THE RELATIONSHIP OF SOME ASSOCIATED FUNGI WITH COLD-HARDINESS OF THE MOUNTAIN PINE BEETLE, *Dendroctonus ponderosae* HOPK.

by

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ABSTRACT

The relationship between six fungi associated with the bark beetle, Dendroctonus ponderosae HOPK., in lodgepole pine from Riske Cr., B.C. was investigated with emphasis on cold-hardiness. A new technique was developed by which third and fourth instar larvae were reared from fourth stage eggs on host phloem and sapwood blocks colonized by a specific fungus. A control group fed exclusively on sterile phloem. Two blue stain fungi, Ceratocystis montia and Europhium clavigerum; two yeasts, Pichia sp. and Hansenula sp.; and an unidentified basidiomycete; the most common fungi; and a deuteromycete, Trichoderma sp. commonly observed in failing broods were selected for study. Beetles were also reared microbe-free (axenic) from egg to adult on an autoclaved diet consisting of ground phloem, water, and brewers' yeast. In another rearing treatment, eggs and larvae in pine bolts were force-reared in the shadehouse to late instar larvae, teneral and adult. Insects from these rearing treatments were subjected to the following acclimation procedure: 1 and 2 weeks at +10°C, then 2 weeks at each temperature: +5°, 0°, -9°, -18° and -26°C.

For insects from each rearing treatment acclimated to each temperature from +5° to -26°C, the developmental stage reached, a survival estimate, average supercooling point, water content, dry weight, sugar and sugar alcohol concentration was recorded. Similar variables were measured on wild larvae and beetles collected from the study area in July, October, February and May, 1981-82. Larval development on blocks colonized by E. clavigerum and the basidiomycete was more successful and faster than development on uncolonized blocks. Development in the presence of C. montia, Pichia sp., and Hansenula sp. was poorer than on uncolonized blocks. Trichoderma sp. was antagonistic to beetle development. Least cold-hardy larvae were produced on fungus-colonized blocks and controls followed by axenics then force-reared with wild larvae most cold-hardy.

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TABLE OF CONTENTS

Page

LIST OF TABLES

LIST OF FIGURES

LIST OF FIGURES (CONT'D)

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DEDICATION

To my wife, Joyce, whose support, encouragement and patience has helped make this possible.

INTRODUCTION

When approaching the subject of symbiology it has become necessary to define the individual authors' useage of the terminology in order to avoid misinterpretation by the reader. My useage of the terminology follows closely the definitions outlined by Henry (1966-67). Symbiosis is defined as the living together of two or more organisms of dissimilar species in a constant, intimate relationship. Mutualism is a symbiotic association that is advantageous to all members. Commensalism describes a relationship where the members are "at table" together with no obvious advantage or disadvantage to any member. A symbiote refers to any member in a symbiotic relationship. There are different terms to describe these and other types of symbiotic relationships but, considering the scope of this study, they are not relevant so have been omitted.

Symbiotic relationships have been reported among a wide variety of organisms such as bacteria, fungi, viruses, protozoans, and plants but probably most widely studied are the relationships between insects and their associated microorganisms (Richards and Brooks, 1958; Henry, 1966-67; Steinhaus, 1967; Graham, 1967; Batra, 1979). The physical relationship between symbiotes can be described as endosymbiotic or ectosymbiotic. An endosymbiotic relationship has a symbiote living inside a partner, and for a detailed description of this type of relationship between microorganisms and insects, consult Richards and Brooks (1958) and Koch (1967). In ectosymbiosis, the symbiotes live in a close physical association outside the bodies of one another except, perhaps, temporarily when a symbiote might be stored in special organs for dissemination. Well documented examples of ectosymbiosis occur between beetles of the family Scolytidae and their respective microorganisms, mainly fungi (Bakshi, 1950; Francke-Grosmann, 1967; French and Roeper, 1972; Batra, 1979; Whitney, 1982). This study deals specifically with the ectosymbiotic relationship between the bark beetle, Dendroctonus ponderosae HOPK., and some associated fungi, with an emphasis on the role of these fungi in the overwintering ability of the insect.

Overwintering ability determines the survival of a population over the seasonal period characterized by cold temperatures during which the insects are in a state of dormancy. Cold temperatures vary in severity and duration according to latitude, altitude, and other factors, and, therefore, insects that live in diverse habitats (eg. exposed vs unexposed) have developed a number of strategies to survive winter conditions (Salt, 1961; Danks, 1978; Ring and Tesar, 1981). Overwintering ability is influenced by a number of factors: 1) annual variation in the onset and severity of cold temperatures 2) the cold-hardiness of the population 3) habitat or hibernaculum characteristics and 4) the overall health and developmental state of the insect population. Ectosymbiotic microbes may be important in 2), 3) and 4). The interaction of these factors will determine population mortality. In the past there have been reports of bark beetle populations substantially reduced by adverse weather conditions (Craighead, 1925; Craighead and St. George, 1940; Safranyik, 1978).

Bark beetles are a serious forest insect pest world-wide and considerable economic losses are attributed to these pests annually. Mountain pine beetle is a serious insect pest in British Columbia. A 1981 survey showed an estimated 19,608,300 trees infested with this beetle over a 158,845 ha. area representing a potential loss of 6,654,100 m^3 of wood (Fiddick and Van Sickle, 1981). According to Manning et al (1982), the 1980 harvest of lodgepole pine in B.C. was 14.4 million m^3 and a potential industry income loss of more than \$35 per m^3 of wood and 3.75 person years per 1000 m^3 sawn by the lumber industry could occur due to mountain pine beetle activity. A reduction in aesthetic value of parks and increased costs for fire management are attributed to bark beetle infestations (Manning et al, 1982). As attempts to manage forest pests by non-chemical methods increase, an understanding of the symbiotic relationship between bark beetles and their associated microbe-tree complex is essential.

In the present study a number of large, relatively distinct areas of biology intersect: entomology, mycology, forestry, ecology and cryobiology. When different disciplines are combined some problems arise, such as conflicts in theoretical ideas and terminology, differences in research priorities, and lack of communication between researchers, which can be detrimental to the study and understanding of a system like the bark beetle-microbe-tree complex.

In the host tree, there is a succession of microbes colonizing the bark beetle gallery system with certain organisms being closer in time and space to the beetles than others (Bramble and Holst, 1940: Robinson, 1962; Coulson, 1979). Also of interest is the difference in abundance of certain microbes within the same tree and between different trees infested with bark beetles (personal observation). Some workers have noted differences in the specific microbial complement between beetles of the same species (Callaham and Shifrine, 1960; Farmer, 1965). The question addressed in this study is: Do differences in the microbial diet affect beetle development and ability to survive cold temperatures?

In the large volume of published work describing the association of bark beetles and microbes, there is some evidence that suggests a mutualistic relationship between bark beetles and at least some of the microbes. Some *Ceratocystis* spp. and their anamorphs and yeasts are always found associated with beetles successfully established in host trees (Shifrine and Phaff, 1956; Shepherd and Watson, 1959; Whitney, 1971; Whitney and Cobb, 1972). For many beetles, fungal transport organs have been described that appeared to be selective for the types and growth form of microorganisms (Barras and Perry, 1971; Whitney and Cobb, 1972; Happ et al, 1975 and 1976). The failure to explain any other function for these organs strengthens the suggestion of a mutualistic relationship between the beetles and fungi in these organs. Even beetles that had no obvious fungal transport organs transmitted fungi to trees that were successfully attacked. Farris (1965) noted fungi in the intersegmental folds of membranes associated with the scutellum and around the coxae of two *Dendroctonus* spp. beetles. Inoculation of symbiotic organisms over a large area of the tree by means of a mass attack behavior in some *Dendroctonus* spp. causes destruction of potential resin-producing host tissue, thereby predisposing the tree to successful brood development (Reid et al, 1967; Coulson, 1979; Whitney, 1982). The luxuriant microbial growth and change to "ambrosial" form of some fungi due to increased aeration of the host tissue and addition of insect-produced chemicals to the substrate illustrates an "intimate relationship" between one organism and another (Bramble and Holst, 1940; Graham, 1967; Barras and Taylor, 1973; Whitney, 1982). The beetles provide the microbes with access to large areas of uncolonized substrate, then fungus-colonized tissue is consumed by developing larvae and the "ambrosia" form is usually eaten by young tenerals. Reid (1961) and Webb and Franklin (1978) reported that host moisture content favoring brood development was related to colonization by "blue-stain" fungi. A microbial role in bark beetle aggregation or antiaggregation pheromone production has been described by Brand et al (1975), Brand et al (1976), Brand et al (1977), and Byers and Wood (1981). A microbe-microbe mutualism has been suggested where yeast or bacterial metabolites enhance the growth of fungi beneficial to the beetle (Graham, 1967). The bark beetle and microbe mutualism is not obligate since both can live separately but neither the insects nor the microbes are as vigorous or successful by themselves (Mathre, 1963-64a; Dowding, 1970; Reid and Shrimpton, 1971; Barras, 1973). Adult bark beetles have been produced free of living microbes on host phloem-based diets but an autoclaved microbe supplement, usually commerical brewers' yeast, was added (Bedard, 1966; Whitney and Spanier, 1982).

One important aspect of overwintering ability that will be considered in this study is cold-hardiness. Cold-hardiness describes a physiological state achieved by an insect which enables it to withstand extended periods of cold temperatures. In areas where temperatures consistently fall far below the freezing point for periods of weeks or months, the degree of cold-hardiness achieved is crucial to long term survival of the species. With respect to cold-hardiness, most insects can be classified into two groups as described by Asahina (1969): 1) those insects that can survive ice formation in the body, termed freezing tolerant, and 2) those insects that cannot survive ice formation in the body, termed freezing susceptible. It is advantageous for freezing tolerant insects to have ice form in the body slowly at high sub-zero temperatures, but in freezing susceptible insects ice formation in the body is lethal. Therefore, the method by which cold-hardiness is achieved is different. Since the bark beetles used in this study are freezing susceptible, the reader is referred to Salt (1961), Baust (1973), Danks (1978), and Ring (1980 and 1981) for details on freezing tolerance in insects. Factors that affect survival of freezing susceptible insects have been reviewed by Salt (1961) and more recently by Ring (1980). Some of the major factors that influence cold-hardiness in freezing susceptible insects are: 1) nucleators: according to Salt (1958) and Zachariassen (1980), these are substances that initiate ice crystal formation in supercooled liquids. Therefore, in supercooled insects the removal of possible nucleators, such as food in the alimentary canal, is crucial, 2) compounds such as sugars, sugar alcohols and proteins: these compounds are believed to have antifreeze properties and have been shown to accumulate in insects exposed to cold and decrease after exposure to warmer temperatures (Salt, 1957; Somme, 1964, 1965, and 1967; Duman, 1977; Danks, 1978). There are a number of theories on the mechanism and source of these protective compounds, but due to observed differences in concentration or even absence in some cold-hardy insects, the role of these protectants is not fully understood (Chino, 1957; Somme, 1967; Ring, 1980; Baust, 1981). 3) Time and probability: The probability that freezing will occur in a supercooled insect becomes closer to one as time spent in the supercooled state increases. Time and probability of freezing are influenced by other factors such as nucleators and temperature (Salt, 1950 and 1961). Farmer (1965) states the importance of food reserves accumulated in the larval stages of bark beetles for successful pupation, beetle development, flight and host attack. The biochemical and physiological changes associated with cold hardiness imply nutrition is a factor in cold hardiness.

Cold-hardiness is a cumulative, physiological process dependent on many factors, therefore, it is difficult to measure directly. In insects that are freezing susceptible the supercooling point has been used as a relative measure of cold-hardiness (Yuill, 1941; Salt, 1956; Green, 1962; Safranyik, 1978). The supercooling point is determined by cooling an insect at a constant rate until the point is reached when the insect freezes. A thermocouple attached to the insect and connected to a potentiometric amplifier and chart recorder detects the heat of crystalization released when ice forms in the body of the insect. For descriptions of various machines used for supercooling point determination consult Sullivan (1965), Salt (1966), or Ring (1981). Supercooling point determination is a good relative measure of cold-hardiness but as Salt (1950, 1953, 1956, 1963, and 1966) indicated, factors such as the cooling rate, desiccation, food in the gut, time at sub-zero temperatures, and inoculative freezing from external sources, changed the supercooling point.

When undertaking the study of a system such as the bark beetle-microbe-tree complex, a logical approach would be to separate the parts and determine their relationships to the system on an individual basis. In this study the approach was taken to study one aspect of overwintering, cold-hardiness, in relation to the nutrition of the developing beetle by altering the microbial content of the diet. Symbiotic fungi were selected from the habitat because developing wild beetles consume these microbes and there are apparent differences in overwintering ability between individuals. In the spring, larvae can be observed close to one another, one of which has failed to overwinter suggesting that differences in microbial diets may have been a factor.

MATERIALS AND METHODS

The beetles used in this study were collected in the fall of 1981 in lodgepole pine, *Pinus contorta* Dougl. var. *latifolia* Englemann., at Riske Creek, B.C. (52°N; 122°30'W) at an altitude of 1050m. Figure 1 shows the major mountain pine beetle infestations in western North America. Brood trees containing *D. ponderosae* and uninfested lodgepole pine trees were felled, cut into bolts approximately 90 cm long and transported to Victoria, B.C. Brood bolts were either put in cold storage at 5°C or in emergence cages in a greenhouse where the temperature was 15°C minimum at night and 24°C maximum during the day. When beetles began to emerge the brood bolts were transferred to an emergence chamber, with a refrigerated collector, similar to the one described by Browne (1972). Adults were stored in a glass jar, with damp paper towel, at +5°C for up to one month. Bolts from uninfested pine trees were stored either in a shadehouse, or a cold room at 10°C. Uninfested pine trees selected were over 30 cm diameter breast height and had phloem 3-5 mm thick. Extremely resinous trees were not selected. All cut surfaces on both infested and uninfested bolts were sealed with smoking-hot paraffin to retard desiccation during storage.

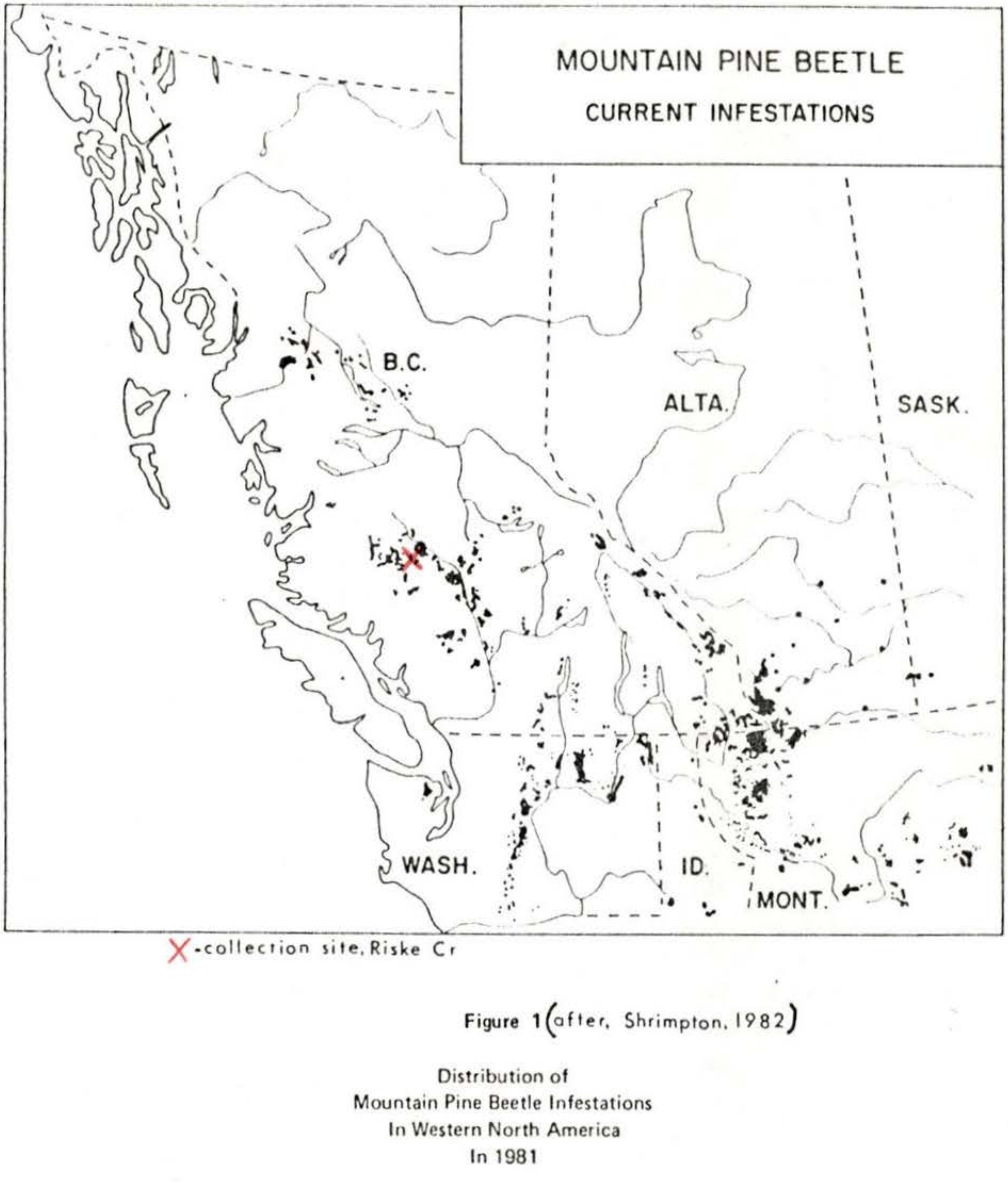


Figure 1(after, Shrimpton, 1982)

Distribution of
Mountain Pine Beetle Infestations
In Western North America
In 1981

Eggs used for laboratory rearing of D. ponderosae were obtained by the method described by Whitney and Spanier (1982) where beetles, exercised overnight in an illuminated flight cage, were paired on fresh lodgepole pine bolts, then incubated at room temperature for ten days. The eggs were harvested and allowed to develop on moist filter paper to the fourth stage, described by Reid and Gates (1970), before being used.

Microbe-fed Technique

D. ponderosae larvae were reared on blocks of lodgepole pine sapwood and phloem colonized by an individual fungus selected from the habitat. Briefly, the technique involved removing the outer bark, then chiselling small blocks of phloem and sapwood out of a fresh, waxed lodgepole pine bolt. After separation of the phloem from the sapwood, the blocks were placed in petri dishes and autoclaved twice for one hour at 85°C. Each block was inoculated, with a selected fungus and two surface sterilized beetle eggs, between the sapwood and phloem. The dishes were sealed and incubated at 24°C for 14-16 days. A more detailed description of the technique follows.

Fungi - The fungi selected for this study are commonly found in the gallery system of *D. ponderosae* and easily isolated by standard microbiological procedures. Two "blue-stain" fungi, *Ceratocystis montia* Rumbold, (1941) and *Europhium clavigerum* Robinson-Jeffrey and Davidson, (1968); two yeasts, *Pichia* sp. and *Hansenula* sp.; a deuteromycete, *Trichoderma* sp. and an unidentified holobasidiomycete were selected. Initial isolations of these fungi were made on yeast extract-malt extract (ym) agar plates diluted approximately ten times with water agar medium. Subsequent transfers were to undiluted ym agar plates. Initial isolations of *C. montia* were made by dispersing ascospores from a single perithecium in pine resin on isolation medium as described by Whitney and Blauel (1972). Conidia of *E. clavigerum* and *Trichoderma* sp., and basidiospores from the basidiomycete were streaked on separate plates of isolation medium. Hyphal tips from several spores were transferred until only the specific fungus was present on the culture medium. The yeasts were isolated by streaking sterile water washings of wild beetles onto isolation medium. Pure cultures were obtained by repeated streaking and transferring from colonies on isolation plates. Cultures were determined to be pure through observation under dissecting and compound microscopes. Identification of the fungi was confirmed by Dr. Stu Whitney, Pacific Forest Research Centre, Victoria, B.C. The fungi were periodically reisolated from sterile wood blocks, inoculated with the specific fungus, on which a surface sterilized egg had been reared to a larva.

Block Preparation - After removal of the outer bark to expose the phloem, blocks approximately 5cmx5cmx1cm were chiselled out of a fresh, waxed lodgepole pine bolt (Fig. 2). The phloem was separated from the sapwood and any blocks with discolored areas on the phloem were discarded. Each block was placed, phloem side down, on moist filter paper in a standard 9 cm glass petri dish with a lid. The blocks were autoclaved for one hour at 85°C in an ®Amsco Lab/isothermal autoclave, then re-autoclaved after 3-4 days at room temperature.

Inoculation - Some sterile blocks were inoculated with each one of the selected fungi. A filamentous fungus was inoculated by squashing two pieces (0.5cmx0.5cmx0.2cm) of culture medium, containing the fungus, between the sapwood and phloem at one end of the block. A yeast was inoculated by scraping enough from the surface of a culture plate to smear across one end of the block. The fourth stage beetle eggs were surface sterilized by gentle agitation in a 0.1%

mercuric chloride solution for four minutes, then rinsed five times with sterile distilled water (Whitney and Spanier, 1982). Two eggs were picked up gently with fine forceps, one at a time, and placed on each block 2 cm apart between the sapwood and phloem, 2 cm from the fungus inoculum (Fig. 3). Five to six drops of sterile water were added to the filter paper, if dry, then the dish was sealed with a rubber petri dish seal or parafilm (Fig. 4). The entire inoculation procedure was done in a laminar flow sterile air cabinet. The blocks were incubated in the dark at 24°C for 14-16 days.

Acclimation - After 14-16 days at 24°C the inoculated blocks were wrapped loosely in black plastic, put in a cardboard box and subjected to the following acclimation procedure in walk-in cold rooms: 1 and 2 weeks at +10°C, then 2 weeks in each cold room at +5°, 0°, -9°, -18° and -26°C. After acclimation at each of the temperatures from +5° to -26°C, a sample (1/5 of total) of blocks containing each fungus and control was removed. The number of frozen and unfrozen larvae and stadium reached were recorded, then fresh weight and supercooling point determined for larvae reared on each fungus and control group. After supercooling point determination, the larvae were freeze-dried and dry weight recorded.

Figure 4 - Sealed petri dish containing inoculated block ready for incubation.

Figure 5 - Axenic rearing units.

Figure 6 - "Ambrosial" growth of the Basidiomycete lining pupal chamber (arrow).
Dark spot in pupal chamber is a shadow.

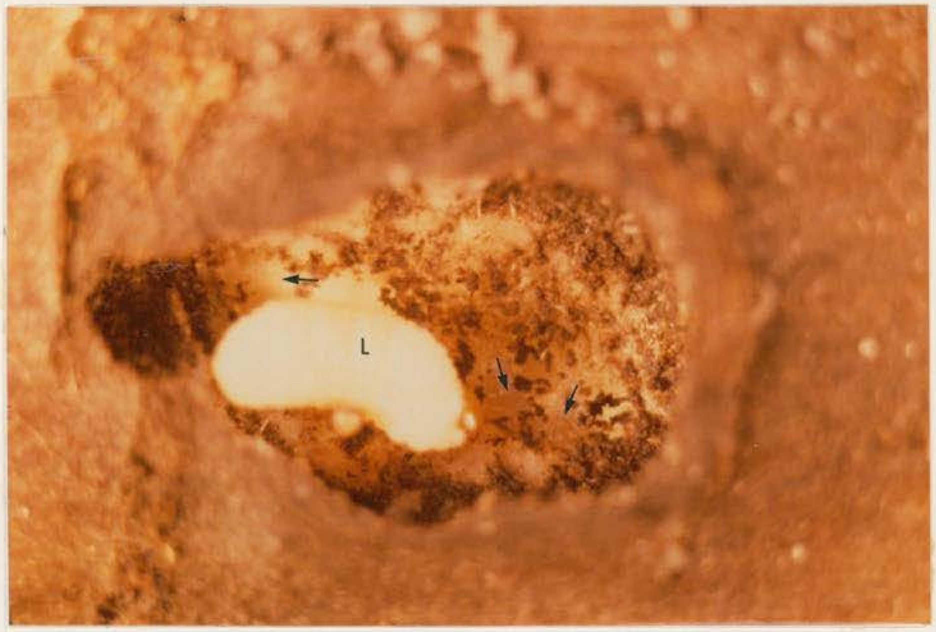


Figure 7 - "Ambrosial growth of E. clavigerum lining pupal chamber.
L - 4th instar larva
Dark material in pupal chamber is frass and debris.
Arrows indicate "ambrosial growth"

Water content was expressed as percentage of fresh weight using the equation: $\frac{\text{fresh weight - dry weight}}{\text{fresh weight}} \times 100$. One to four insects were combined to determine water content. Dried larvae were stored at -25°C until biochemical analysis was performed.

Supercooling point determination - The apparatus used to determine supercooling point was a ®Cole-Parmer electronic cryobath with a 30 gauge copper-constantan thermocouple attached to a recording potentiometer. Four insects were placed in individual compartments of a foil cup inside a 3.5 gram (1/4 dram) shell vial. The thermocouple and thermometer probe were lowered into the shell vial adjacent to the insects in the foil cup, then the vial was put inside a larger glass tube submerged in an acetone:methanol; (1:1) mixture in the cryobath cooling chamber. The cooling rate was between 0.5° and 1.0°C/min. The supercooling point was determined to the nearest 0.5°C by an upward deflection of the thermometer needle and corresponding rebound in the cooling curve, recorded on a strip chart recorder, caused by heat released when ice formed in the insects' body. The insects were kept frozen for 5 minutes, then removed from the cryobath and a few rewarmed at room temperature for 24 hours to check for recovery. The insects were kept at or near the acclimation temperature until supercooling point was determined.

Axenic Rearing

D. ponderosae was reared from egg to adult in axenic culture. The beetles were reared individually on a sterile diet of ground host phloem, dehydrated brewers yeast, and water (1:0.1:0.5 by weight) according to the method reported by Whitney and Spanier (1982) (Fig. 5). The ground phloem used for diet preparation in this study was harvested from a single uninfested lodgepole pine tree (collected in April, 1981), mixed thoroughly and kept frozen until used. Individuals were reared to each of the developmental stages (four larval instars, pupa, teneral, and adult) according to the development schedule for *D. ponderosae* in this diet (Table 1) then acclimated as described in the microbe-fed technique.

TABLE 1

Developmental schedule for *D. ponderosae* Hopk. in axenic culture on Whitney and Spaniers' (1982) diet.*

	DEVELOPMENTAL STAGE									
	Egg Stage				Larval Instars					
	I	II	III	IV	1	2	3	4	Pupae	Teneral
Days[a]	1	2	2	1	4	4	4	11	8	8

* - From Strongman unpublished report, 1981.

a - ±0.5 days

The number of frozen and unfrozen insects, fresh weight, supercooling point, dry weight, and water content was recorded for the various developmental stages at each acclimation temperature. The freeze-dried insects were stored at -25°C for biochemical analysis. At the end of an acclimation period cultures were made from the diet, frass and insect on aerobic media, ym agar and plate count agar, and on Poinar and Thomas' (1978) AC medium for anaerobic organisms to determine if the insects were microbe-free. Samples of diet, frass and insect were viewed under the compound microscope.

To determine if there were any effects of inoculative freezing, from non-ingested diet, on supercooling point, some second, third and fourth instar larvae acclimated to 0°C were removed from the diet and acclimation continued on pieces of dried bark. Data for first instar are missing due to development to second instar during acclimation at +10° and +5°C.

Cannibalism Observations - Evidence of cannibalism was occasionally observed in the microbe-fed group. To test the nutritional effects, if any, of cannibalism, 80 axenic rearing units were prepared as described above except no brewers yeast was added. The yeastless rearing units were implanted with 1, 2, 3, or 5 surface sterilized fourth stage beetle eggs per unit, then incubated at 24°C. Larval development was observed after 2 days, 19-21 days, than at 7 day intervals until observation was terminated at 48 days. Throughout the incubation period rearing units conspicuously contaminated were discarded. After 48 days cultures were made from diet and tenerals on ym agar plates and AC medium to determine if they were microbe-free. The microbe-free tenerals were paired on fresh pine bolts and their performance (gallery length and number of eggs deposited) was recorded after incubation in a growth chamber at 24°C ± 1°C for 10 days.

Force-Reared

Two fresh, waxed bolts, one collected in July and one in October, 1981, containing D. ponderosae eggs and young larvae were force-reared to the later developmental stages. The bolt collected in July, 1981 contained mostly eggs that were reared to tenerals and adults after about one month in the shadehouse, where air temperatures measured nearby ranged from 22° to 33°C, and about one week in the greenhouse where temperatures were 15°C minimum at night and 24°C maximum during the day. The other bolt contained mostly young larvae and was force-reared to late instar larvae in the shadehouse from October to December when air temperatures measured nearby ranged from 2°C to 9°C. The force-reared bolts were acclimated as described in the microbe-fed technique. At the end of each acclimation period from +5° to -26°C, a section (1/5) of a bolt was peeled, the insects collected and the number of frozen and unfrozen insects, fresh weight, supercooling point, dry weight, and water content recorded. Freeze-dried insects were stored at -25°C for biochemical analysis.

Wild Collections

D. ponderosae was collected from Riske Cr., B.C. in August and October, 1981 and February, 1982. In May, 1982 larvae were collected from an area 50 km east of Riske Cr. The insects were transported in a styrofoam container to Victoria where fresh weight, supercooling point, dry weight and water content were recorded. The insects were freeze-dried and stored at -25°C for biochemical analysis.

Brood bolts collected in October, 1981 were subjected to the same acclimation procedure described in the microbe-fed treatment and the same variables measured.

Survival Estimates

At the lower acclimation temperatures (-18°, -26°C) it was difficult to determine if an insect was dead or alive or if mortality occurred due to freezing or other causes. The bodies of frozen larvae were white, hard and punctured easily; after thawing larvae appeared "watery", the integument had lost its resiliency and remained indented if poked with a blunt probe. There is a rapid loss of supercooling ability if cold acclimated insects are rewarmed (Somme, 1965) therefore, only a few larvae from each sample were rewarmed to determine survival at an acclimation temperature. Survival estimates in this study were made from observation of acclimated insects rewarmed at room temperature for 15 min. to 48 hr., the number of frozen and unfrozen insects, and from supercooling points.

Gut Dissections

Throughout the acclimation procedure a few insects from each rearing treatment were dissected and the gut examined for food particles since food in the gut is known to influence supercooling point (Salt, 1953).

Biochemical Analysis

Whole insects from the above groups were analyzed for sugars and sugar alcohols by high pressure liquid chromatography (HPLC). Sugars, polyols, and amino acids were extracted by the following procedure modified from Ring and Tesar (1980): 1) 25-100 mg dry weight of insect was thoroughly homogenized in 3 ml of water:chloroform:methanol (0.8:1:2), 2) the homogenate was poured into a centrifuge tube, then the pestle and homogenizer tube rinsed well with approximately 2 ml of the water:chloroform:methanol mixture, 3) the homogenate was centrifuged at 6000g for 15 minutes and the supernatant decanted into test tube A, 4) the pellet was resuspended in 3 ml of the water:chloroform:methanol mixture and centrifuged at 6000g for 15 minutes and the supernatant combined in test tube A, 5) step 4 was repeated, then the pellet discarded, 6) 2 ml of water and 2 ml of chloroform were added to test tube A and the contents thoroughly mixed by inverting the test tube 3-4 times, 7) the mixture was allowed to partition for approximately 18 hours, 8) the water layer containing sugars, polyols, and amino acids was removed with a pipette and the chloroform layer discarded, and, 9) the sample was evaporated to dryness at 55°-60°C in a stream of nitrogen gas. The dried samples were stored at -25°C until ready for injection into the chromatograph. For samples in which only 5-25 mg dry weight of insect were obtained, the procedure was the same except one half the volume of solvents was used throughout. All solvents used were HPLC grade.

The biochemical analysis was carried out using a ®Waters high pressure liquid chromatograph system (HPLC). The dried, frozen samples were resuspended in water (1 ml/100 mg dry weight of insect), then a 15 microliter sample was injected into the HPLC and passed through a cation-anion exchange resin precolumn. The carbohydrate column was a ®Bio-rad HPX-87P heavy metal kept at a constant temperature of 75°C. The mobile phase was 15% acetonitrile in water at a flow rate of 0.4 ml/min. Absorbance was measured on a ®Waters Lambda-max 480 U-V detector at 192 nm and a sensitivity of 0.05 absorbency units full strength (AUFS). Sugars and sugar alcohols were identified by retention time compared to standards, and concentration was calculated automatically, by the integrator, from peak height.

Samples of autoclaved phloem and axenic diet were analyzed for sugars and sugar alcohols. Concentrations of sugars and sugar alcohols in all samples were converted to percentage of dry weight of insect tissue.

Initially, a few samples were checked against standard sugars by thin layer chromatography. Five microliters of each sample and standard were spotted on a silica gel 7G plate then developed in water:chloroform:methanol (0.8:1:2) and visualized with thymol-H_2SO_4 reagent (Kartnig and Wegschaider, 1971) or sodium metaperiodate reagent (Lemieux and Bauer, 1954).

Statistical Analysis

A one way analysis of variance (ANOVA) was used to determine if the mean supercooling points between the different rearing treatments at a given acclimation temperature were significantly different at the $\alpha = 0.05$ level. The population from which the samples were drawn was assumed to be normally distributed and the variances equal, but according to Zar (1974), the ANOVA test performs well even if these assumptions are false as long as the sample size (n) is equal or nearly equal. Therefore, comparisons were made only between samples with similar sample size. If the ANOVA test showed a difference in means where more than two means were tested, a Student-Newman-Keuls (SNK) multiple range test was done to determine which means differed (Zar 1974).

RESULTS AND DISCUSSION

Microbe-Fed Technique

The microbe-fed technique was developed in an attempt to elucidate the role of some ectosymbiotic fungi in the beetle-microbe-tree complex under study. The objective, using the technique, was to present a type of fungus in a growth form that larvae would likely ingest in the wild condition. The situation where only one fungus is available may simulate the wild condition in at least part of larval development. Although a complex of microorganisms is inoculated by the adult during gallery construction and egg deposition, there is a definite succession of microorganisms with certain fungi able to colonize the substrate faster than others (Bramble and Holst, 1940; Coulson, 1979). According to Whitney (1971) late instar larvae may even mine out of fungus-colonized host tissue. Pure cultures of the "ambrosial" growth form, of some fungi, that is observed in the wild gallery system (Graham, 1967; Whitney, 1971), were produced on blocks colonized by the basidiomycete, *Europhium clavigerum* (Fig. 6 and 7) and *Ceratocystis montia.* This "ambrosial" growth was only observed in conjunction with larval mines or pupal chambers and was not produced on blocks colonized by *Pichia* sp., *Hansenula* sp., *Trichoderma* sp. or on the controls.

Yeast growth is similar to "ambrosial" growth in that yeast colonies exist as a large mass of individual cells compared to the mycelial growth form of filamentous fungi. The change in fungal growth form to "ambrosia" may be in response to larval secretions or excretions. According to Barras and Taylor (1973) larvae of *D. frontalis* stimulated "ambrosial" growth of the mycangial *Ceratocystis minor* on wood. Chemicals produced by larvae and pupae of *D. frontalis* are believed to produce the "ambrosial" form of a *Sporothrix* sp. and a basidiomycete (Happ et al, 1975 and 1976). Larvae and pupae may stimulate "ambrosial" growth through physical activity. Fruiting in culture of a fungus, *Schizophyllum commune*, was induced by mechanical injury (Leonard, 1973). No attempt was made to determine which, if any, of these insect activities stimulated the change in growth form of fungi associated with *D. ponderosae*. More pupae and fourth instar larvae were produced and dry weight of fourth instar larvae was larger, than production and dry weight of larvae from the control group, when reared on blocks colonized by the ambrosial-type fungi, *E. clavigerum* and the basidiomycete (Fig. 8, 9 & 12; Tables 2 & 3). This could indicate better larval nutrition on these two fungi. Actual production of larvae on microbe-fed blocks was 45%, mortality was about

30%, and the remaining 25% were larvae and unhatched eggs not found due to dense fungal growth or larvae that were cannibalized. Table 2 shows the percentage of live insects observed.

Although *C. montia* exhibited the "ambrosial" growth form fewer pupae were produced, compared to the other ambrosial-type fungi (Fig. 8), there was high mortality of fourth instar larvae (Table 2), and total production was lower than the control group (Fig. 11). The cause of larval mortality could not be determined but might indicate a dietary deficiency or toxic fungal metabolite production . There was no difference in dry weight of third and fourth instar larvae reared with *C. montia* or on controls (Fig. 12 & 13). Therefore, one might speculate that the symbiotic role of *C. montia* may not be nutritional. This agrees with the profuse reports that describe the pathogenicity of *Ceratocystis* sp. symbiotes in trees attacked by bark beetles (Craighead, 1928; Shepherd and Watson, 1959; Reid et al, 1967; Francke-Grosmann, 1967; Coulson, 1979). Another explanation of the results might be that *C. montia* may require the presence of another microbe(s) to supply necessary nutrients or to break down toxic products. Bramble and Holst (1940) described a relationship where *C. pini* and *Zygosaccharomyces* (=*Pichia*) *pini* together were more pathogenic to pine trees than *C. pini* alone.

TABLE 2

Percentage of live *D. ponderosae* observed in each developmental stage on autoclaved phloem and sapwood blocks colonized by each fungus after 5208°hr. at or above 10°C.

FUNGUS	DEVELOPMENTAL STAGE					TOTAL[b] PRODUCTION
	Pupa	4th instar	3rd instar	2nd instar	1st instar	
Control[a]	0	14.6(28)[+]	18.8(15)	5.3(4)	0	10.0(47)
Basidiomycete (Basidio)	26.5(9)	23.0(44)	8.8(7)	2.7(2)	0	13.2(62)
Europhium clavigerum (E.C.)	35.3(12)	18.8(36)	2.5(2)	1.3(1)	1.1(1)	11.1(52)
Ceratocystis montia (C.M.)	14.7(5)	13.6(26)	10.0(8)	0	0	8.3(39)
Pichia sp.	5.9(2)	10.5(20)	17.4(14)	2.7(2)	0	8.1(38)
Hansenula sp. (Hans.)	5.9(2)	10.5(20)	17.4(14)	13.3(10)	0	9.8(46)
Trichoderma sp.	0	0	0	0	0	0
Total live observed in each developmental stage	88.3(30)	91.1(174)[c]	75.0(60)	25.3(19)	1.1(1)[d]	

[+] Numbers in parenthesis indicate actual number observed.

[a] Control had no fungus inoculum.

[b] Total production of live insects observed on blocks colonized by each fungus.

[c] 58.6% of the dead 4th instar larvae came from blocks colonized by *C. montia*.

[d] 56% of dead 1st instar were on yeast colonized blocks.

FIGURE 8- Percentage of live *D. ponderosae* pupae produced on blocks colonized by each fungus.

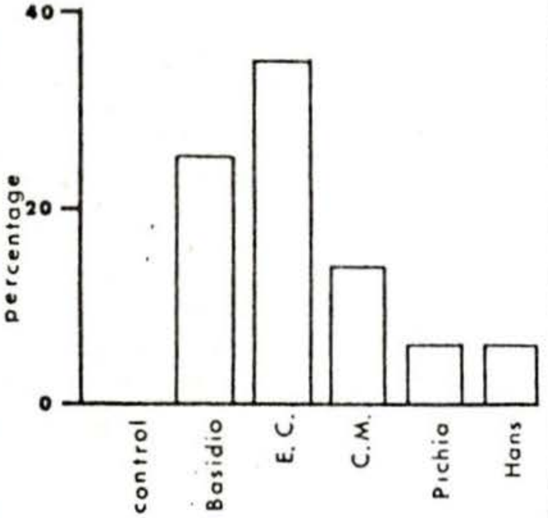


FIGURE 9- Percentage of fourth instar larvae of *D. ponderosae* on blocks.

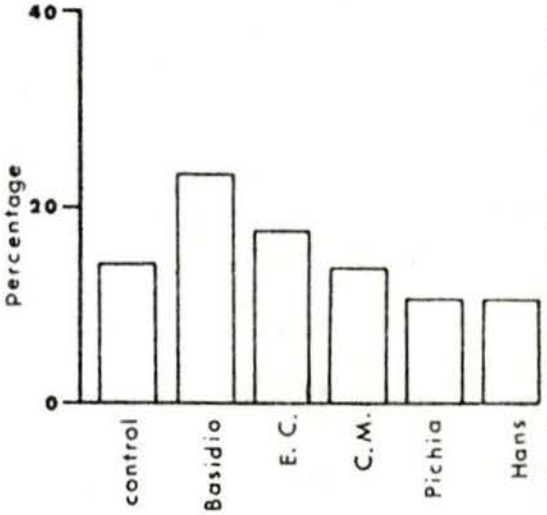


FIGURE 10-Percentage of third instar larvae of *D. ponderosae* on blocks.

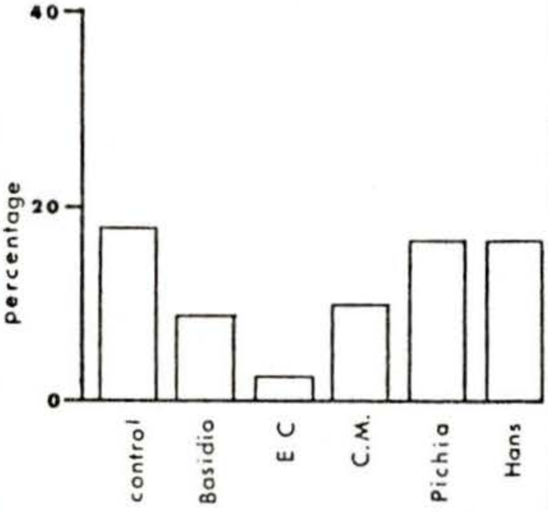


FIGURE 11-Percentage of total production of larvae and pupae of *D. ponderosae* on blocks.

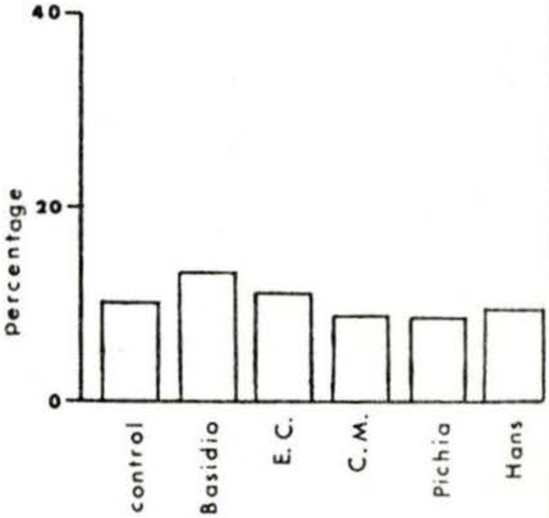


TABLE 3

Dry weight of _D. ponderosae_ larvae from each rearing treatment.

	REARING TREATMENT										
	AXENIC		FORCE-REARED	MICROBE-FED*						WILD ACCLIMATED	WILD
Developmental Stage	Dry Weight	Time[3]	Dry Weight	Control	Basidio	E.C.	C.M.	Pichia	Hans	Dry Weight	
	mg	°hr	mg	mg	mg	mg	mg	mg	mg	mg	mg
3rd instar	$2.0 \pm 0.6(103)^{a}$	3360	$1.7 \pm 0.8(38)^{a}$	$1.3 \pm 1.0(17)^{1,2}$	-	-	$1.5 \pm 0.6(6)^{1}$	$1.2 \pm 0.8(9)^{1,2}$	$1.0 \pm 0.5(12)^{2}$	-	-
4th instar	$6.6 \pm 1.8(128)^{a}$	4368	$4.4 \pm 1.9(102)^{bd}$	$4.4 \pm 2.0(21)^{bd}$	$4.9 \pm 1.9(38)^{bd}$	$6.4 \pm 2.2(35)^{a}$	$4.4 \pm 2.2(23)^{bd}$	$3.5 \pm 2.0(14)^{cd}$	$3.0 \pm 1.9(18)^{c}$	$4.6 \pm 1.7(89)^{bd}$	$5.2 \pm 1.6(55)^{b}$

* - All microbe-fed groups had 5208 degree hours at or above +10°C.
Numbers in parentheses indicate sample size (n).
Dry weights with the same letters or numbers for a given instar indicate no significant difference. ($\alpha = 0.05$)

3 - Development time above +10°C.

FIGURE 12- Dry weight of fourth instar larvae of *D. ponderosae* in each rearing treatment.

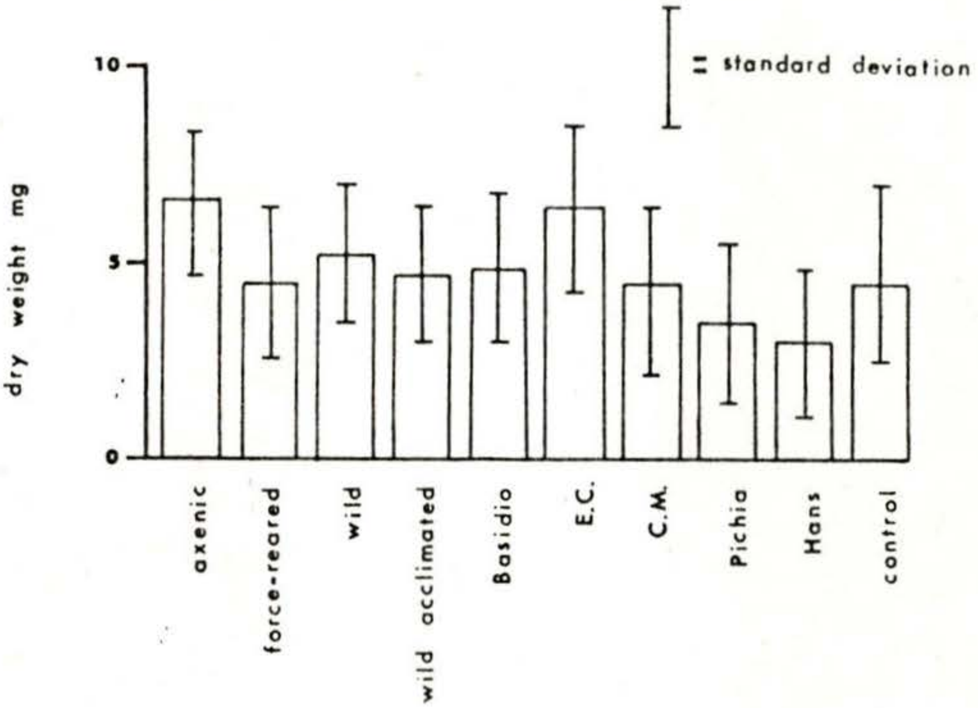
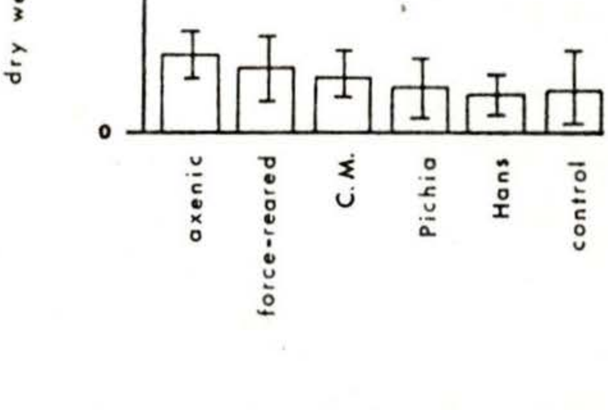


FIGURE 13- Dry weight of third instar larvae of *D. ponderosae* in each rearing treatment.



The number of live larvae observed on yeast colonized blocks was low (Table 2). Larval development may have been inhibited by oxygen deficiency or toxic metabolites produced by profuse yeast growth. Some young larvae appeared to get stuck in the yeast colony and were not able to feed in the phloem. Graham (1967), Francke-Grosmann (1967) and Farmer (1965) state that yeast may be ingested as food in developing beetles but could also modify the host substrate and release nutrients beneficial to fungal and/or beetle development. The results of this study suggest that profuse yeast growth inhibits larval development.

Trichoderma sp. was antagonistic to larval development (Table 2). Fourth stage eggs hatched and larvae began to feed, on most blocks, but few reached the late instars. Characteristically, there was massive spore production along the larval mine which terminated in a dense spore mass on, in and around the dead larvae. Whether the fungus depleted nutritional components of the phloem or was the direct cause of larval mortality could not be determined but mortality was always 100%. Some Trichoderma sp. are known to be mycoparasitic (Elad et al, 1980; Abd-el-moity and Shatla, 1981), can produce volatile fungistatic metabolities (Upadhyay, 1981), and are fast colonizers of fresh substrate (Smith et al, 1981).

Except where blocks were conspicuously contaminated, only the inoculated fungus was recultured from the blocks.

According to the results (Table 4) the effect of cannibalism on beetle development depended on the level of cannibalism. In ground phloem most larvae developed only to fourth instar unless other larvae were consumed. Most larvae developed to tenerals at the highest cannibalism level (Table 4) but development took 16,128 degree hours above 10°C compared to 8,340 degree hours for development to tenerals in the wild (Powell, 1968). Tenerals produced by cannibalism performed poorly on pine bolts when compared to wild adults (Table 5). Cole (1973) tested the effects of crowding on D. ponderosae larvae in a phloem-based diet containing brewers' yeast. In Cole's study, larval deaths were attributed to "entomocide", defined as the act of one larva killing another but not necessarily as a cannibalistic act for food. Larval deaths due to entomocide never exceeded 17.86% and usually were less than 10% at crowd levels of 3, 6, and 9 larvae/3.6 cm^3 of diet. In this study only one individual was produced in each unit at crowding levels of 2, 3 and 5 larvae/1 cm^3 of diet. Cannibalism may only occur if the larval diet is deficient or if crowding levels are high. In the microbe-fed treatment 2 eggs/block were used. The effects of cannibalism on sapwood and phloem blocks were assumed to be negligible based on cannibalism observations (Table 4) and the fact that, usually, both eggs could be accounted for.

TABLE 4

Effects of cannibalism on D. ponderosae development in an autoclaved ground phloem diet.*

No. of 4th stage egg/rearing unit	DEVELOPMENTAL STAGE REACHED†										No. of insects produced/unit
	2nd instar		3rd instar		4th instar		Pupa		Teneral		
	#	%	#	%	#	%	#	%	#	%	
1	-	-	-	-	7	64	1	9	3	27	1
2	-	-	1	8	6	46	1	8	5	38	1
3	2	13	2	13	4	27	1	7	6	40	1[+]
5	-	-	-	-	3	20	1	7	11	73	1

* - Whitney and Spanier (1982) diet without brewers yeast.

† - After 48 days, incubated at 24°C. 16,128 °hr. above 10°C.

[+] - 1 unit had one 2nd instar and one 3rd instar, the rest had 1 insect/unit.

\# - Actual number observed in each developmental stage.

TABLE 5

Performance of adults from different rearing treatments on fresh pine bolts.[1]

	REARING TREATMENT					
Pair No.	Cannibalism[2]		Axenic[3]		Wild	
	Gallery (mm)	Eggs	Gallery (mm)	Eggs	Gallery (mm)	Eggs
1	6.0	0	185	26	280	32
2	DNE[4]	-	220	24	250	51
3	DNE	-	200	43	270	79
4	3.0	0	145	9	180	30
5	11.5	12	235	34	205	42
6	DNE	-	45	0	110	23
7	8.5	0	40	0	75	0
8	5.5	0	145	32	225	51
9	8.5	0	250	38	120	17
10	-	-	80	2	225	17
11	-	-	135	25	265	56
12	-	-	215	37	170	18
13	-	-	260	46	280	50
14	-	-	310	62	10	0
15	-	-	65	0	190	30

1 - Bolts inoculated at room temperature for 10 days then frozen.

2 - Teneral adults from cannibalism experiment - females for pairs 1 and 2 came from 1 egg/unit, pairs 3 and 4 from 2 eggs/unit, males from 3 eggs/unit; pair 5 from 2 eggs/unit, pair 6 from 3 eggs/unit and pairs 7, 8, and 9 from 5 eggs/unit. Only 9 pairs were obtained from cannibalism treatment.

3 - Axenic beetles produced on autoclaved phloem and sapwood blocks. Data for axenic and wild beetles from Dr. S. Whitney (unpublished).

4 - Did not enter.

Webb and Franklin (1978) reported that microbe-free larvae of *D. frontalis* reared on pine bolts constructed long mines, were smaller than normal and did not pupate. Farmer (1965) emphasizes the importance of food reserves accumulated in the larval stages for successful bark beetle metamorphosis. The larvae that were reared on sterile phloem did not produce any pupae in the same time that pupae were produced by larvae reared on fungus colonized blocks. There were differences in the percentage of pupae produced on each fungus (Fig. 8). If poor larval nutrition inhibits pupation, then these results indicate a difference in nutritional value between the fungi used in this study. Pupae were produced in the cannibalism experiment but only after incubation for approximately twice as long as the microbe-fed group. Also, more pupae were produced, in a shorter time, at the highest cannibalism level. Whitney[1] (per. comm.) has produced microbe-free adults on sterile phloem and sapwood blocks as described in this study except the phloem and sapwood were left intact, only one egg/block was implanted in a niche in the phloem and the phloem-side was up in the petri dish. Development to adult took 20,496 degree hours above 10°C, production was 48%, and performance of the axenic adults was

[1] Dr. H. S. Whitney, Pacific Forest Research Centre, Victoria, B.C.

comparable to wild adults (Table 5). The results of larval development on fungus-colonized blocks, tenerals produced by cannibalism, and adults reared on microbe-free phloem and sapwood blocks suggest that host phloem contains the necessary nutrients for successful metamorphosis but addition of certain habitat fungi, or other larvae, improves the nutritional value of the substrate which is reflected in a shorter incubation period.

Production of larvae on the yeasts may be improved by a reduction in the amount of inoculum and subsequent growth of the yeast colony. The normal physical association of phloem appressed to sapwood was disrupted to provide aeration for fungal growth. This close physical association might be important for successful establishment of first instar larvae and subsequent feeding of later instars in the phloem. Reducing this physical disruption, therefore, might improve beetle production. Also, reduced physical disruption may limit fungal blooms that could inhibit larval development. Furthermore, there likely are differences in phloem nutrient levels between trees and at different heights on the stem. Standard use of phloem from the most nutritious part of a tree could improve production.

No microorganisms were cultured from the diet, frass or beetle in axenic rearing units not conspiciously contaminated.

Cold Hardiness

Freezing was lethal in all developmental stages of this beetle. Beetle larvae were most cold-hardy followed by eggs then pupae, adults and tenerals. Safranyik (1978) reported wild D. ponderosae larvae were most cold-hardy followed by adults, pupae then eggs with supercooling points for larvae from -34°C to -38'C and eggs supercooled to -18°C. Reid and Gates (1970) determined supercooling points of -17°C (1.3°F) for eggs acclimated to room temperature and -18°C (-0.6°F) after acclimation to -5°C (23°F). In this study the supercooling points for non-acclimated eggs in all stages of development, produced by wild adults in pine bolts after incubation at room temperature for ten days, ranged from -20°C to -24°C. No explanation can be offered for the difference between supercooling points of these eggs and the reported supercooling points except that the eggs came from different geographical areas. Tenerals, adults and pupae supercooled to about the same temperature after acclimation to -9°C (Fig. 20 & 23, Table 9) below which survival was nil. The difference in supercooling points of pupae, tenerals and adults could be attributed to the artificial rearing of these stages compared to reported supercooling points for wild collections. This study concentrated on third and fourth instar larvae because, although all instars are present, these instars are predominant during the winter months. Adults are also present in the habitat over the winter months but according to Reid (1962 and 1963) they do not contribute substantially to the overwintering population.

The results of this study showed that a decrease in supercooling point of 5°-6°C or more corresponded with a decrease in water content and an increase in glycerol content (Fig. 14-19). Where supercooling points varied only a few degrees, water content and glycerol content were variable (Fig. 14-24). Somme (1964) described a relationship where decreased supercooling points in wild D. monticolae (=ponderosae) larvae were related to increased glycerol content with acclimation to low temperatures. Ring and Tesar (1981) published data for freezing susceptible insects from the arctic that showed when supercooling point decreased so did water content and a corresponding increase in glycerol concentration occurred in some insects. Hansen et al (1980) working with the bark beetle Ips typographus and Dubach et al (1959) with ants described the same relationship between supercooling point, water content and glycerol.

FIGURE 14- Supercooling points for fourth instar larvae of *D. ponderosae* in each rearing treatment.

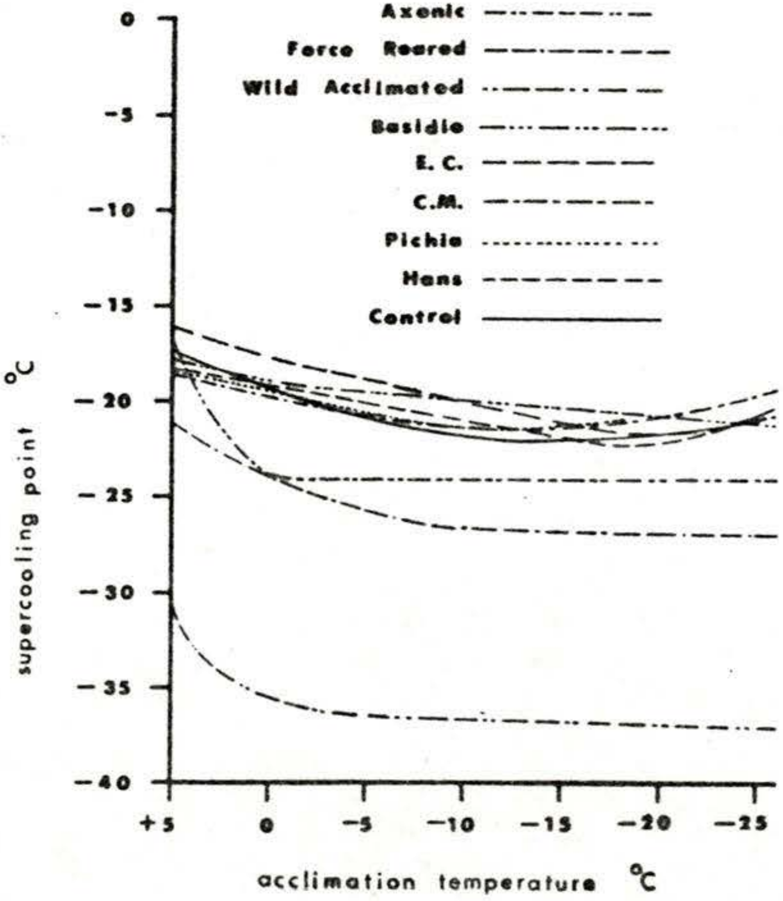
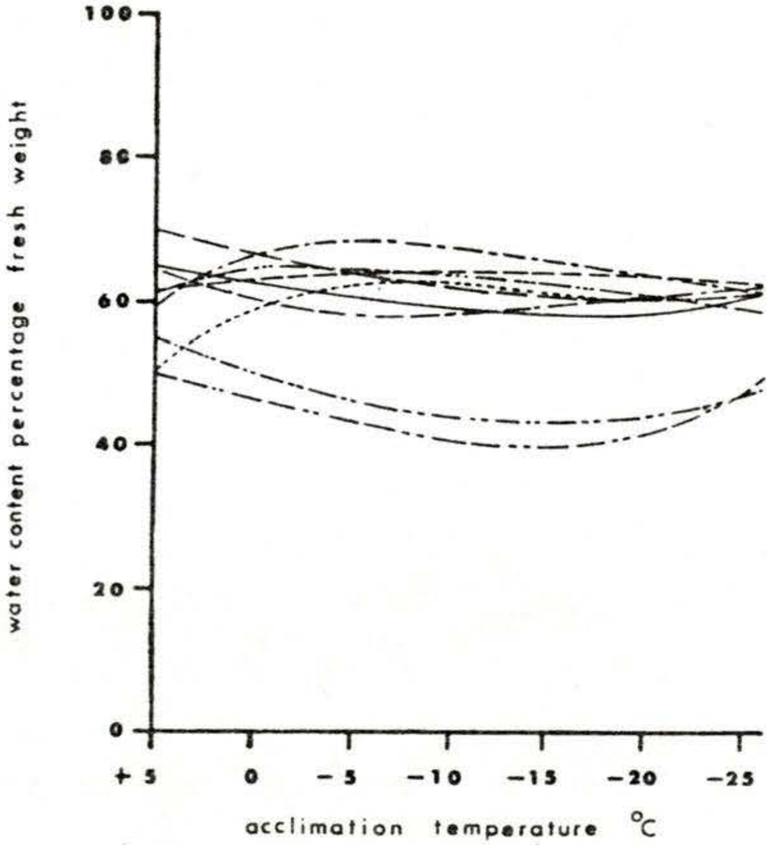
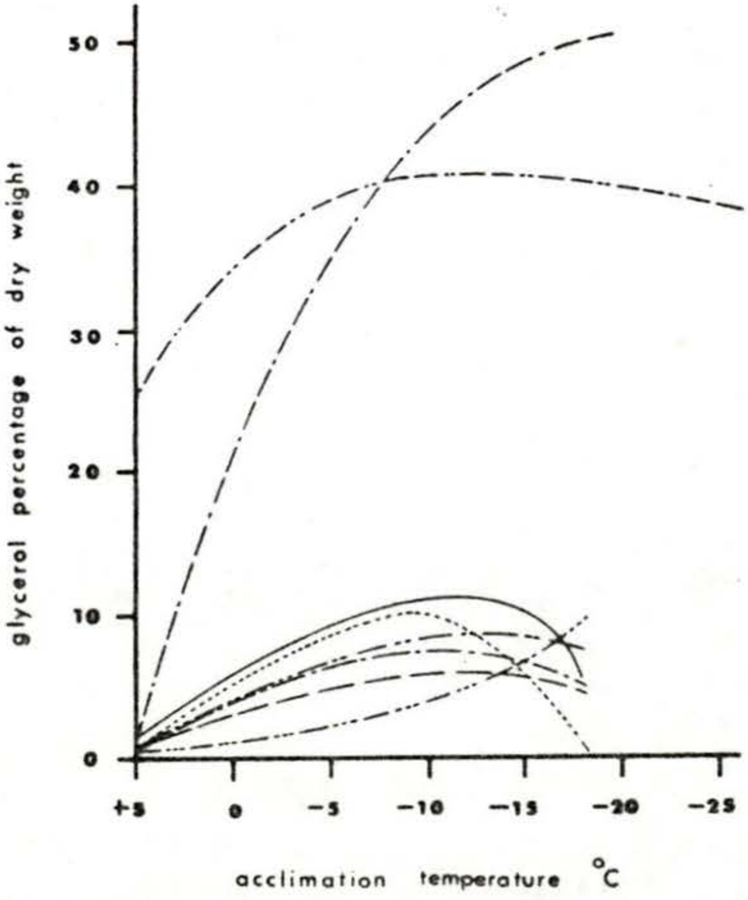


FIGURE 15[1]- Water content of fourth instar *D. ponderosae*.



[1] 1- same legend as fig. 14

FIGURE 16 - Glycerol concentration in fourth instar larvae of *D. pondersae.*



1- same legend as fig. 14

FIGURE 17- Supercooling points for third instar larvae of *D. ponderosae* in each rearing treatment.

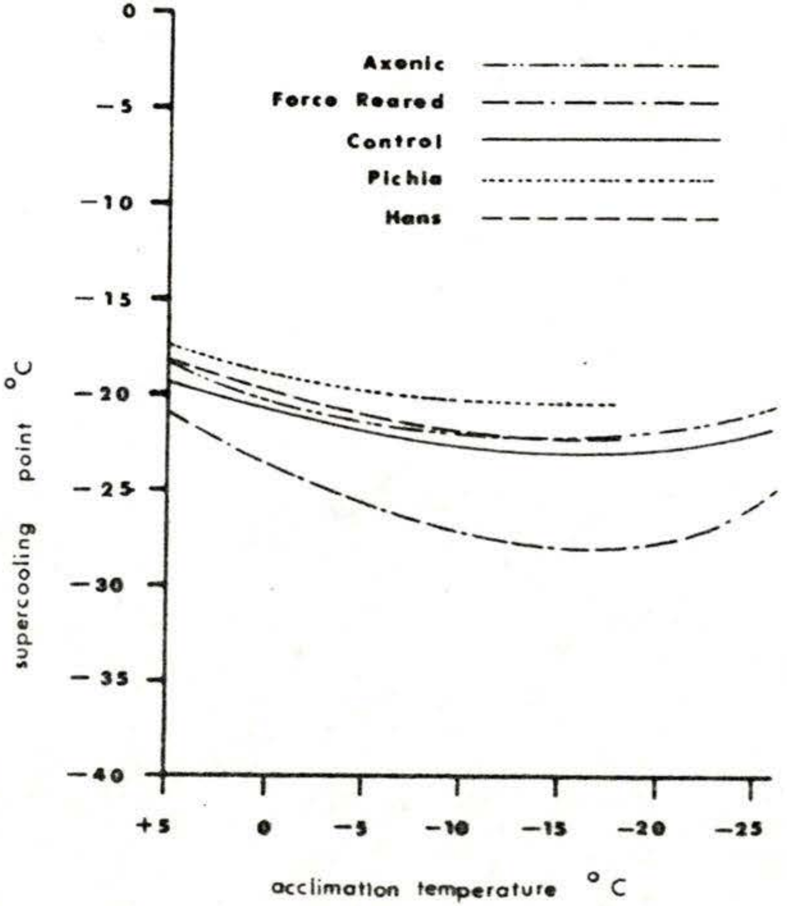
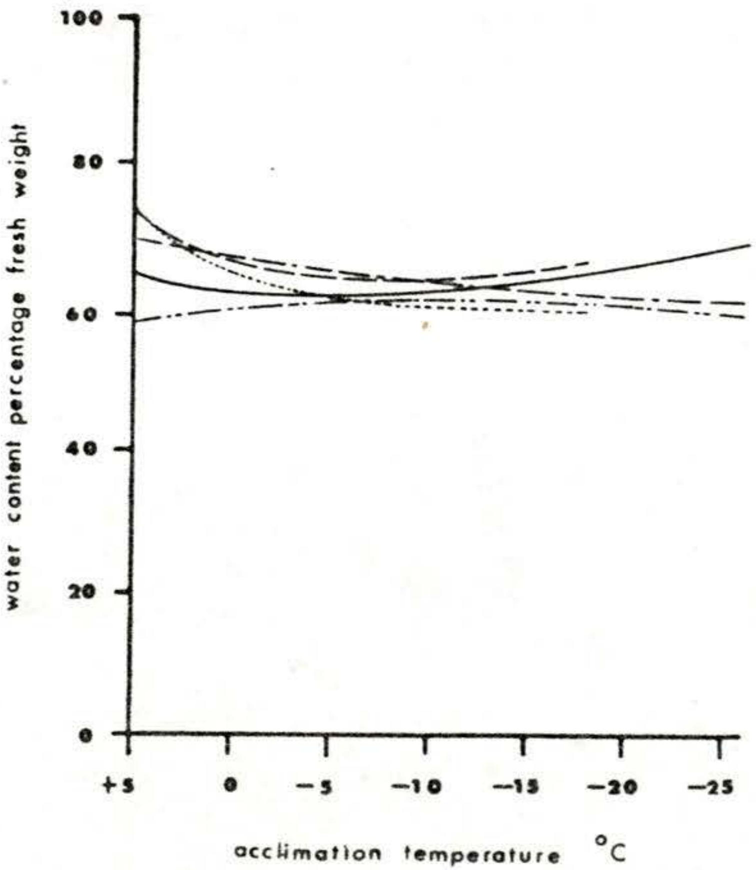
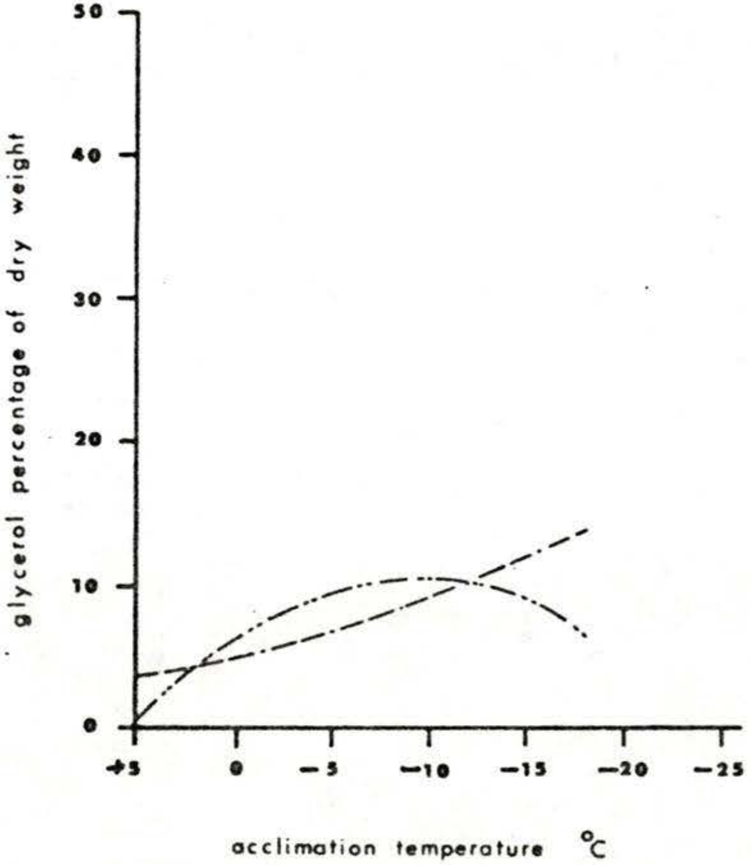


FIGURE 18[1] **-** Water content of third instar larvae of *D. ponderosae*.



[1]- same legend as fig. 17

FIGURE 19[1] - Glycerol concentration in third instar larvae of *D. ponderosae.*



[1] same legend as fig. 17

FIGURE 20- Supercooling points for tenerals of *D. ponderosae* in each rearing treatment.

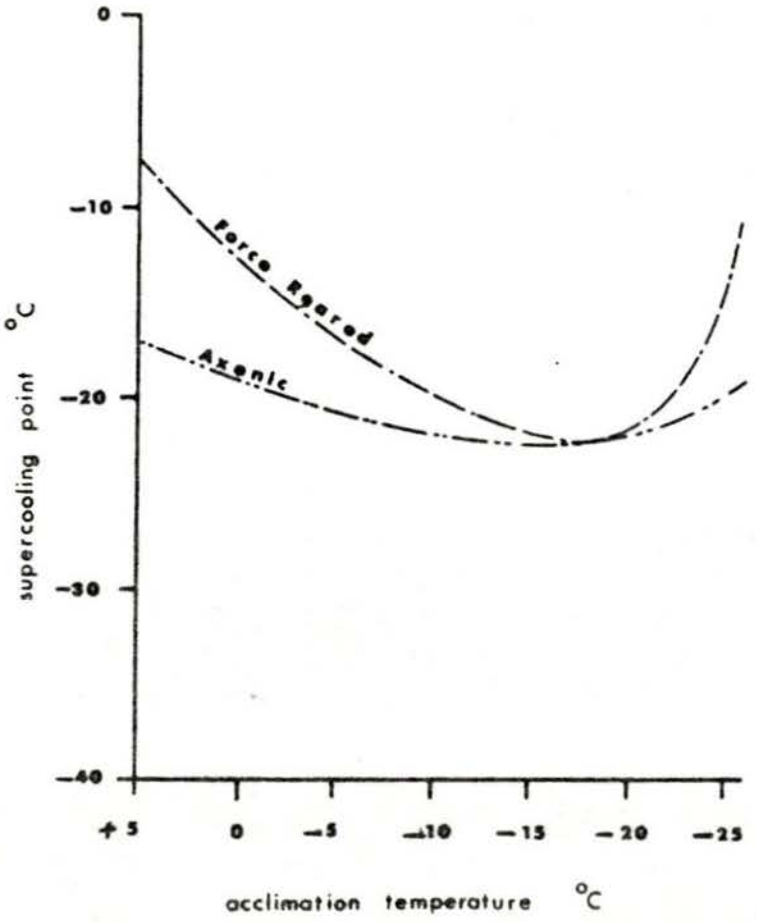


FIGURE 21 - Water content of *D. ponderosae* tenerals.

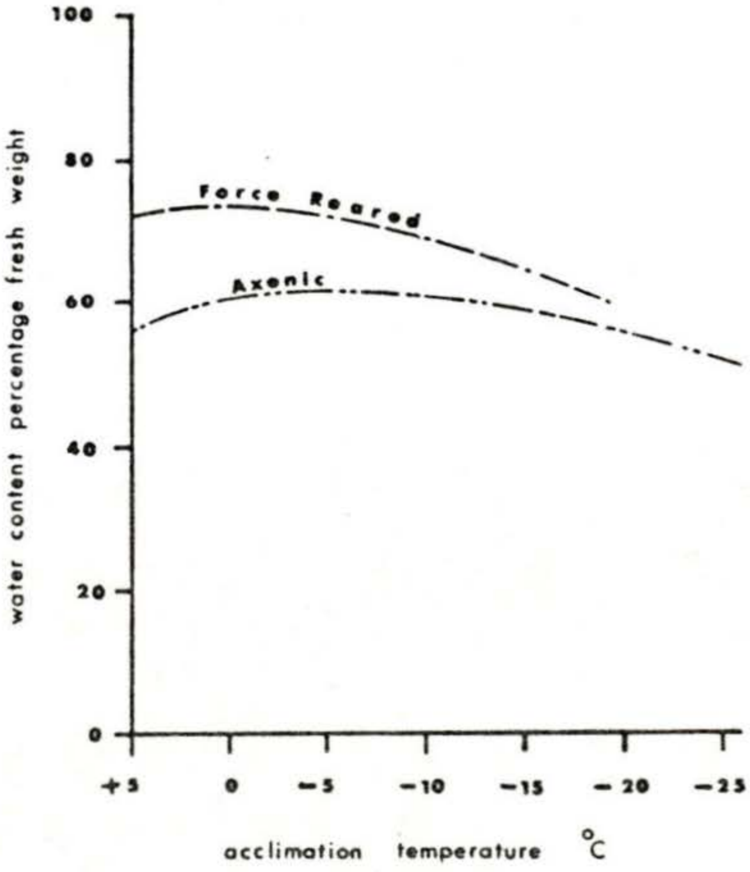


FIGURE 22- Glycerol and sorbitol concentrations in axenic *D. ponderosae* tenerals.

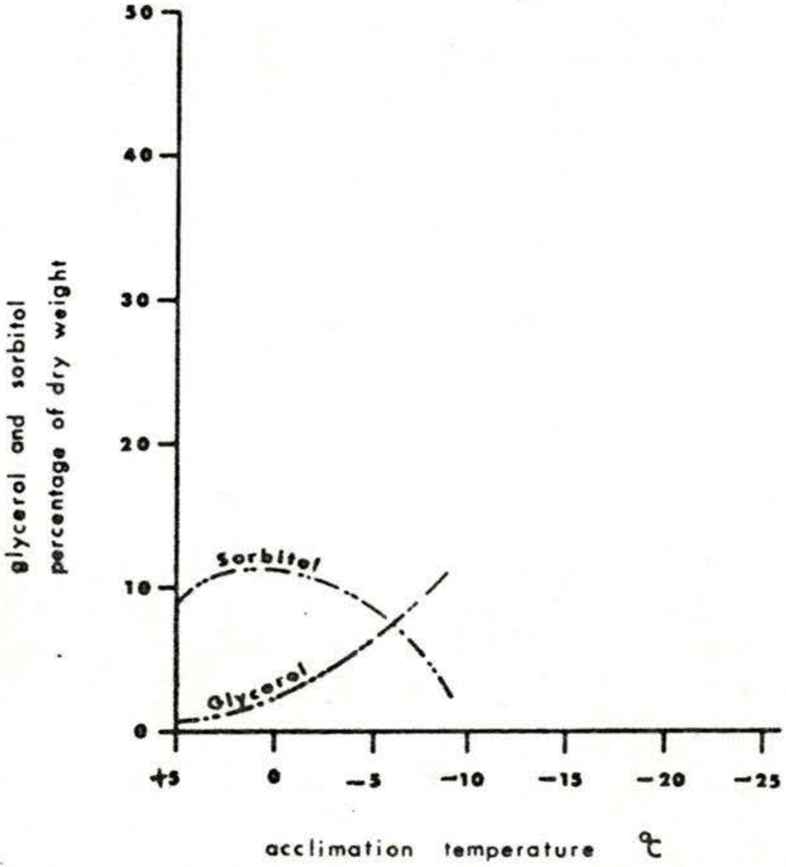


FIGURE 23- Supercooling points for *D. pandersae* adults in each rearing treatment.

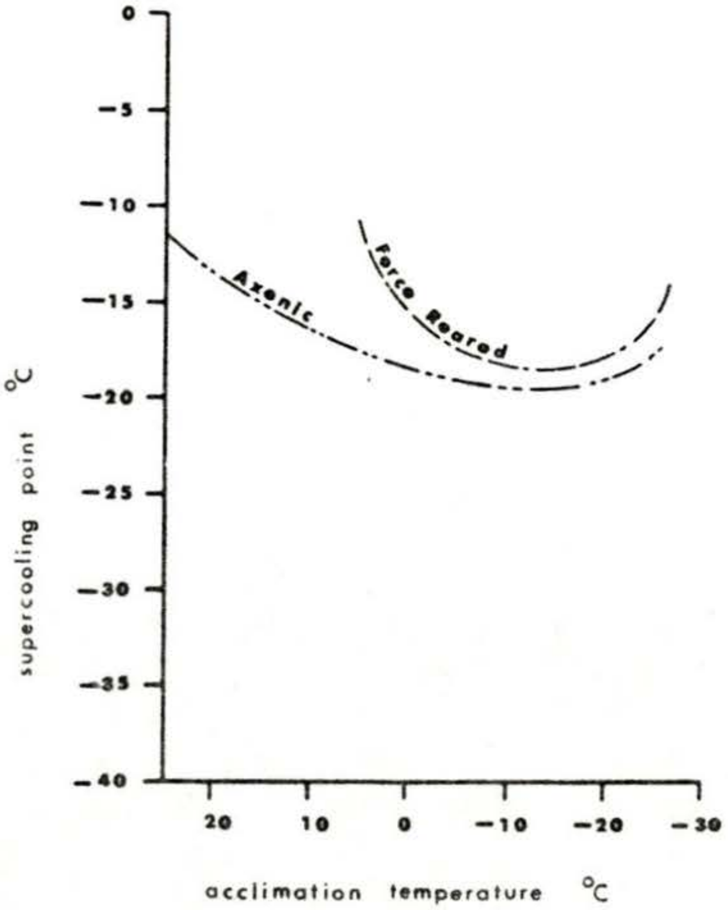
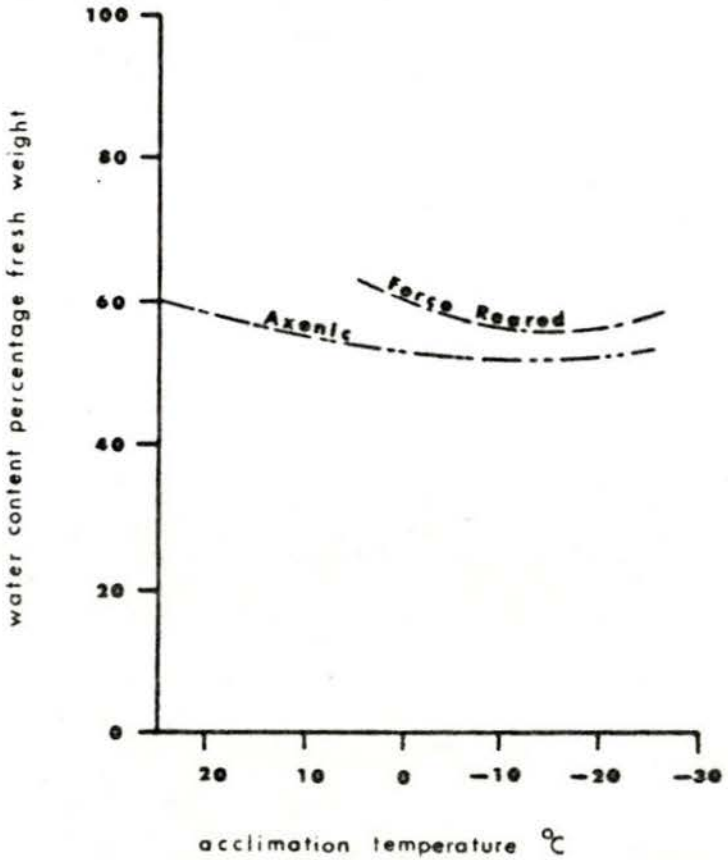


FIGURE 24- Water content of *D. ponderosae* adults.



According to Salt (1956 and 1961) lethal or near-lethal dehydration must occur before there is an appreciable amount of cold-hardening. There is, however, a small decrease in supercooling point attributed to concentration of solutes by dehydration. Ring (1980) states that dehydration is an integral part of diapause preparation and in some insects where dehydration is extreme supercooling ability is increased substantially. Since D. ponderosae does not enter a diapause and the reduction in water content is not large, increased cold-hardiness probably is due, for the most part, to glycerol accumulation.

The major sugar detected was trehalose, with small amounts of glucose also present in some samples (Tables 6-11). These results show no clear relationship between sugar concentration and supercooling ability (Tables 6-11). Increased concentrations of sugars in the hemolymph, mainly trehalose and glucose, has been related to a decrease in supercooling point in some insects (Asahina and Tanno, 1964; Somme, 1966 and 1967). Increased sugar concentration has been noted in some insects that do not accumulate glycerol at low temperatures while decreased concentration occurs in some insects that accumulate glycerol (Ring, 1981).

Sorbitol was detected in most samples but there appeared to be no direct relationship between sorbitol and supercooling abilities.

Sorbitol concentration increased with acclimation in some samples and decreased in others (Tables 6-11). As far as this author is aware, sorbitol production has not been previously reported in this beetle. In other insects, a lowering of supercooling points has been attributed to increased sorbitol concentration alone or in conjunction with glycerol or other solutes (Chino, 1957; Somme, 1967; Ring, 1981; Ring and Tesar, 1981).

Hansen et al (1980) have suggested a dual system of protective reactions against cold in _Ips typographus_ where glucose was the protective agent at temperatures above 0°C, below which, glycerol was accumulated and glucose concentration decreased. Somme (1964) reported that larvae of _D. monticolae_ (=_ponderosae_) lost glycerol at 0°C. The results of this study show lowest glycerol levels above 0°C (Fig. 16, 19 and 22; Table 11). If a dual protective system exists for _D. ponderosae_ like the one suggested for _I. typographus_ it was not detected in this study. Such a system may not have been detected because data were not recorded until insects were acclimated to +5°C by which time the switch to another protective agent may have been underway.

TABLE 6

Supercooling points, percentage water, sugar and polyol concentrations for fourth instar larvae of <u>D. ponderosae</u> from different rearing treatments.

		REARING TREATMENT									
				MICROBE-FED†							
Acclimation Temperature	Variable Measured	Axenic	Force-Reared	Control	Basidio	E.C.	C.M.	Pichia	Hans	Wild Acclimated	Wild
°C +5	Supercooling Pt (°C)	-17.0±1.2(34)^{a}	-21.1±4.8(15)^{b}	-17.2±2.4(3)	-17.8±1.8(8)	-16.2±2.9(7)	-18.7±1.2(7)	-18.4±1.1(4)	-18.2±1.3(6)	-30.3±3.8(18)^{c}	May -5.1±1.8(19
	Water Content (%)	55.5±2.8(11)	65.6±4.0(5)	66.4(1)	63.8±5.3(3)	67.8±5.6(3)	59.0±2.8(3)	50.9(1)	61.5±4.9(2)	50.1±4.1(5)	65.7±1.2(5)
	Trehalose (%)			4.70	2.0	7.50	4.14	-	5.00	3.10	1.35
	Glucose (%)			1.33	0.55	-	2.00	Trace	3.00	0.60	0.05
	Sorbitol (%)	1	1	7.17	1.25	4.40	8.77	-	12.00	11.01	8.36
	Glycerol (%)			1.05	Trace	1.60	0.86	-	-	20.38	2.19
0	SCP	-24.2±4.8(32)^{a}	-20.6±5.2(22)^{b}	-19.2±0.6(4)	-19.1±1.7(8)	-19.7±1.6(13)	-18.0±2.3(4)	-19.3±1.2(3)	-19.1±0.7(5)	-35.4±2.0(15)^{c}	Oct. -26.1±3.7(15
	H_2O (%)	49.7±7.2(9)	61.3±5.7(6)	51.5±15.9(3)	66.9±6.3(3)	58.9±4.2(5)	61.6±1.0(2)	60.9±2.3(2)	64.3±0.5(3)	46.6±5.8(4)	54.8±3.8(5)
	Trehalose		6.43	5.10	5.67	2.20	5.87	10.70	5.06	2.36	4.10
	Glucose		-	-	0.56	-	1.64	1.80	0.75	0.05	0.63
	Sorbitol	1	9.13	5.70	3.10	2.96	4.50	7.30	5.86	10.05	8.23
	Glycerol		7.33	4.85	1.13*	3.24	1.19*	2.95	- *	33.87	16.95
-9	SCP	-24.1±3.1(37)^{a}	-26.6±4.9(19)^{b}	-21.8±2.1(4)	-19.6±2.8(9)	-16.5±4.9(2)	-21.5±4.6(3)	-21.5±1.1(4)	-21(1)	-36.5±3.3(18)^{c}	Feb. -31.7±3.2(19
	H_2O (%)	44.9±4.3(10)	58.8±5.2(5)	60.2±0.8(2)	63.4±7.1(4)	64.8±6.5(2)	68.6±9.7(3)	62.1±2.4(2)	60.7(1)	41.0±6.3(5)	53.0±1.5(5)
	Trehalose	3.70	5.86	5.56	3.48	-	4.10	8.30	-	-	
	Glucose	-	-	-	1.82	-	-	4.86	-	-	
	Sorbitol	5.0	19.46	10.16	1.50	-	2.30	11.50	-	9.75	
	Glycerol	8.74	43.07	11.80	3.83	-	8.95	12.08	-	45.75	1
-18	SCP	-25.0±3.3(29)^{a}	-27.0±3.8(22)^{a}	-21.8±1.6(7)	-20.6±2.8(6)	-21.7±2.8(6)	-20.3±3.0(5)	-21.0±2.0(3)	-22.5±0.5(3)	-36.8±1.9(18)^{b}	
	H_2O (%)	49.4±5.6(8)	60.1±4.2(6)	57.8±0.4(2)	63.3±3.0(3)	62.5±5.7(3)	64.9±7.6(2)	60.1(1)	64.2±2.8(2)	40.2±4.1(5)	
	Trehalose	4.25	6.84	3.57	4.45	9.70	8.50	-	6.30	-	
	Glucose	-	-	1.90	1.49	5.60	1.67	-	1.36	-	
	Sorbitol	8.15	25.20	10.02	6.90	2.60	3.46	0.34	5.20	8.67	
	Glycerol	7.77	50.40	5.90	7.65	4.10	4.90	0.68	6.73	39.39	
-26	SCP	-24.1±4.0(34)^{a}	-27.0±6.2(20)^{b}	-21.0±1.5(3)$^{+}$	-22.1±1.9(6)$^{+}$	-20.9±3.1(6)$^{+}$	-19.8±2.1(6)$^{+}$	-	-20.5±0.7(2)$^{+}$	-37.1±1.7(15)^{c}	
	H_2O (%)	47.2±6.9(4)	62.8±6.3(5)	62.0(1)	60.7±4.0(2)	64.6±1.3(2)	63.4±3.7(2)	-	81.8(1)	47.6±1.5(4)	
	Trehalose									1.30	
	Glucose									-	
	Sorbitol									7.15	
	Glycerol									38.45	

The same letter after SCP at a given temperature indicates no significant difference ($\alpha = 0.05$).
Month above data for wild group indicates time of year larvae were collected.
ǂ - Biochemical analysis missing because samples were contaminated.
† - No significant difference in supercooling point between numbers of this group at a given acclimation temperature.
( ) - Numbers in parenthesis indicate sample size (n).
* - Small amounts of erythritol (≈0.4 mg/ml) measured.
\+ - 100% mortality.

TABLE 7

Supercooling points, percentage water, sugar and polyol concentrations for third instar larvae of D. ponderosae from different rearing treatments.

		REARING TREATMENT								
				MICROBE-FED†						
Acclimation Temperature	Variables Measured	Axenic	Force-Reared	Control	Basidio	E.C.	C.M.	Pichia	Hans	Wild
°C +5	Supercooling Pt.	-18.3±1.6(30)^{a}	-20.8±2.8(15)^{b}	-19.1±1.5(4)	-17.5(1)	-	-18(1)	-17.2±2.5(2)	-18.4±1.4(5)	
	Water Content (%)	58.2±2.8(9)	69.4±2.8(15)	65.0±1.6(2)	60.6(1)		58.1(1)	74.7(1)	74.1±8.5(3)	
	Trehalose (%)	7.69	4.20					-	-	
	Glucose (%)	-	-					-	-	
	Sorbitol (%)	22.26	8.27							
	Glycerol (%)	1.96	4.36							
0	SCP	-19.3±1.0(27)^{a}	-20.9±2.7(16)^{b}	-19.8±0.3(3)	-19.0(1)	-	-19(1)	-19.5±0.7(4)	-19.0±0.7(2)	Oct. -21.7±3.6(12)^{b}
	H_2O (%)	57.8±4.4(8)	66.1±7.5(4)	62.0±1.7(2)	52.9(1)		80.0(1)	65.5±0.4(2)	63.9(1)	60.0±4.5(3)
	Trehalose	9.45	5.21							5.10
	Glucose	-	-							0.57
	Sorbitol	13.72	8.03							5.05
	Glycerol	0.84	5.48							3.90
-9	SCP	-22.1±2.2(43)	-27.2±2.9(8)^{a}	-22.4±3.4(4)	-	-	-21(1)	-20(2)	-21.5(1)	Feb. -32.0±2.6(4)^{b}
	H_2O (%)	61.2±3.8(11)	64.8±7.9(2)	63.5(1)			64.4(1)	63.4±9.3(2)	66.7(1)	57.1(1)
	Trehalose	7.24								-
	Glucose	-								-
	Sorbitol	7.87								11.78
	Glycerol	10.54								13.50
-18	SCP	-22.0±2.1(35)^{a}	-23.9±2.6(14)^{b}	-20.9±2.0(5)	-	20.0(1)	-18(1)	-20.5(1)	-22.1±1.0(4)	
	H_2O (%)	61.3±2.5(10)	66.5±1.7(4)	73.0±7.9(2)		76.2(1)	67.2(1)	63.3(1)	65.4±1.9(2)	
	Trehalose	8.15	2.40							
	Glucose	-	-							
	Sorbitol	10.86	10.50							
	Glycerol	6.66	14.20							
-26	SCP	-21.7±2.0(37)$^{+}$	-24.8±7.6(4)	-21.5(1)$^{+}$	-22.2±1.1(2)$^{+}$	-	-20(2)$^{+}$	-	-	
	H_2O (%)	59.9±4.5(10)	61.4(1)	64.7(1)	64.2(1)		65.4(1)			

The same letter after supercooling point at a given temperature indicates no significant difference ($\alpha = 0.05$).
Abbreviated month above wild data indicates larvae they were collected.
+ - 100% mortality.
† - No significant difference was measured between supercooling points at a given temperature.

TABLE 8

Supercooling points and percentage water for second instar larvae of _D. ponderosae_.

		REARING TREATMENT		
		AXENIC		
Acclimation Temperature °C	Variables Measured	Rearing Units	Bark*	Wild
+5	Supercooling Point('C)	-19.6±1.2(27)		
	Water Content (%)	60.2±14.4(8)		
0	SCP	-21.2+1.3(35)		Oct. -22.4±0.7(11)
	H_2O (%)	61.8±9.7(11)		57.5±0.9(3)
-9	SCP	-22.9±1.5(16)	-23.5±1.0(12)	
	H_2O (%)	67.1±7.4(5)	57.3±5.0(3)	
-18	SCP	-22.7±1.3(16)	-24.9±1.5(12)	
	H_2O (%)	63.3±7.2(3)	60.5±5.2(3)	
-26	SCP	-21.1±3.9(10)$^{+}$	-25.6±1.0(12)$^{+}$	
	H_2O (%)	71.9±9.9(3)	60.5±3.9(3)	

* - Acclimated on dry bark.
$^{+}$ - 100% mortality.

TABLE 9

Supercooling points, percentage water, sugar and polyol concentration for *D. ponderosae* pupae.

		REARING TREATMENT						
			MICROBE-FED					
Acclimation Temperature °C	Variables Measured	Axenic	Control	Basidio	E.C.	C.M.	Pichia	Hans
+5	Supercooling Point (°C)	-18.2±2.7(26)	-	-19(1)	-20(2)	-16(1)	-16(1)	-
	Water Content (%)	72.3±4.6(9)		73.5(1)	80.1(1)	80.5(1)	79.8(1)	
	Trehalose (%)	6.90						
	Glucose (%)	-						
	Sorbitol (%)	2.50						
	Glycerol (%)	1.06						
0	SCP	-19.6±1.3(18)	-	-19.1±1.6(4)	-18.2±0.4(2)	-19.8±0.4(2)	-	-
	H_2O (%)	73.7±2.1(5)		77.2±1.2(2)	75.2±1.6(2)	77.8(1)		
	Trehalose	9.40						
	Glucose	-						
	Sorbitol	4.10						
	Glycerol	0.57						
-9	SCP	-18.9±2.7(19)	-	-	-19.8±0.3(3)	-20(1)	-15(1)	-
	H_2O (%)	75.1±4.9(6)			75.0±2.2(2)	77.6(1)	80.2(1)	
	Trehalose	13.9						
	Glucose	-						
	Sorbitol	4.95						
	Glycerol	0.77						
-18	SCP	-15.7±4.4(18)[†]	-	-	-19.8±1.1(2)	-	-	-
	H_2O (%)	73.4±2.7(5)			75.9(1)			
-26	SCP	-14.1±4.9(20)[†]	-	-	-	-	-	-
	H_2O (%)	68.1±6.6(6)						

[†] - 100% mortality.

TABLE 10

Supercooling points, percentage water, sugar and polyol concentration for male and female *D. ponderosae* tenerals.

Acclimation Temperature °C	Variables Measured	REARING TREATMENT	
		Axenic	Force-reared
+5	Supercooling point (°C)	-17.3±1.8(32)[a]	-7.8±1.5(14)[b]
	Water content (%)	56.0±6.2(10)	72.8±7.7(4)
	Trehalose (%)	4.68	2.26
	Glucose (%)	0.80	0.90
	Sorbitol (%)	8.54	2.15
	Glycerol (%)	0.62	-
0	SCP	-17.7±1.5(25)[a]	-7.8±2.5(6)[b]
	H_2O (%)	59.5±6.5(7)	74.3±10.5(2)
	Trehalose	7.77	4.56
	Glucose	1.41	-
	Sorbitol	10.71	2.10
	Glycerol	1.61	-
-9	SCP	-20.6±2.9(19)	-15.9±6.3(4)
	H_2O	60.5±8.7(5)	65.0(1)
	Trehalose	5.68	
	Glucose	-	
	Sorbitol	1.43	
	Glycerol	8.91	
-18	SCP	-22.4±2.4(16)[+]	-22.6±3.4(7)[+]
	H_2O (%)	52.4±1.0(4)	62.8±1.8(2)
-26	SCP	19.4±0.6(8)[+]	-10.7±4.6(3)[+]
	H_2O	53.2±1.8(2)	-

[+] - 100% mortality.

TABLE 11

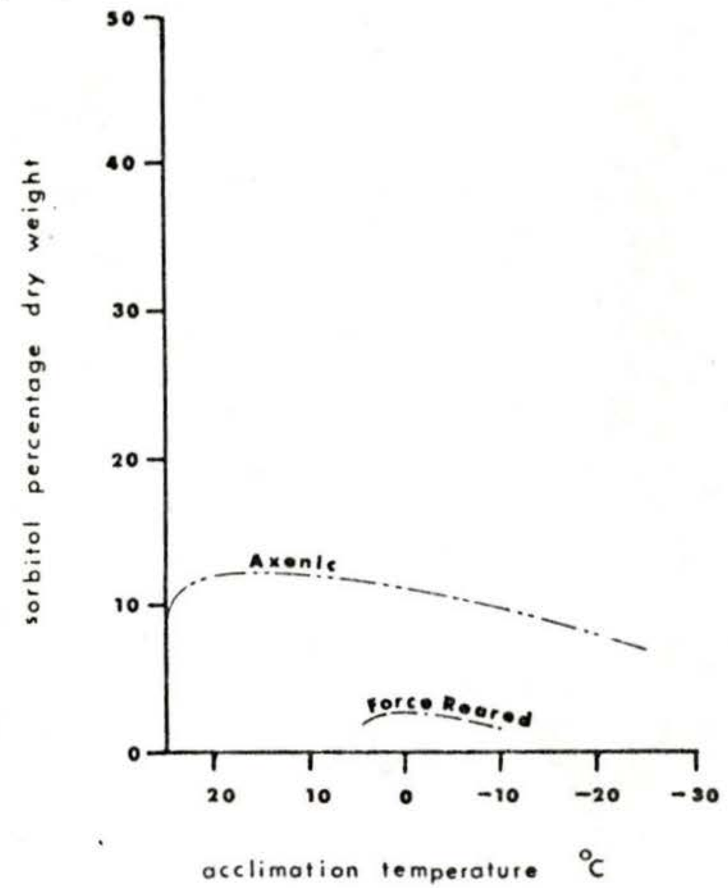
Supercooling points, percentage water, sugar and polyol concentration for male and female D. ponderosae adults.

Acclimation Temperature °C	Variables Measured	REARING TREATMENT Axenic	 Force-reared	 Wild (July)
+24	Supercooling point (°C)	-11.3±2.6(18)		-12.1±1.7(49)
	Water Content (%)	60.6±5.9(10)		62.8±3.7(25)
	Trehalose (%)	– –		– –
	Glucose (%)	– –		– –
	Sorbitol (%)	3.4 15.82		6.42 17.24
	Glycerol (%)	– –		– –
		♂ ♀ *		♂ ♀*
+ 5	SCP	-17.1±1.2(23)^{a}	-10.7±3.2(20)^{b}	
	H_2O (%)	55.5±2.7(6)	64.8±4.9(8)	
	Trehalose	–	–	
	Glucose	1.47	–	
	Sorbitol	12.80	3.40	
	Glycerol	–	–	
0	SCP	-17.4±1.4(22)^{b}	-10.8±4.2(22)^{b}	
	H_2O (%)	57.7±3.4(7)	57.9+3.4(7)	
	Trehalose	2.75	4.96	
	Glucose	1.15	–	
	Sorbitol	11.73	5.70	
	Glycerol	–	–	
-9	SCP	-19.2±3.0(20)^{a}	-19.8±5.0(11)^{a}	
	H_2O (%)	54.8±4.3(6)	56.0±2.2(3)	
	Trehalose	3.59	2.0	
	Glucose	0.46	–	
	Sorbitol	7.70	3.80	
	Glycerol	6.92	8.99	
-18	SCP	-18.2±1.2(15)$^{+}$	-19.3±5.8(8)$^{+}$	
	H_2O	54.3±2.8(4)	53.8±3.0(2)	
-26	SCP	-17.7±1.2(15)$^{+}$	-14.9±3.4(6)$^{+}$	
	H_2O (%)	55.2±1.8(4)	57.2±3.2(2)	

The same letter after supercooling point at a given temperature indicates no significant difference ($\alpha = 0.05$).

* ♂ and ♀ split up for biochemical analysis. Sorbitol content was averaged for this value in Fig. 25.

FIGURE 25-Sorbitol concentration in *D. ponderosae* adults.



A larger sample size for biochemical analysis might have reduced the variation measured in sugar and sorbitol concentrations so a better assessment of their function could have been made. It is of interest that in the biochemical analysis of axenic adults acclimated to +24°C only sorbitol was detected, at a high level in females, then decreased with acclimation from +5°C to -9°C (Fig. 25). There was an increase in sugar concentrations and glycerol was detected in beetles only after acclimation to -9°C (Table 11). The biochemical analysis showed high sorbitol levels in wild adults collected in July that were comparable to sorbitol levels measured in axenics at +24°C (Table 11). Analysis of force-reared beetles showed a fluctuation in sorbitol and sugar concentrations with acclimation from +5°C to -9°C and glycerol was detected only after acclimation to -9°C (Table 10).

When survival was low in the samples (Tables 12-14) average supercooling point increased and water content usually increased (Table 6-11). This increase in supercooling point agrees with the findings of Somme (1964). No significant levels of sugars and sugar alcohols were detected in frozen insects probably due to mixing of body fluids after thawing. Therefore, all samples with zero survival at -18°C and -26°C were discarded. An unidentified

TABLE 12

Survival estimates for microbe-fed *D. ponderosae* larvae and pupae.

Acclimation Temperature	Control	Basidio	E.C.	C.M.	Pichia	Hans	Trichoderma sp.
°C	%	%	%	%	%	%	%
+5	100	100	100	100	100	100	0
0	100	100	100	100	100	100	0
− 9	62	33	0	60	38	43	0
−18	15	10	20	29	29	29	0
−26	0	0	0	0	0	0	0

TABLE 13

Survival estimates for axenic beetles.

	DEVELOPMENTAL STAGE						
	Larval instars						
Acclimation Temperature	1	2	3	4	Pupa	Teneral	Adult
°C	%	%	%	%	%	%	%
+5	100	100	100	100	100	100	100
0	100	100	100	93	100	100	100
− 9	50	75	82	100	14	100	100
−18	0	21	50	65	0	0	0
−26	0	0	0	5	0	0	0

TABLE 14

Survival estimates for force-reared beetles.

	DEVELOPMENTAL STAGE			
	LARVAL INSTARS			
Acclimation Temperature	3	4	Teneral	Adult
°C	%	%	%	%
+5	100	100	100	100
0	100	100	100	100
− 9	100	100	–	92
−18	13	50	0	0
−26	25	35	0	0

Estimated 100% survival for wild acclimated larvae at all acclimation temperatures.

compound with a retention time between 59-61 minutes measured in the biochemical analysis of about 50% of the samples was not detected in most microbe-fed samples. The compound was not detected in phloem or diet samples. The concentration varied with no apparent relationship to acclimation or supercooling ability so no further attempt was made to identify the compound. The concentration of sugars and sugar alcohols measured was probably low due to loss in the extraction procedure. Variation in concentrations could have been introduced by small differences in dry weight of the sample extracted, low survival in some samples and experimental error in techniques and equipment.

None of the artificially reared insects were as cold-hardy as the wild ones (Fig. 14, Tables 7, 8 & 11). Microbe-fed larvae were least cold-hardy followed by axenics then force-reared. Wild acclimated and wild larvae collected in the winter months were most cold-hardy (Fig. 14). Within the microbe-fed treatment supercooling points did not differ significantly between larvae in a given instar fed phloem or different fungi. There was also no significant difference in supercooling points between larvae fed different fungi (Tables 6 & 7). The water content and biochemical components varied with acclimation to lower temperatures.

There were only slight differences in supercooling points between pupae reared on different diets or at different acclimation temperatures (Table 9). Axenic tenerals and adults supercooled better than force-reared beetles at the higher acclimation temperatures but at -9°C there was not much difference (Fig. 18 & 21). No satisfactory explanation can be given for this observation except, perhaps, because the beetles were force-reared at high temperatures (Methods) they took longer to acclimate to lower temperatures. The groups that fed on the full complement of microbes and host tissue were more cold-hardy than the groups that fed on a single fungus, sterile phloem, or axenic diet. The observed differences in cold-hardiness may be due to a deficiency in the artificial diets that produced insects not able to synthesize or utilize compounds necessary for increased cold-hardiness. This dietary deficiency could be due to the lack of normal microbial content, physical and chemical changes that occurred during diet and block preparation, or a combination of both these factors. The microbes associated with bark beetles modify the biochemical composition of host tissue during the colonization process. Effects such as water loss and changes in carbohydrate levels in pine tissues colonized by fungi have been described by Mathre (1963-64b). Reid et al (1967) and Francke-Grosmann (1967) have suggested that the microbes add a nutritional component to the host tissue. The importance of larval nutrition to the success of many beetle functions was stated by Farmer (1965) and these results indicate nutrition is important in cold-hardiness as well.

Another explanation for the observed differences between artificially reared insects and wild ones may be that the acclimation procedure did not allow artificially reared insects enough time to accumulate protective compounds, or the alimentary canal was not evacuated which could result in higher supercooling points (Salt, 1953). The wild insects from the study area were subjected to a gradual acclimation over 3-4 months with diurnal temperature fluctuation and a mean air temperature close to -9°C in the coldest month (see Appendix). Wild larvae responded to the artificial acclimation procedure (wild acclimated, Fig. 14-16) but appeared to have been already prepared for extreme cold temperatures when collected in October. Larvae, in bolts from the same collection, that were force-reared in the shadehouse (see Methods) had higher supercooling points than wild acclimated larvae (Fig. 14) and some had food in the gut. *D. monticolae* (=*ponderosae*) eliminated the contents of the alimentary canal when exposed to a temperature fluctuation of -6.5°C to +10°C (20-50°F) for several days then were more cold-hardy but another species, *D. brevicomis*, did not eliminate the gut contents when exposed to temperature fluctuation (Miller, 1931). Particulate matter was observed in the alimentary canal of most adults and tenerals. Food was observed in the gut of some larvae but most contained an opaque, brown, viscuous fluid in the foregut and a clear viscuous fluid in the mid and hindguts. The alimentary canal of wild larvae also contained a clear, viscous fluid. Somme (1966) reported that larger particle size of material in the insect gut raised the supercooling point more than smaller particle size. Although no particulate matter was observed, with a dissecting microscope, in this viscous fluid it is possible that nucleators were present that could raise supercooling point. The artificial acclimation procedure may have had some effect but the results indicate a difference due to rearing treatment between groups subjected to this acclimation procedure.

The supercooling points of axenic larvae acclimated on dried bark did not differ substantially from larvae acclimated in the diet (Table 8). This result suggests that inoculative freezing did not occur from the diet. Occasionally ice crystals were observed on larvae and extended from the month of larvae. Inoculative freezing may have occurred in these larvae from contact moisture or regurgitated fluids (Salt, 1958).

Glycerol and glucose were detected in biochemical anlysis of sterile phloem and axenic diet. Sorbitol was also detected in axenic diet samples.

TABLE 15

Biochemical analysis on axenic diet and autoclaved pine phloem.

	Axenic Diet	Autoclaved Phloem
Trehalose (%)	-	-
Glucose (%)	0.66	0.75
Sorbitol (%)	1.09	-
Glycerol (%)	1.00	1.18

It is not known if the glycerol and sorbitol were used directly by the insect but nucleation is suppressed in viscous solutions (Salt, 1961) so ingestion of these compounds with food may have some effect on cold-hardiness if gut contents have not been excreted. Small amounts of glycerol and sorbitol may be used in beetle processes not related to cold-hardiness. The major insect sugar trehalose is synthesized from glucose (Wigglesworth, 1972).

Differences in dry weight had no apparent effect on supercooling ability.

Conclusions

1. Larval development in phloem colonized exclusively by *E. clavigerum* or the unidentified basidiomycete was faster and more larvae were produced than on sterile phloem.
2. Larval development was poorer in phloem colonized by *C. montia*, *Pichia* sp., and *Hansenula* sp. than in sterile phloem.
3. *Trichoderma* sp. was antagonistic to beetle development.
4. Larval development time on blocks colonized by the basidiomycete and *E. clavigereum* was comparable to development time in the wild.
5. Dry weight of larvae produced on blocks colonized by *E. clavigerum* and the basidiomycete was larger than dry weight of larvae reared on phloem alone or the other fungi.
6. The symbiotic relationship between the beetles and microbes is mutualistic since larval development on blocks colonized by some fungi was better than on phloem alone.

7. The "ambrosial" growth form of some fungi was observed on blocks colonized by E. clavigerum, C. montia and the basidiomycete.
8. The change to "ambrosial" growth form was stimulated by the presence of larvae and/or pupae.
9. Production of larvae on phloem and sapwood blocks was 45%, mortality was 30%, and 25% were unhatched eggs not found due to dense fungal growth or larvae that were cannibalized.
10. Cannibalism at a crowding level of 5 larvae per cubic centimeter of diet enabled more larvae to complete metamorphosis compared to crowding levels of 3, 2 or 1 larvae per cubic centimeter of diet.
11. Tenerals produced by cannabalism did not perform as well as wild adults when paired on pine bolts.
12. Beetle larvae were nost cold-hardy followed by pupae, adults and tenerals.
13. Microbe-fed larvae were not as cold-hardy as larvae that fed on a sterile diet containing brewers' yeasts or wild larvae that fed on the full microbial complement.
14. No difference was observed in cold-hardiness between larvae reared on blocks colonized by the different fungi.

15. A decrease in supercooling point of 5°-6°C or more through acclimation corresponded with a decrease in water content and increased glycerol concentration.
16. A decrease in supercooling point of less than 5°-6°C through acclimation showed no clear relationship between supercooling points, water content and glycerol concentration.
17. Trehalose and small amounts of glucose were detected in the biochemical analysis but concentrations varied through acclimation.
18. High concentrations of sorbitol were detected in some samples acclimated to temperatures above 0°C, but no clear relationship was observed between sorbitol concentrations and supercooling points through acclimation.
19. High mortality at the lower acclimation temperatures resulted in elevated supercooling points and water content.
20. An unidentified compound was detected in biochemical analysis of about 50% of the samples. No relationship was observed between concentration of the unknown compound and supercooling point through acclimation.

21. Glycerol and glucose were detected in biochemical analysis of sterile phloem and axenic diet. Sorbitol was also detected in axenic diet samples.

The microbe-fed technique is a valuable tool for the study of the bark beetle-microbe-tree complex to the extent that it allows the complex to be manipulated in a controlled laboratory environment. There likely are interactions between many different organisms in the complex which may be antagonistic or beneficial to brood success depending on where and when they occur in the beetles' life cycle. Further investigations along this line might determine the effects of combined microorganisms grown together with the beetle throughout its development.

Cold-hardiness is a dynamic process affected by many factors (eg. nutrition) throughout the entire life cycle which are expressed as brood survival or failure after the overwintering period. Therefore, cold-hardiness might best be studied in the field where the microhabitat could be scrutinized for many factors (eg. temperature, humidity, type and abundance of microorganisms) over the entire life cycle then related to brood survival over winter. A better understanding of the microhabitat would enable the researcher to simulate the natural conditions in the more controlled laboratory environment.

LITERATURE CITED

Abd-el-moity, J. H., and M. N. Shatla. 1981. Biological control of white rot disease (*Sclerotium cepivorum*) of onion by *Trichoderma harzianum.* Phytopathol. Zool. 100(1): 29-35.

Asahina, E. 1969. Frost resistance in insects. *In* Advances in Insect Physiology Ed. by J. W. L. Beament, J. E. Treherne, V. B. Wigglesworth. 1969. P 1-49. Academic Academic Press, N.Y. and London.

Asahina, E. and K. Tanno. 1964. A large amount of trehalose in a frost-resistant insect. Nature 204: 1222.

Bakshi, B. K. 1950. Fungi associated with ambrosia beetles in Great Britain. Trans. Brit. Mycol. Soc. 33: 111-112.

Barras, S. J. 1970. Antagonism between *Dendroctonus frontalis* and the fungus *Ceratocystis minor.* Ann. Ent. Soc. Amer. 63: 1187-1190.

Barras, S. J. 1973. Reduction of progeny and development in the southern pine beetle following removal of symbiotic fungi. Can. Entomol. 105B: 1295-1299.

Barras, S. J., and T. Perry. 1971. Gland cells and fungi associated with prothoracic mycangium of *Dendroctonus adjunctus* (Coleoptera: Scolytidae). Ann. Ent. Soc. Amer. 64: 123-126.

Barras, S. J., and J. J. Taylor. 1973. Varietal *Ceratocystis minor* identified from mycangium of *Dendroctonus frontalis.* Mycopath. et. Mycol. Appl. 50: 293-305.

Batra, L. R. (ed.). 1979. Insect-fungus symbiosis. Allanheld, Osmun and Co. New Jersey. p. 276.

Baust, J. G. 1973. Mechanisms of cryoprotection in freezing tolerant animal systems. Cryobiology 10: 197-205.

Baust, J. G. 1981. Biochemical correlates to cold-hardening in insects. Cryobiology 18: 186-198.

Bedard, W. D. 1966. A ground phloem medium for rearing immature bark beetles. Ann. Ent. Soc. Amer. 59: 931-937.

Bramble, W. C. and E. C. Holst. 1940. Fungi associated with *Dendroctonus frontalis* in killing shortleaf pines and their effect on conduction. Phytopath. 30: 881-899.

Brand, J. M., J. W. Bracke, A. J. Markovetz, D. L. Wood and L. E. Brown. 1975. Production of verbenol pheromone by a bacterium isolated from bark beetles. Nature. 254: 136-137.

Brand, J. M., J. W. Bracke, L. N. Britton, A. J. Markovetz and S. J. Barras. 1976. Bark beetle pheromones: Production of verbinone by a mycangial fungus of *Dendroctonus frontalis.* Jour. Chem. Ecol. 2(2): 195-199.

Brand, J. M., J. Shultz, S. J. Barras, L. J. Edson, T. L. Payne and R. L. Helden. 1977. Bark beetle pheromones: enhancement of *Dendroctonus frontalis* aggregation pheromone by yeast metabolites in lab bioassays. Jour. Chem. Ecol. 3(6): 657-666.

Browne, L. E. 1972. An emergence cage and refrigerated collector for wood-boring insects and their associates. Jour. Econ. Ent. 65(5): 1499-1501.

Byers, J. A. and D. L. Wood. 1981. Antibiotic induced inhibition of pheromone synthesis in a bark beetle. Science 213: 763-764.

Callaham R. Z. and M. Shifrine. 1960. The yeasts associated with bark beetles. For. Sci. 6: 146-154.

Chino, H. 1957. Conversion of glycogen to sorbitol and glycerol in the diapause egg of the *Bombyx* silkworm. Nature. 180:606-607.

Cole, W. E. 1973. Crowding effects among single-age larvae of the mountain pine beetle, *Dendroctonus ponderosae* (Coleoptera: Scolytidae). Envir. Ent. 2: 285-293.

Coulson, R. N. 1979. Population dynamics of bark beetles. Ann. Rev. Ent. 24: 417-447.

Craighead, F. C. 1925. Bark beetle epidemics and rainfall deficiency. Jour. Econ. Ent. 18: 577-586.

Craighead, F. C. 1928. Interrelation of the tree-killing bark beetles (*Dendroctonus*) and blue stains. Jour. For. 26: 886-887.

Craighead, F. C., and R. A. St. George. 1940. Field observations on the dying of pines infected by the blue stain fungus *Ceratostomella pini.* Phytopath. 30: 976-979.

Danks, H. V. 1978. Modes of seasonal adaptation in the insects. I. Winter survival. Can. Ent. 110B: 1167-1205.

Dowding, P. 1970. Colonization of freshly bared pine sapwood surfaces by staining fungi. Trans. Brit. Mycol. Soc. 55: 399-412.

Dubach, P., D. Pratt, F. Smith, C. M. Stewart. 1959. Possible role of glycerol in the winter hardiness of insects. Nature. 184: 288-289.

Duman, J. G. 1977. The role of macromolecular antifreeze in the darkling beetle, Meracantha contracta. Jour. Comp. Physiol. 115: 279-286.

Elad, Y., I. Chet, and J. Katon. 1980. Trichoderma harzianum: a biocontrol agent effective against Sclerotium rolfsii and Rhizoctonia solani. Phytopath. 70(2): 119-121.

Farmer, L. J. 1965. The phloem-yeast complex during infestations of the mountain pine beetle in lodgepole pine. Disser. Abst. 26: 1304-1305.

Farris, S. H. 1965. A preliminary study of mycangia in the bark beetles, Dendroctonus ponderosae Hopk., Dendroctonus obesus Mann., and Dendroctonus pseudotsugae Hopk. Can. Dept. of For. Bi-monthly prog. Rept. 21(5): 3-4.

Fiddick, R. L., and G. A. VanSickle. 1981. Forest insect and disease conditions. British Columbia and Yukon/1981 Canadian Forestry Service BC-X-225.

French, J. R. J. and R. A. Roeper. 1972. Interactions of the ambrosia beetle Xyleborus dispar (Coleoptera: Scolytidae) with its symbiotic fungus Ambrosiella hartigii (F.I.) Can. Ent. 104: 1635-1641.

Francke-Grosmann, H. 1967. Ectosymbiosis in wood-inhabiting insects. In Symbiosis Vol. II Ed. by S. M. Henry. 1967. P. 141-205. Academic Press. N.Y. and London.

Graham, K. 1967. Fungal-insect mutualism in trees and timber. Ann. Rev. Ent. 12: 105-126.

Green, G. W. 1962. Low winter temperatures and the European pine shoot moth, Rhyacionia buoliana (Schiff) in Ontario. Can. Ent. 94: 314-336.

Hansen, T. E., M. O. Viyk and A. K. Luyk. 1980. Biochemical changes and cold-hardiness in overwintering bark beetles Ips typographus. Ent. Rev. 59(2): 9-12.

Happ, G. M., C. M. Happ, S. J. Barras. 1975. Bark beetle - fungal symbiosis III. Ultra Stucture of conidiogenesis in a Sporothrix ectosymbiont of the southern pine beetle. Can. Jour. Bot. 53D: 2702-2711.

Happ, G. M., C. M. Happ, S. J. Barras. 1976. Bark beetle - fungal symbiosis. II. Fine stucture of a basidiomycetous ectosymbiont of the southern pine beetle. Can. Jour. Bot. 54: 1049-1062.

Henry, S. M. (ed.) 1966-67. Symbiosis (2 Vols). Academic Press, New York and London. Vol. I P. ix-xi.

Kartnig, T. and O. Wegshaider. 1971. Eine moglichkeit zur identifizierung von zuckern auskleinsten meugen von glycosiden oder aus zucker gemischen. J. Chromatogr. 61: 375-377. (in German).

Koch, A. 1967. Insects and their endosymbionts. In Symbiosis Vol II Ed. S. M. Henry. 1967. P. 1-106. Academic Press, N.Y. and London.

Lemieux, R. U., and H. F. Bauer. 1954. Spray reagent for the detection of carbohydrates. Analytical Chem. 26: 920.

Leonard, T. J. 1973. Introduction of haploid fruiting by mechanical injury in Schizophyllum commune. Mycol. 65: 809-822.

Manning, G. H., L. Safranyik, G. H. VanSickle, R. B. Smith, W. A. White, and E. Hetherington. 1982. A review of mountain pine beetle problems in Canada. Canadian Forestry Service, Pacific Forest Research Centre, 506 W. Burnside Rd., Victoria, B.C. V8Z 1M5

Mathre, D. E. 1963-64a. Pathogenicity of Ceratocystis ips and Ceratocystis minor to Pinus ponderosa. Contrib. Boyce. Thomp. Inst. 22: 363-388.

Mathre, D. E. 1963-64b. Effect of Ceratocystis ips and Ceratocystis minor on the free sugar pool in ponderosa pine sapwood. Contrib. Boyce. Thomp. Inst. 22: 509-511.

Miller, J. M. 1931. High and low lethal temperatures for the western pine beetle. Jour. Agr. Res. 43: 303-321.

Poinar, G. O. Jr. and G. M. Thomas. 1978. Diagnostic Manual for the Identification of Insect Pathogens. Plenum Press, N.Y. 218. p.

Powell, J. M. 1968. A study of habitat temperatures of the bark beetle *Dendroctonus ponderosae* (Hopkins) in lodgepole pine. Agric. Meteorol. 4: 189-201.

Reid, R. W. 1961. Moisture changes in lodgepole pine before and after attack by mountain pine beetle. For. Chron. 37(4): 368-375.

Reid, R. W. 1962. Biology of mountain pine beetle, *D. monticolae*: life cycle, brood development and flight. Can. Ent. 94: 531-538.

Reid, R. W. 1963. Biology of the mountain pine beetle *Dendroctonus monticolae* (Hopkins) in the East Kootenay region of British Columbia. III. Interaction between the beetle and its host with emphasis on brood mortality and survival. Can. Ent. 95: 225-238.

Reid, R. W., H. S. Whitney, J. A. Watson. 1967. Reactions of lodgepole pine to attack by *Dendroctonus ponderosae* (Hopkins) and blue stain fungi. Can. Jour. Bot. 45: 1115-1125.

Reid, R. W. and H. Gates. 1970. Effect of temperature and resin on hatch of eggs of the mountain pine beetle *Dendroctonus ponderosae*. Can. Ent. 102: 617-622.

Reid, R. W. and D. M. Shrimpton. 1971. Resistant response of lodgepole pine to inoculation with *Europhium clavigerum* in different months and at different heights on stem. Can. Jour. Bot. 49A: 349-351.

Ring, R. A. 1980. Insects and their cells. *In* Low Temperature Preservation in Medicine and Biology Edited by M. J. Ashwood Smith and J. Farrant. Pitman Medical Limited, 57 High St., Tunbridge Wells, Kent.

Ring. R. A. 1981. The physiology and biochemistry of cold tolerance in arctic insects. Jour. Therm. Biol. 6: 219-229.

Ring, R. A. and D. Tesar. 1980. Cold-hardiness of the arctic beetle, *Pytho americanus* Kirby (Coleoptera, Pythidae (Salpingidae)) J. Insect Physiol. 26: 763-774.

Ring, R. A. and D. Tesar. 1981. Adaptations to cold in Canadian arctic insects. Cryobiology. 18: 199-211.

Robinson, R. C. 1962. Blue stain fungi in lodgepole pine (*Pinus contorta* Dougl. var. *latifolia* Englemann.) infested by the mountain pine beetle *Dendroctonus monticolae* (Hopkins) Can. Jour. Bot. 40: 609-614.

Robinson-Jeffrey, R. C. and R. W. Davidson. 1968. Three new *Europhium* species with *Verticicladiella* imperfect states on blue stained pine. Can. Jour. Bot. 46: 1523-1527.

Richards, G. A., and M. A. Brooks. 1958. Internal symbiosis in insects. Ann. Rev. Ent. 3: 37-56.

Rumbold, C. T. 1941. A blue stain fungus, *Ceratostomella montium* N.Sp. and some yeasts associated with two species of *Dendroctonus*. Jour. Agr. Res. 62: 589-601.

Safranyik, L. 1978. Effects of climate and weather on mountain pine beetle populations. *In* Theory and Practice of Mountain Pine Beetle Management in Lodgepole Pine Forests. Symposium proceedings Dec. 1978. Forest, Wildlife, and Range Experiment Station. U. of Idaho.

Salt, R. W. 1950. Time as a factor in the freezing of undercooled insects. Can. Jour. Res. 28 (section D): 285-291.

Salt, R. W. 1953. The influence of food on cold-hardiness of insects. Can. Ent. 85: 261-269.

Salt, R. W. 1956. Influence of moisture content and temperature on cold-hardiness of hibernating insects. Nature 34: 283-294.

Salt, R. W. 1957. Natural occurrence of glycerol in insects and its relation to their ability to survive freezing. Can. Ent. 89: 491-494.

Salt, R. W. 1958. Application of nucleation theory to the freezing of supercooled insects. J. Insect Physiol. 2: 178-188.

Salt, R. W. 1961. Principles of insect cold-hardiness. Ann. Rev. Ent. 6: 55-74.

Salt, R. W. 1963. Delayed inoculative freezing of insects. Can. Ent. 95: 1190-1202.

Salt, R. W. 1966. Effect of cooling rate on the freezing temperatures of supercooled insects. Can. J. Zool. 44: 655-659.

Shepherd, R. F. and J. A. Watson. 1959. Blue stain fungi associated with the mountain pine beetle. Can. Dept. Agr. For. Biol. Div. Bimont. Prog. Rept. Vol 15(3): 2-3.

Shifrine, M. and H. J. Phaff. 1956. The association of yeasts with certain bark beetles. Mycologia 48: 41-55.

Shrimpton, D. M. (ed.) BC-X-230. 1982. Proceedings of the joint Canada/USA workshop on mountain pine beetle related problems in western North America.

Smith, K. J., R. O. Blanchard, and W. C. Shortle. 1981. Postulated mechanism of biological control of decay fungi in red maple (Acer rubrum) wounds treated with Trichoderma harzianum. Phytopathol. 71(5): 496-498.

Somme, L. 1964. Effects of glycerol on cold-hardiness of insects. Can. J. Zool. 42: 87-101.

Somme, L. 1965. Further observations on glycerol and cold-hardiness in insects. Can. Jour. Zool. 43:765-770.

Somme, L. 1966. The effect of temperature, anoxia, or injection of various substances on haemolymph composition and supercooling in larvae of Anagasta kuehniella. J. Insect Physiol. 12: 1069-1083.

Somme, L. 1967. The effect of temperature and anoxia on haemolymph composition and supercooling in three overwintering insects. J. Insect Physiol. 13: 805-814.

Steinhaus, E. A. 1967. Insect Microbiology. Hafner Publishing Co. New York and London.

Sullivan, C. R. 1965. Laboratry and field investigations on the ability of eggs of the European pine sawfly, Neodiprion sertifer (Geoffrey) to withstand low winter temperatures. Can. Ent. Vol. 97: 978-993.

Upadhyay, R. K. 1981. Effect of volatile phylloplane fungal metabolites on the growth of a foliar pathogen (Pastalotiopris fumerea). Experientia (Barel) 37(7): 707-708.

Webb, J. W. and R. T. Franklin. 1978. Influence of phloem moisture on brood development of the southern pine beetle (Coleptera: Scolytidae). Environ. Ent. 7: 405-410.

Whitney, H. S. 1971. Association of D. ponderosae with blue stain fungi and yeasts during brood development in lodgepole pine. Can. Ent. 103: 1495-1503.

Whitney, H. S. 1982. Relationships between bark beetles and symbiotic microorganisms. In Bark beetles in North American conifers: Ecology and evolution. Edited by J. B. Mitton and K. B. Sturgeon. Univ. of Texas Press.

Whitney, H. S. and R. A. Blauel. 1972. Ascospore dispersion in Ceratocystis spp. and Europhium clavigerum in conifer resin. Mycologia Lxiv(2): 410-414.

Whitney, H. S. and F. W. Cobb Jr. 1972. Non-staining fungi associated with the bark beetle Dendroctonus brevicomis (Coleoptera: Scolytidae) on Pinus ponderosa. Can. Jour. Bot. 50: 1943-1945.

Whitney, H. S. and O. J. Spanier. 1982. An improved method for rearing axenic mountain pine beetles, Dendroctonus ponderosae Hopk. (Coleoptera: Scolytidae). Can. Ent. (submitted 1982).

Wigglesworth, V. B. 1972. The Principles of Insect Physiology. 7th edition. Chapman and Hall, London.

Yuill, J. S. 1941. Cold-hardiness of two species of bark beetles in California forests. Jour. Econ. Ent. 34(5): 702-709.

Zachariassen, K. E. 1979. The mechanism of the cryoprotective effect of glycerol in beetles tolerant to freezing. J. Insect Physiol. 25A: 29-32.

Zar, J. H. 1974. Biostatistical Analysis. Prentice-Hall, Inc. New Jersey.

APPENDIX

B.C. Monthly Weather Summary - LORSTA, Williams Lake*

	Average			Extreme	
Month	Max	Min	Mean (°C)	Max	Min (°C)
			1978		
Sept.	15.3	3.4	9.4	20.0	- 4.0
Oct.	12.2	- 1.2	5.5	20.5	11.0
Nov.	- 2.5	-13.1	-7.8	-12.0	-31.5
Dec.	- 4.6	16.2	-10.4	7.0	-42.0
			1979		
Jan.	-11.0	-23.9	-4.1	5.0	-40.5
Feb.	1.6	-4.9	-3.3	10.0	-26.0
Mar.	7.8	-6.3	-	17.0	-19.0
Apr.	10.8	-1.5	13.1	24.0	- 6.0
Aug.	25.9	-	-	31.0	3.5
Sept.	21.0	5.1	4.7	26.0	- 1.0
Oct.	-	-	0.8	24.0	- 9.5
Nov.	2.5	-9.1	- 1.7	10.0	-17.0
Dec.	1.1	-9.3	-17.5	7.0	-36.5
			1980		
Jan.	- 8.3	-19.6	-14.0	3.0	-36.0
Feb.	- 0.6	-11.1	- 5.9	-	23.0
Mar.	3.3	- 5.5	- 1.1	10.0	-24.0
Apr.	15.2	- 0.3	6.1	22.5	- 7.0
Aug.	20.1	6.9	13.5	25.0	2.5
Sept.	18.0	4.5	11.3	27.0	1.5
Oct.	13.0	- 0.5	6.3	25.0	- 7.0
Nov.	6.5	- 5.6	0.5	15.0	-17.5
Dec.	- 4.3	-11.6	- 8.0	11.2	-32.8
			1981		
Jan.	2.9	- 5.8	- 1.4	12	-13
Feb.	1.4	- 8.6	- 3.6	9	-30
Mar.	9.8	- 3.1	3.3	14	-10.5
Apr.	11.1	- 3.1	4.0	22	-10.5
Aug.	27.1	8.6	17.9	34.5	1.0
Sept.	19.5	4.5	12.0	32	4.0
Oct.	11.3	0.0	5.7	18	9.5
Nov.	5.7	- 2.9	1.4	15	11.5
Dec.	- 4.0	-14.7	- 9.3	5	-30
			1982		
Jan.	- 8.6	-19.2	-13.9	4	-39.5
Feb.	-2.4	-13.5	- 7.9	6	-30.5
Mar.	3.9	-10.2	- 3.2	9	-22
Apr.	7.4	- 4.8	1.3	-	-

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* This weather station was located within 2-5 km. of the experimental area at Riske, B.C.

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