus by increasing the surface area of endometrium and myometrium. It stimulates uterine contractibility by increasing the degree and rate of contractions at parturition. This stimulation is largely accomplished by the ability of estrogen to increase the sensitivity of the uterus to oxytocin (Hafez, 1968). Under normal conditions, estrogen levels are low during early pregnancy and gradually increase until the last trimester of pregnancy at which time estrogen levels rapidly increase until parturition (Asdell, 1964; Nalbandov, 1964).

The uterus is prepared for implantation through the joint stimulation of progesterone and estrogen. A potentiator, probably coumesterol, and an inhibiter of estrogen have been shown to be present in alfalfa by Bickoff et al. (1959) and Adler (1962). Ostrovsky and Kitts (1963) studied effects of estrogenic plant extracts upon uteri of rats. It appeared that infertility resulted from the inability of the uterus under constant stress from plant estrogens to respond to progesterone priming. Therefore the embryo development cannot be maintained because the uterine environment is not proper.

Four substances pinoslyvin, its monomethyl ether, pinocembrin and pinobanksin are very common polyphenols present in the heartwood of the Pinus genus. An excellent series of investigations concerned with the separation of the polyphenolic substances was done by Lindstedt (1950). Since other stilbenes such as diethylstilbesterol have marked effects on reproductive processes, Cook (1960) investigated the possibility that ponderosa pine needles contained certain stilbenes. An unidentified stilbene compound different from pinoslyvin was found to be present in an ethyl alcohol eluent after partition chromatography. It is still not known whether this stilbene compound would cause reproductive disturbances in cattle and laboratory animals.

Toner (1971) further investigated the possible estrogenic like action of ponderosa pine needles. Three bred heifers were drenched via stomach tube with 5 pounds of fresh pine needles for 3 days. Blood samples collected on the third day after receiving needles had elevated plasma estrogens levels in comparison to levels prior to treatment ($P<.05$). Estrogen levels remained high for approximately two more days. Progesterone steadily decreased as estrogen increased. Approximately 24 hours after estrogen first peaked, progesterone levels reached their lowest point. This was immediately followed by a significant progesterone surge while estrogen levels declined. Ponderosa pine needles with their ability to increase estrogen synthesis could possibly upset the normal uterine progesterone controlled environment necessary to maintain pregnancy.

Progesterone is secreted by the corpus luteum of the ovary, placenta and to lesser extent from the adrenal cortex and testes. As long as progesterone is the predominant hormone in the uterus, the myometrium is blocked and the fetus cannot be expelled (Ganong, 1969). Progesterone levels rise in cows soon after ovulation and remain high after conception until one month prior to parturition at which time levels rapidly decline (Nalbandov, 1964). It is the progesterone block that maintains pregnancy and the withdrawal of progesterone induces parturition. The consumption of ponderosa pine needles may somehow have the ability to upset the proper hormonal checks necessary for the normal development of the fetus.

An anti-estrogen as defined by Ostrovsky (1960) is any substance that can cause a decrease in uterine weight or prevent vaginal cornification. According to Adler (1962), alfalfa contains an anti-estrogen which inhibits the physiological responses of estradiol and diethylstilbesterol injections. The compound present in alfalfa must have structural similarities to the different forms of estrogen and is capable of inhibiting the actions of all of them.

In an attempt to solve the problem of abortion following the consumption of ponderosa pine needles, it was discovered that the needles may also contain an anti-estrogenic factor. This research employed a six-hour rat bioassay for uterine weight (Allen and Kitts, 1961; Cook and Kitts, 1964; Allison and Kitts, 1964). It was demonstrated by Allen and Kitts (1961) that an aqueous fraction of ponderosa pine needles contained a factor which depressed the uterine weight of immature weanling mice. An ether fraction made from the needles increased the mortality rate in the mice under study. The aqueous, acetone and ether fractions of ponderosa pine needles appeared to depress reproductive performance in female mice.

Cook and Kitts (1964) further studied the possible anti-estrogenic effect of pine needles and verified that the aqueous fraction depressed uterine weight in laboratory rats and mice by oral and parenteral administration. Allison and Kitts (1964) found that aqueous and chloroform extracts prepared from pine needles when fed or injected decreased uterine weight in immature mice. The mice had received prior priming doses of estrone and estradiol-17β. Allen and Kitts (1961) prepared an amorphorus material from pine needles that showed slight estrogenic activity on uterine weights of immature mice. Increases in embryonic death or resorption were noted in pregnant mice experiments. The toxic effect appeared to be accumulative. The longer that the aqueous fraction was fed during the gestation period, the lower the live weights of the mice were at birth.

Mode of action of the proposed anti-estrogen present in ponderosa pine needles is unknown. Cook (1960) postulated three possible pathways. First, the agent may inactivate the naturally occurring estrogens circulating in the blood. Second, the agent may render the uterus nonresponsive to estrogen stimulation. Third, it may decrease uterine size directly without interfering with estrogen activity. The action must occur in one of these manners and not by influencing the pituitary, since this possible route would take longer to show its effect than the time allowed in the six-hour bioassay.

Mineral Imbalance Relationship. Range cattle often are maintained on sublevels of nutrition during the winter months. This is primarily because range forages lose much of their protein, energy and phosphorus content with maturity and leaching (Stoddart and Smith, 1955; Watkins and Knox, 1945). Nutritional deficiencies lead to lowered metabolism efficiency by the animal.

Phosphorus derivatives are involved in the formation of many enzymes and coenzymes involved in the metabolism of carbohydrates, proteins and lipids. A deficiency of phosphorus results in decreased protein synthesis and energy utilization by decreasing the efficiency of the energy transport system (Mahler and Cordes, 1966; Maynard and Loosli, 1969). Animals suffering from a phosphorus deficiency exhibit the characteristic symptom of a depraved appetite. Animals chew on boards, bones, and other uncommon materials including pine needles. Feed intake is reduced in a phosphorus deficiency. Inadequate phosphorus levels result in decreased fertility in cattle (Rombauts, et al., 1961).

Toner (1971) conducted a series of experiments to determine if protein or phosphorus deficiencies were a factor in ponderosa pine needle consumption. Results indicated that rats deficient in both protein and phosphorus consumed a greater percentage of their total feed when a pine needle extract was added ($P < .05$). Control animals did not tend to crave their ration as much as rats on deficient rations. Eighteen bred heifers were also studied for depraved appetite in relation to greater pine needle consumption. Heifers deficient in phosphorus consumed significantly greater amounts of fresh pine needles on the one month study. Unfortunately, the study was discontinued before any abortion occurred.

Toxic or abortive principles. The ponderosa pine native to western North America is extremely hardy and drought resistant. In South Dakota, the ponderosa pine is found principally near or in the National Black Hills Forest. The tree has also been described as the yellow pine and bull pine. Under usual range conditions the tree reaches a height of 70 to 80 feet, although it may grow 160 to 170 feet. Needles are usually in fascicles of two to three. The stout needle is 4 to 7 inches long and gives off a spicy turpentine odor when crushed (Ferrell, Collins and Macksam, 1965).

At present there is a great amount of information on the chemistry of many pine products such as cellulose, lignin, phenolic compounds of wood and bark, turpentine and rosin, essential oils of foliage and minor components such as alkaloids. However, prior research has principally been conducted for industrial uses. Very little is known about the toxic effects present in pine needles that are harmful to man and animals.

Pine needles have varied numbers of resin ducts. When served these ducts exude oleoresins that appear to be much thinner, i.e., richer in volatiles oils than the oleoresins obtained from the resin ducts of sapwood. The resin ducts of needles are not connected with the resin ducts of the pine wood. This is the reason why the composition of volatile oil obtained from the pine needles differs considerably from the composition of volatile oil obtained from the wood. Hunt (1912) distilled needle oil of several western North American pines and found that the yield of the oil was very poor. Pinus ponderosa yielded an oil 0.11% of the weight of twigs and needles. When the needles were carefully separated from the twigs, the yield of volatile oil in the needles was 0.75%. Schorger (1919) analyzed material distilled by Hunt (1912) and found the following components in the needles and twigs of ponderosa pine:

1 - α - pinene	2%
1 - β - pinene	75%
dipentine	6%
borneol	7%
bornyl acetate	2%
"green oil" (probably alcohols and sesquiterpenes)	3%

Early investigations of pine needle oil were unacceptable for biological purposes. Either the botanical source of material was not certain or as in the case of Schorger's (1919) work the analyses were suggestive but not conclusive.

Age of the needles is important in the amount of needle oil. Primary leaves or needles appear above the cotyledons very shortly after germination and assume the functions of foliage in the winter growth period. The leaves are always single and different in appearance from secondary needles, occasionally even during the first growing season. Within two to three years primary leaves are completely replaced by secondary needles, remaining on the shoot only as scarious bracts. The shedding of needles is influenced by the environment. Weidman

(1939) reported on a plantation in northern Idaho. Ponderosa pine needles remained on the tree three to four years. In its natural habitat, in low altitudes, needles act in the above manner. In medium altitudes, they remain on the tree for six years, and in high altitudes, they remain on the tree for six years, and in high altitudes the needles may remain on the trees for eight years (Mirov, 1967).

Zavarin *et al.* (1971) investigated the influence of season and needle age on the composition of ponderosa pine oil. Qualitatively their results agreed with Schorger (1919). They did find several other minor monoterpenoids with the only large difference in the identification of methyl chavicol as the major pine oil constituent. It sometimes made up 40% of the total needle oils.

Biosynthetically, methyl chavicol is more closely related to the formation of lignin than are the terpenoids. The difference could be associated with the fact that common precursors are involved in the formation of plant cell walls. Some processes of growth such as lignin accumulation in photosynthesis are known to continue in pines well beyond the point of maximal needle extension (Kramer and Kozlowski, 1960). Methyl chavicol (Zavarin *et al.*, 1971) content of the young developing needle is much lower than neighboring mature needles. This difference was present in October, well beyond the time needles reach their maximum growth extension. In general, the amount of methyl chavicol and total monoterpenoids was highest in the summer. They were lower in juveniles than in the first year needles. A possible intermediate in the formation of methyl chavicol has recently been suggested as the toxic component causing pine needle abortion (Zavarin, personal communication).

Volatile oils present in pine needles contain turpentine. In sufficient quantities turpentine is capable of inducing abortion (Dreisbach, 1971). Turpentine is the steam volatile fraction of needle oleoresin. It is not always a chemically pure substance. Generally, turpentine consists of cyclic hydrocarbons, monoterpenes of an empirical formula $C_{10}H_{16}$. There is almost always an admixture of sesquiterpenes ($C_{15}H_{24}$) and sometimes nonterpene substances (Mirov, 1967).

One may wonder whether the turpentine composition changes during the year and whether this variation corresponds to the season when ponderosa pine needle abortion is the greatest. So far it is not known, since turpentine composition varies greatly among individual pine trees (Herty, 1908). Black and Thronson (1934) found that during a single season the optical rotatory properties of individual pine tree species did not follow any definite pattern. All evidence seems to indicate that turpentine composition which is almost pure limonene in ponderosa pine trees varies little throughout the year (Mirov, 1967). Previous research in the area of pine needle abortion has provided little evidence that turpentine alone is the toxic factor.

Recently published work from Colorado by Chow et al. (1972) indicated the "unknown abortive agent in pine needles neither acts as a contraceptive nor affects fertilization and implantation. The disruption of pregnancy seemed to occur during the stage when the placenta begins to grow." Klopper and Diczfalusy (1969) defined the placenta as an association of the endometrium and the chorion. It serves as an organ of resorption, excretion and exchange of material, provides nourishment and respiration for the fetus and provides the transport of raw material and waste products. Placental membranes also play a considerable role in the exchange of material between the dam and fetus. It stands to reason that if a toxic substance damaged the placenta there would be termination of pregnancy. Cell division is very rapid at the time that the placenta begins to grow. An investigation to assess the pathological effects of the abortive factor on placental cell structure of placental tissue of laboratory animals would be a worthwhile continuation of the work by Chow et al. (1972).

EXPERIMENTAL PROCEDURES

Ponderosa Pine Collections

Monthly collections of approximately 4.5 kg of fresh ponderosa pine needles were collected in a pine enclosure area for a year. The pasture was located immediately east of the Fort Meade Veterans Administration Hospital at Sturgis, South Dakota. Only the lower branches from the trees were hand clipped. The branches were comparable in height to what the grazing cow consumed. Needles were stripped from their branches, divided into individual 100 g bags and stored as received at -20° C. These samples were used to prepare the pine needle extracts for the biological assays.

Needle and branch samples used for chemical composition and *in vitro* dry matter digestibility determinations were cut into 2.54 cm long segments and dried in a large force draft oven at 40° C for 48 hours. Branch samples were ground to pass a 20 mesh screen in an Intermediate Size Wiley mill. The needles were ground to pass a 40 mesh screen.

The collection dates were:

Collection 1 - July 30, 1971
Collection 2 - August 27, 1971
Collection 3 - September 30, 1971
Collection 4 - October 27, 1971
Collection 5 - November 30, 1971
Collection 6 - January 5, 1972
Collection 7 - January 21, 1972
Collection 8 - February 28, 1972
Collection 9 - March 23, 1972

Collection 10 - April 26, 1972

Collection 11 - June 5, 1972

Collection 12 - June 27, 1972

Experimental Animals

Mature virgin female rats (Rattus norvegicus) of the Sprague-Dawley strain were used for the preliminary gestation experiment. Female rats were randomly mated using two females per male in a cage. The vaginal smear technique was used to determine estrus.

Virgin female mice (Mus musculus) of the BALB/c strain (Jackson Laboratory, Bar Harbor, Maine) were used for experiments conducted on embryonic growth and mortality during both implantation and gestation.

Preparation of Pine Needle Fractions for the Rat Studies

1. Water-soluble fraction

Each 100 g of needles was cut into 2.54 cm segments and macerated in a Waring blender with 400 ml distilled water. The liquid portion was decanted into a Buchner funnel and the residue extracted again with an additional 400 ml distilled water. Filtrate and washings were concentrated in a flash evaporator and designated as the aqueous fraction.

2. Acetone-soluble fraction

The solids, a green mass retained from the aqueous extraction were transferred into the Waring blender. The material was extracted with a total of 400 ml acetone following the same procedure used for the water extraction. The filtrate and washings were concentrated in a flash evaporator and designated as the acetone fraction.

Preparation of Pine Needle Fractions for the Mouse Studies

1. Water-soluble fraction

The water-soluble fraction was prepared essentially as before except that the filtrate and washings were freeze-dried. During the initial freeze drying period, the thermostat was set at 0° C for the first 4+ hrs and then set at 20° C for an additional 12+ hours. After completion of freeze drying, the remaining crystalline extracted material was weighed into plastic bags and stored in a desiccator.

2. Acetone-soluble fraction

The same procedure was used as in the rat experiment except that the green filtrate was freeze-dried after the removal of acetone by the flash evaporator. The material was freeze-dried at 0° C for the first 4+ hrs and then the thermostat was set at 20° C for the next 8+ hours. After completion of freeze drying, the green fraction was weighed into plastic bags and stored in a desiccator.

Rat Experiment

The objective of this experiment was to ascertain if the feeding of ponderosa pine needle extracts to bred rats was detrimental to embryonic development. Feeding of the pine needle extracts began three days after mating. Needles used for the rat experiments were collected on July 30, 1971, October 27, 1971 and January 5, 1972.

The amount of either pine needle fraction added to the basal feed, Purina Laboratory Chow, was calculated according to the amount of each fraction obtained from a known weight of fresh needles. For consumption purposes, the amount of concentrated extract obtained from 25 g of fresh needles was added to each 100 g of the basal feed. Control rats were fed only the ground basal feed throughout the experiment.

Twenty-eight bred female rats were used for the experiment. They were randomly allotted by approximate equal weight basis into groups of 4 rats per treatment. Females were fed the individual test ration until their expected date of parturition.

EXPERIMENTAL DESIGN FOR RAT GESTATION STUDY

Test Ration	Type of Extract	Number of Rats
Control		4
July 30, 1971 Collection	Aqueous	4
	Acetone	4
October 27, 1971 Collection	Aqueous	4
	Acetone	4
January 5, 1972 Collection	Aqueous	4
	Acetone	4

Consumption of each feed and the amount wasted or refused was recorded daily and fresh feed was added. The number of females giving birth, average litter size and total number of young rats surviving after 3 days post partum was recorded. Initial weight at birth and at 3 days post partum was recorded for each young rat.

Mouse Experiments

1. Preparation of the extract rations

The amount of freeze-dried fractions added to the basal feed, Purina Mouse Chow, was as follows:

1:1 ration means that an amount of the freeze-dried extract equivalent to 1 g of fresh pine needles was added to 1 g of basal feed.

2:1 ration means that an amount of the freeze-dried extract equivalent to 2 g of fresh pine needles was added to 1 g of the basal feed.

2. Collection periods concerned with mouse experiments

Pine needles used for this phase of experimentation were collected during the suspected months when pine needle abortion seemed to be more prevalent in the ponderosa pine grazed ranges. Collection dates were: November 30, 1971; January 5, 1972; January 21, 1972; February 28, 1972; March 23, 1972 and April 26, 1972.

3. Gestation Study

Thirty-eight virgin female mice 8 to 10 weeks old were randomly mated using two females per male. Females were examined for the presence of a vaginal plug daily. Post coital timing began at 4:00 A.M. Bred mice were not placed on experiment until 1 day post coitum (pc).

Two bred mice were fed only the ground basal feed, Purina Mouse Chow, throughout gestation and served as controls. Two bred mice were caged together and placed randomly according to equal weight basis on any one of the following treatments: aqueous 1:1, aqueous 2:1 and acetone 1:1 for every collection studied. Weight of the mice was recorded twice a week. Data was also obtained on the number of mice giving birth, average litter size, average birth weight of young in a litter, and total number of young dead after birth. If a mouse did not deliver at the expected date of parturition, the mouse was sacrificed by axial dislocation. An autopsy was performed to examine the gastro-intestinal and reproductive tract.

4. Implantation study

Twenty-four virgin females were randomly mated and placed on experiment for each one of the collection dates under study. At 1 day pc, the bred mice were allotted into groups of six mice per treatment. The treatments were: Control (ground Purina Mouse Chow); aqueous 1:1; aqueous 2:1 and acetone 1:1. Two mice per treatment were sacrificed consecutively at 124 hr, 148 hr and 172 hr pc by axial dislocation. Each uterus from the mice on experiment was excised, trimmed and placed in 0.8% physiological saline (8 g NaCl per 1 l distilled H_2O) for microscopic examination. The total number of viable embryos in each uterine horn, presence of abnormalities, occurrence of resorption and overall physiological condition of the vital organs was recorded on the sacrificed mouse. Adrenal glands were excised, trimmed and weighed.

Chemical Analysis Procedures

The method of Van Soest (1963) was used to determine acid-detergent fiber and acid-detergent lignin content of the needle and branch samples. The only change made in the procedure was the use of an asbestos padded Gooch crucible for filtering.

A.O.A.C. (1970) methods of analysis were used for the determination of crude protein, ether extract, ash and moisture.

In Vitro Dry Matter Digestibility

In vitro dry matter digestibility was carried out based on the procedure of Tilley and Terry (1963). *In vitro* dry matter digestibility (IVDMD) was calculated by the following formula:

$$\text{IVDMD} = 100 \times \frac{\text{sample dry matter} - \text{residue of fermentation} - \text{residue of inoculum}}{\text{sample dry matter}}$$

All statistical analyses presented were according to the methods outlined by Steele and Torrie (1960).

RESULTS AND DISCUSSION

Preliminary Experimentation—Rat Gestation

Previous research by Allen and Kitts (1961) indicated that when an aqueous extract of ponderosa pine needles was fed to bred mice more than 50% of the mice on the experimental ration completely reabsorbed their young before term. Other groups of mice on acetone and ether extracts delivered no viable fetuses. In their research, the acetone extract was the parent liquor from which the other two extracts were prepared. This may have been the reason that the acetone extract was so toxic in its effect on reproduction.

An aqueous 1:1 fraction of pine needles fed to bred mice in the research conducted by Chow et al. (1972) resulted in a 50% reduction in the number of mice giving birth. Average litter size was almost reduced to one-half in comparison to the number of young born to control mice that were fed no pine needle extracts. While an acetone 1:1 fraction appeared to have no effect on the number of bred mice giving birth or average litter size, it should be realized that this particular concentration of pine needle extract was much less than the volatile fractions used in the study. Volatile 3:1 and volatile 4:1 fractions which were three and four times more concentrated than 1:1 fraction caused only an increase in the number of young dead after birth. Possible resorption was also more apparent at sacrifice in some of the bred mice fed the volatile fractions.

Based upon prior research, it appeared evident that the aqueous fraction derived from ponderosa pine needles was detrimental to normal embryo development. Questionable results using the acetone fraction and its possible toxicity on *in utero* development appeared to be a logical basis for its use in this study. Initial testing through the use of various other fractions, different bioassays, methods of administrations and concentration of extract for desirable feed intake by small laboratory animals led to the selection of the aqueous and acetone fractions. It is known that the water extract contains the sugars and pinitol present in the needles. Important components present in the acetone extraction are pinosylvin, its monomethyl ether, small amounts of pinobanksin and unidentified membrane substances (Erdtman and Rosengren, 1968; Lindstedt, 1949).

Results from the effect of the two pine fractions on pregnancy in rats are presented in table 1. The feeding of the aqueous and acetone fractions of ponderosa pine needles was toxic to both the dam and the fetus. There was a significant ($P < .01$) decrease in average litter size from both pine needle fraction treated rats compared to the control rats. The average litter size from bred rats fed the January aqueous fraction was reduced to almost one-half the average number of young that were born to the control rats. A somewhat lesser effect upon reproduction was obtained after the feeding of the acetone fraction. Resorption appeared to occur in bred rats on both pine needle fractions, however, since average litter size was reduced.

Statistical analysis of results testing the effect of the two pine fractions upon pregnancy in rats are presented in table 2. There was a significant ($P < .05$) difference in the average litter size from the bred rats fed pine needle fractions collected during the three

TABLE 1. EFFECT OF PINE NEEDLE FRACTIONS ON PREGNANCY IN RATS

Test Ration	No. of Bred Rats	No. of Rats Giving Birth	Avg Litter Size	Total No. of Stillborn	Avg Birth Wt (g)	Wt at 3 Days of Age (g)
Control	4	4	11.2	3	5.3	5.5
July Collection						
aqueous	4	3	8.7	5	4.8	5.1
acetone	4	4	7.8	3	5.0	5.2
October Collection						
aqueous	4	3	7.3	5	4.9	5.1
acetone	4	4	6.8	1	4.9	5.2
January Collection						
aqueous	4	2	5.5	5	4.8	5.1
acetone	4	2	7.0	4	5.0	5.3

TABLE 2. ANALYSIS OF VARIANCE FOR TREATMENT EFFECT UPON RAT GESTATION

Source of Variation	d.f.	s.s.	m.s.
Total	17	35.7778	
Treatment	1	1.0030	1.0030
Date	2	17.9206	8.9603*
Treatment x date	14	16.8542	1.2039

*P $<$ .05

different months. Aqueous and acetone test rations prepared from the January collection of pine needles caused a greater reduction in average litter size than the July and October pine needle collections. This would appear to indicate that the detrimental factor present in ponderosa pine needles may be a seasonal occurrence since the reproductive disturbances were more severe in the winter collection.

A high incidence of gastro-intestinal irritation occurred with the feeding of both pine needle fractions to bred rats during all of the collection periods. An autopsy was conducted on each of the six rats that died during experimentation. Severe ulceration of the entire intestinal tract was observed in the bred rats. Some of the embryos examined at autopsy appeared to be in stages of resorption since they were less developed than other embryos in the same uterus.

Average birth weight of the young born from bred rats fed the aqueous and acetone pine needle fractions was significantly ($P < .01$) lower than weight of the young born to the control rats. Birth weight and weight of the pups at three days of age was significantly ($P < .01$) lower in litters from the pine needle treated rats for all collection months used in the study. Birth weight of the pups born to the control rats was always within the expected normal birth weight ranges of from 5 to 6 gram. Birth weight of pups from rats fed both of the pine fractions often averaged under 5.0 gram. All pups regardless whether they were from the control or from pine needle treated dams gained approximately 0.2 gram in weight by three days of age.

Significance between pine needle test treatments was not apparent in this study. Both the aqueous and acetone fractions obtained from pine needles appeared to act with equal strength in causing a reduction in the number of bred females that did not survive until term. There was also no apparent difference in average litter size, survival rate, birth weight and weight at three days of age after dams had been maintained on one of the pine needle rations throughout gestation.

Effect of Ponderosa Pine Needle Fractions Upon Implantation in the Mouse

The purpose of this investigation was to determine whether the causative toxic factor(s) present in ponderosa pine needles exerts a detrimental effect at the time of implantation. Bred mice were used for this phase of the study since their feed consumption was considerably less than larger laboratory animals. Least squares analysis of variance was used in this study to determine if differences existed between treatments and the number of viable embryos at days 5 to 7 pc. Control mice fed only the Purina Mouse Chow had a ($P < .01$) greater number of viable embryos at 5 to 7 days pc than mice fed either the aqueous or acetone fractions of pine needles (table 3). There was also a significant ($P < .05$) difference in the number of viable embryos from mice fed the two pine needle fractions obtained at different collection dates. A general conclusion obtained from this phase of study was that the average litter size in controls was larger and fairly constant throughout the six conducted experiments than any of the pine needle treated groups of bred mice.

Average litter size for controls, aqueous 1:1, aqueous 2:1 and acetone 1:1 treated mice at 5 days pc (table 4) was, respectively, 6.33, 5.42, 1.08 and 2.00. Mice on the aqueous 2:1 fraction in particular

TABLE 3. ANALYSIS OF VARIANCE FOR EFFECT OF PINE NEEDLE EXTRACTS UPON EARLY EMBRYOLOGY IN THE MOUSE

Source of Variation	d.f.	s.s.	m.s.
Mean	1	1694.6944	
Month	5	91.0556	18.2111*
Treatment	3	730.3611	243.4537**
Age	2	19.0556	9.5278
Month x treatment	15	177.8889	11.8592
Month x age	10	115.6944	11.5694
Treatment x age	6	49.0556	8.1759
Month x treatment x age	30	209.1944	6.9731
Error	72	529.9000	7.3472

*$P < .05$

**$P < .01$

TABLE 4. NUMBER OF VIABLE MOUSE EMBRYOS AT FIVE DAYS POST COITUM

Collection Date	Treatment			
	Control	Aqueous 1:1	Aqueous 2:1	Acetone 1:1
November 30	16	18	0	0
January 5	14	3	0	0
January 21	7	17	0	9
February 28	12	11	0	0
March 23	15	13	11	15
April 26	12	3	2	0

Each number of viable embryos represents results from two mice/treatment.

suffered loss of almost all embryos within 5 days pc. It was evident by their physical appearance, especially of diarrhea, that their nutritional performance was somewhat depressed. There was a very slight loss of weight in most of the aqueous 2:1 treated mice. At the time of sacrifice, the uterus obtained from bred mice on the double (2:1) strength aqueous fraction was usually very pale and thin.

At 6 days pc the average number of viable embryos per litter for controls, aqueous 1:1, aqueous 2:1 and acetone 1:1 was, respectively, 6.58, 4.42, 0.17 and 3.25 (table 5). In addition, there was a further reduction in average litter size at 7 days pc (table 6) from bred mice fed the pine needle fractions. Many of the mice fed pine needle fractions that recorded an absence of any viable embryos present in their uterus at days 5 to 7 pc did have apparent resorption sites. Hemorrhage filled uterine horns were common at autopsy. Questionable resorption sites were recorded as such in the data.

Results in this phase of the study indicated that the biological assay used here could accurately estimate the intensity of the abortive factor present in ponderosa pine needles. It had been suggested by Chow et al. (1972) that the water-soluble fraction from pine needles in particular caused a detrimental effect upon pregnancy at about the time that the placenta begins to grow in mice. It is at this time that cell division is very rapid. In this study, both the aqueous and acetone fractions appeared to interfere with normal embryo development at the time of implantation. However, bred mice on the stronger aqueous fraction had less viable embryos than either of the groups fed the aqueous 1:1 or acetone 1:1 fractions.

TABLE 5. NUMBER OF VIABLE EMBRYOS AT SIX DAYS POST COITUM

Collection Date	Treatment			
	Control	Aqueous 1:1	Aqueous 2:1	Acetone 1:1
November 30	12	9	2	10
January 5	15	8	0	0
January 21	12	10	0	11
February 28	14	10	0	8
March 23	14	10	0	3
April 26	12	6	2	7

Each number of viable embryos represents results from two mice/treatment.

TABLE 6. NUMBER OF VIABLE MOUSE EMBRYOS AT SEVEN DAYS POST COITUM

Collection Date	Treatment			
	Control	Aqueous 1:1	Aqueous 2:1	Acetone 1:1
November 30	17	10	4	0
January 5	10	7	0	0
January 21	12	0	0	15
February 28	20	16	0	3
March 23	11	0	0	3
April 26	12	0	0	0

Each total of the number of viable embryos represents results from two mice/treatment.

Weight of the Adrenal Gland as Affected by the Consumption of Ponderosa Pine Needle Fractions

The adrenal gland weight was used as a confirmatory measure of the interference of normal pregnancy after bred mice had consumed ponderosa pine needle fractions. Use of the weight of the adrenal gland was based upon the suggestion of Dr. Thomas Dunn (professor in the Animal Science Division at the University of Wyoming, Laramie) that changes in adrenal gland weight might result after bred mice consumed ponderosa pine needle fractions. The gland is very important in maintaining normal carbohydrate, fat and nitrogen metabolism in the body (Hafez, 1968). Therefore, adrenal gland weights were obtained from the 144 bred mice used for the implantation phase of study. As indicated in table 7, there was a significant difference ($P < .01$) in weight of the gland obtained from mice fed pine needle fractions at each different collection date. There was also a significant ($P < .01$) treatment month interaction. Results appeared to not conclusively explain whether treatment or the collection period exerted the greatest change in adrenal weights. Mice on the aqueous 2:1 fraction, although nonsignificant, generally had lower adrenal weights than mice on any of the other treatments.

There also was a significant ($P < .05$) difference in the weight of the gland between treatments, plus a month x age x treatment x age interaction. No definite trend existed in the weight of the adrenal gland as affected by age of the embryo, treatment or collection period. A loss in adrenal weight on a specific treatment, from a certain collection period, at an exact date of gestation was not

TABLE 7. ANALYSIS OF VARIANCE FOR EFFECT OF PINE NEEDLE EXTRACTS UPON ADRENAL WEIGHT IN THE MOUSE

Source of Variation	d.f.	s.s.	m.s.
Mean	1	0.599592	
Month	5	0.007980	0.001598**
Treatment	3	0.000954	0.000318*
Age	2	0.000459	0.000230
Month x treatment	15	0.0040959	0.000273**
Month x age	10	0.002482	0.000248*
Treatment x age	6	0.001235	0.000206
Month x treatment x age	30	0.006391	0.000213*
Error	72	0.0078910	0.000110

**P < .01

*P < .05

necessarily a general trend. Individual mice on the different rations fluctuated in adrenal gland weights. Great care is required in excising the pair of glands since any excess adipose tissue would greatly change the weight of the gland.

Gestation Study in Relation to Ponderosa Pine Needle Consumption in the Bred Mouse

Thirty-eight virgin female mice were mated and used for this phase of the research. Numbers were limited because of an inadequate amount of fresh pine needles to prepare the different fractions for each of the six collection periods. Test rations of the aqueous 1:1 and acetone 1:1 pine needle fractions were readily consumed by mice on these two treatments throughout gestation. Obtaining adequate intake with mice on the aqueous 2:1 ration was sometimes difficult particularly from needles collected in February and April. In general, mice on the aqueous 2:1 ration did not consume as much as mice on all the other treatments and diarrhea was frequently observed. Almost all mice on the aqueous 2:1 fraction did not gain weight throughout gestation.

Because of the limitation in numbers of bred mice for the gestation phase of this study, the data was analyzed using Chi Square. There was only a significant difference ($P < .05$) in the total number of embryos born at each collection period (table 8). Normal gestation of mice fed the aqueous and acetone pine needle fractions were equally affected.

Only one mouse fed the acetone fraction delivered a litter of eight mice as indicated in table 9. The mouse was fed an acetone fraction obtained from needles collected on January 21, 1972. As

TABLE 8. CHI-SQUARE ANALYSIS FOR SIGNIFICANCE BETWEEN PINE NEEDLE-TREATMENTS COMPARED TO CONTROLS

	No. Females Delivering	No. of Females Not Delivering
Control	2	0
Aqueous 1:1	4	8
Aqueous 2:1	1	11
Acetone 1:1	1	11

*x^2 3 d.f. 10.92

TABLE 9. EFFECT OF PINE NEEDLE FRACTIONS ON PREGNANCY IN MICE

Treatment	No. of Pregnant Mice	No. of Mice Giving Birth	Total No. Born	No. of Young Dead 3 Days After Birth
Control	2	2	17	0
November 30				
Aqueous 1:1	2	1	6	1
Aqueous 2:1	2	0	0	0
Acetone 1:1	2	0	0	0
January 5				
Aqueous 1:1	2	1	5	3
Aqueous 2:1	2	0	0	0
Acetone 1:1	2	0	0	0
January 21				
Aqueous 1:1	2	0	0	0
Aqueous 2:1	2	0	0	0
Acetone 1:1	2	1	8	0
February 28				
Aqueous 1:1	2	1	3	1
Aqueous 2:1	2	*1	3	0
Acetone 1:1	2	0	0	0
March 23				
Aqueous 1:1	2	1	7	0
Aqueous 2:1	2	0	0	0
Acetone 1:1	2	0	0	0
April 26				
Aqueous 1:1	2	0	0	0
Aqueous 2:1	2	0	0	0
Acetone 1:1	2	0	0	0

*died 3 days after parturition

noted in the earlier implantation research, bred mice fed the acetone fraction from the same collection date were the only acetone treated females that had a normal number of viable embryos comparable to control fed mice. Only one bred mouse in each pair fed the aqueous 1:1 test ration collected on November 30, January 5, February 28 and March 23 delivered a litter at term. A bred mouse fed the aqueous 2:1 test ration obtained from needles collected in February delivered a litter of three mice. Three days after parturition the female died from apparent toxemia. The entire gastro-intestinal and reproductive tracts observed at autopsy were inflammed and blood filled. Therefore, no viable litters were actually obtained from bred female mice fed the aqueous 2:1 pine needle rations.

Initial weights were obtained on all bred mice as they were put on experiment and bred mice were weighed weekly until 18 days pc. In general, the bred mice on the lower concentrations of pine needle fractions maintained more stable weights throughout their pregnancy. It could be expected, of course, that bred females carrying a viable litter to term would show an increase in weight accounting for litter, tissues and fetal fluids. Other than a slight decrease in weight in the aqueous 2:1 treated mice, all pine needle test rations appeared to be palatable and satisfy nutritional requirements. A certain amount of tail biting was noted, however, in the caged pairs of mice on the concentrated aqueous treatment and the ration was not as well accepted as other rations.

Chemical Composition and In Vitro Dry Matter Digestibility of Needles and Branches

As emphasized in the literature review, the complete chemical composition of the ponderosa pine is not known. It is true that a great deal of commercial use has been made of the ponderosa pine. Why an animal will choose pine needles and other slash material as part of its diet is not well defined. The protein, ash, ether extract content and in vitro dry matter digestibility certainly does not categorize this material as highly nutritious feed even for the roughage consuming ruminants.

The composition of the needles and branches throughout the year is somewhat variable as measured on a month by month basis. Analyses comparisons were made with both branches and needles. Moisture, protein, ether extract, ash, acid-detergent fiber and acid-detergent lignin content were significantly ($P < .01$) different from a month to month basis in both the ponderosa pine needles and branches.

Moisture Content. The moisture content of the needles and branches of the ponderosa pine exhibited no great differences throughout the year (tables 10 and 11). Branches collected November 30 contained a somewhat larger percentage of moisture than the needles obtained at other collection dates. Moisture content in the pine branches and needles is low in the winter and spring followed by a gradual moisture increase in the summer (Philpot and Mutch, 1971). In general, the moisture content remained quite stable in both the pine needles and branches. Seasonal fluctuations were very small.

Protein Content. Ponderosa pine needles and branches are very low in crude protein content. Grazing ruminants would not be expected to survive on the needles and branches for long term feeding. Crude protein content was very low as indicated in table 10 and 11. Needle content of crude protein ranged from a low of 5.33% in March to 8.00% in a February collection. The crude protein content in the needles appeared to more or less follow seasonal climatic and growing conditions. However, the same was not true for the branches where only slight erratic changes in crude protein content existed throughout the year.

Ether Extract. In agreement with Mirov (1967), the needles and branches were found to be rich in the ether fraction (tables 10 and 11). The branches contained nearly twice the percentage of ether extract than the needles. Branches are the storage area for yearly growth of new needles and seed cones. In both the needles and branches, the ether extract content increased from January to June. After that time, the percentage of ether extract remained constant and could be directly related to the dormant growth period of ponderosa pine trees in the summer.

Ash Content. Perhaps the most stable chemical component present in pine needles and branches was ash content. Over the entire collection year, the percentage of ash in either the needles or the branches varied less than 2% (tables 10 and 11). The amount of ash was very low and contributed very little to the total grouping of chemical components. Branches in this study did, however, contain a greater percentage of ash than the needles.

TABLE 10. PROXIMATE ANALYSES PARAMETERS OF PONDEROSA PINE NEEDLES

Date	Moisture %	Ash %	Protein %	Ether Extract %
7-30	51.42	1.86	5.58	7.98
8-27	50.52	1.74	5.86	8.58
9-30	51.52	2.22	6.16	9.61
10-27	52.35	2.50	7.32	8.52
11-30	49.48	1.89	7.38	8.17
1-5	53.34	2.12	6.82	8.66
1-21	45.47	2.52	7.40	9.20
2-28	48.54	2.06	8.00	10.21
3-23	48.02	1.92	5.33	10.54
4-26	51.40	2.24	6.66	9.16
6-5	51.53	2.21	6.19	11.05
6-27	49.98	2.64	7.41	8.27

TABLE 11. PROXIMATE ANALYSES PARAMETERS OF PONDEROSA PINE BRANCHES

Date	Moisture %	Ash %	Protein %	Ether Extract %
7-30	50.28	1.83	3.00	12.20
8-27	49.48	2.28	3.48	13.28
9-30	51.60	2.45	3.08	17.23
10-27	52.58	2.18	4.78	16.54
11-30	56.40	3.82	4.29	14.60
1-5	50.18	3.55	3.58	18.12
1-21	43.57	3.55	3.69	16.26
2-28	41.90	2.93	4.78	16.11
3-23	44.30	2.28	3.15	16.80
4-26	48.73	2.59	4.85	15.68
6-5	51.60	2.44	5.78	14.22
6-27	55.23	2.74	4.16	13.48

Acid-Detergent Fiber and Acid-Detergent Lignin Content. In general, all cell wall tissues of the pine may make up 45% cellulose and approximately 30% lignin (Mirov, 1967). In this study, acid-detergent fiber and acid-detergent lignin content was higher in the branches than in the needles for every collection period (table 12). The apparent woody structure of the branches would suggest that needles would contain less fiber and lignin.

In Vitro Dry Matter Digestibility. Ponderosa pine needles and branches are only fair to poorly digestible when compared to hay. However, digestibility is better than the expected 7-10% *in vitro* dry matter digestibility obtained from bark and sawdust. The branches at most collection periods were generally more digestible than the needles (table 13). An inverse relationship between digestibility and lignin content in both the needles and branches was evident. It is well known that such an inverse relationship exists between lignin content and *in vitro* dry matter digestibility in forages.

TABLE 12. ACID-DETERGENT FIBER AND ACID-DETERGENT LIGNIN CONTENT OF PONDEROSA PINE NEEDLES AND BRANCHES

Date	Needles ADF %	Needles ADL %	Branches ADF %	Branches ADL %
7-30	35.11	15.29	42.86	19.42
8-27	35.20	14.66	39.21	17.72
9-30	34.75	14.84	41.22	17.88
10-27	36.90	14.20	38.59	19.80
11-30	35.78	14.54	40.06	19.04
1-5	32.97	13.59	37.55	17.28
1-21	33.78	14.13	38.46	18.14
2-28	28.82	12.60	35.00	16.48
3-23	33.37	14.74	40.08	18.50
4-26	32.95	13.51	37.47	17.72
6-5	32.25	13.37	33.50	13.92
6-27	35.67	15.10	44.70	21.98

TABLE 13. IN VITRO DRY MATTER DIGESTIBILITY OF PONDEROSA PINE NEEDLES AND BRANCHES

Date	Needles %	Branches %
7-30	35.43	38.45
8-27	33.22	43.40
9-30	30.69	41.93
10-27	32.61	36.80
11-30	31.83	41.38
1-5	30.23	38.60
1-21	37.75	45.64
2-28	41.15	47.48
3-23	42.17	48.03
4-26	40.77	40.19
6-5	41.45	39.71
6-27	37.81	26.84

SUMMARY AND CONCLUSIONS

A study was made to develop a reliable method to measure not only the presence but intensity of the toxic factor present in ponderosa pine (Pinus ponderosa) needles. Special emphasis was to determine the sensitivity of the biological assay during implantation and gestation in laboratory animals. Samples of all needles and branches were collected from 1971 to 1972 in a pine enclosed Bureau of Land Management pasture located near Sturgis, South Dakota. Only the fresh frozen needle samples were used to prepare the pine needle fractions for the biological studies. Oven dried needles and branch samples were used for the chemical composition and *in vitro* dry matter digestibility analyses.

According to results of this study, the component present in ponderosa pine needles that is detrimental to normal embryo development of mice and rats was present in both the aqueous and acetone fractions. In the rat gestation trial, there was a significant ($P < .05$) reduction in average litter size among collection periods but no significant difference between the two pine needle fractions and the reduction of average litter size. A high incidence of gastrointestinal inflammation occurred in many rats fed pine needle fractions.

There was a significant ($P < .01$) difference between treatments at the stage of implantation in bred mice fed pine needle fractions. Control mice fed only a basic mouse laboratory chow had a ($P < .01$) greater number of viable embryos at 5 to 7 days post coitum than mice fed aqueous 1:1, aqueous 2:1 and acetone 1:1 fractions. There was also a significant difference ($P < .05$) in number of viable embryos as affected by the feeding of pine needle fractions at different collection periods. Average litter size of controls 5 to 7 days post coitum was larger than the litter numbers from mice fed the pine needle fractions. Weight of the adrenal gland obtained from the dam was also significantly different ($P < .01$) between collection periods. Although not significant mice on the strong aqueous concentration generally had lighter adrenal gland weights than any other mice throughout the six experiments. A significant ($P < .05$) difference in the weight of the adrenal gland was obtained for mice fed the control, aqueous 1:1, aqueous 2:1 and acetone 1:1 test rations. Weights of adrenal glands from mice on different treatments fluctuated erratically throughout the six months of needle collections used.

The gestation study proved to be a verification of results obtained in the earlier implantation research. Obtaining adequate feed intake was sometimes difficult using the bred mice fed the aqueous 2:1 pine needle fraction. It appeared evident that a biological assay at implantation could be used and results obtained at that time would be as accurate as feeding ponderosa pine needle fractions to bred laboratory animals through the entire gestation period.

In general, in vitro dry matter digestibility changed very little between collection periods in both the ponderosa pine needles and branches. Digestibility of the needles and branches was comparatively greater than ponderosa pine sawdust and bark. The grazing ruminant selects the less lignified and more digestible components of the ponderosa pine tree. However, the low protein content hinders any long term maintenance of animals on pine needles and branches. Perhaps future research will be conducted to further define the chemistry of ponderosa pine needles. At this time, the use of the biological assay in early gestation research would be an excellent screening tool to eventually isolate the active component present in ponderosa pine needles.

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