COLD-ACCLIMATION OF THE WOOD COCKROACH *Cryptocercus punctulatus* (Scudder) (Dictyoptera: Cryptocercidae)

by

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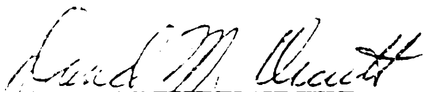
APPROVED:

Donald E. Mullins, Chairman



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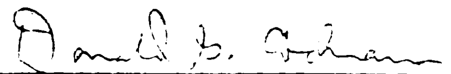

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(ABSTRACT)

The general overwintering strategy employed by the woodroach, *Cryptocercus punctulatus* (Scudder) appears to be fairly typical among freeze-tolerant insects. In this species, as well as others, heat-labile hemolymph ice nucleating factors induce extracellular ice formation at a high subzero temperature (around -6 °C), there is a seasonal accumulation of a cryoprotective substance (polyol) and internal ice is tolerated only during the winter. In addition, body water content does not fluctuate seasonally.

Hemolymph Ice Nucleating Factors (INF's) have been previously described from members of the following orders of insects: Diptera, Coleoptera, and Hymenoptera. This study documents the occurrence of INF's in a fourth order: Dictyoptera.

Cryptocercus punctulatus contains hemolymph ice nucleating factors through out the year. In this species, hemolymph serum freezes at -5.5 °C all year long and appears to be initiated by a heat-labile proteinacious nucleating factor. These proteins may function primarily as nucleators in the winter, and perform other function (s)

during the other seasons of the year.

A sugar alcohol (ribitol), contained in the hemolymph of *Cryptocercus punctulatus* was found to fluctuate seasonally. Ribitol levels were highest in the winter months, and declined to undetectable levels in the summer. To date, five polyols have been reported as occurring in insect hemolymph. They are: glycerol, sorbitol, mannitol, erythritol, and threitol. There is one report of the occurrence of an unidentified 5-carbon polyol, possibly either arabitol or ribitol from whole body extracts of winter-acclimated collembolans. This thesis documents the occurrence of ribitol in insect hemolymph. It is assumed that ribitol, like other polyols acts as a cryoprotectant to stabilize protein structure, membrane integrity, and reduce osmotic fluctuations in freezing tissues.

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1. INTRODUCTION

Overwintering biology is an extremely important aspect of the life history of insects inhabiting temperate zones. In order for insects to colonize and survive in cold climates, they must be able to withstand subzero temperatures. This phenomenon has been of interest to entomologists for many years, but little was known about the mechanisms which are involved. Recently, however, a considerable amount of progress has been made in gaining an understanding of insect cold-acclimation mechanisms. Many insects have the potential to withstand ice crystal formation within their bodies. Since these organisms can tolerate the presence of ice crystals within their body fluids, they are known as freeze-tolerant insects. These insects can survive many months in the frozen state with no apparent damage. The wood cockroach, (Cryptocercus punctulatus), is an insect which can withstand freezing. Studies of the mechanisms employed by this cockroach to avoid freezing damage (metabolic and physiological) may provide a means for the experimental and commercial applications in tissue freezing technology. Possible biotechnological applications include: freezing and storage of embryos, sperm, eggs, and other tissues. The objectives of this research were to: 1) determine certain aspects regarding the overwintering strategy of Cryptocercus

punctulatus, and, 2) to study hemolymph-borne factors and mechanisms which allow for cold-hardiness in *C. punctulatus*.

2. LITERATURE REVIEW

2.1 Description, Habitat and Distribution

The woodroach, *Cryptocercus punctulatus* was first described by Scudder in 1862, based on the observation of 3 dried specimens. There are only 3 species belonging to the genus *Cryptocercus* and the family Cryptocercidae. *Cryptocercus punctulatus* is the only species found in North America. This genus *Cryptocercus* was somewhat of a taxonomic curiosity and relatively unknown until Cleveland et. al., (1934) published an extensive study on the biology of this primitive insect.

Cryptocercus punctulatus inhabits downed hardwood timber (oak, chestnut) in various stages of decay, ranging from freshly downed (2-3 yrs) to fully rotted (25-30 yrs) logs. These insects burrow through the logs, forming an extensive network of galleries. Much like termites, they consume wood, utilizing cellulose as a food source with the aid of symbiotic gut organisms (protozoans and bacteria) (Cleveland et. al., 1934). This species is believed to be ancestral to termites because its members contain wood-feeding symbionts and live in similiar habitats (Seelinger and Seelinger, 1983).

Members of this species are found in scattered mountainous regions of North America at elevations ranging between 3,000 and 5,000 ft. These regions include the Appalachian Mountains between Southern Pennsylvania and

Northern Georgia, and the mountains of California, Oregon, and Washington. In the East, they are most abundant in Virginia and West Virginia, and are relatively scarce at the upper- and lower-most boundries of their distribution. Cleveland et. al., (1934) postulated that their distribution is limited in the Eastern United States because of an inability of both cockroach and gut symbionts to withstand extremes in temperature (ie above 30 ^{o}C, or "harsh" winters).

Cryptocercus punctulatus has a long and unique life cycle. Upon reaching sexual maturity, adult males and females pair for life. They will produce only 1 brood of young in their 10 yr. life span (Nalepa, 1984). A mated female will produce an ootheca in late May or early June which generally contains 32 eggs (Cleveland et. al., 1934). The ootheca hatches in July and the young nymphs molt several times during the first season and overwinter as third or fourth instars. After the first winter, the nymphs will molt once per year, (in the summer) for 4-5 years. Throughout the maturation period, nymphs and parents live together in distinct family units (Nalepa, 1984). The adults care for (brood) and protect the young for 3-5 years (Seelinger and Seelinger, 1983). After 5 years, the nymphs become sexually mature, leave the brood, find a mate, and reproduce.

Cleveland et. al., (1934), and more recently several other workers (Bloodgood and Fitzharris, 1976; Grave, 1980; Martin, 1983; Yamin, 1979) have reported on the symbiotic, cellulose-digesting organisms in the gut of C. punctulatus. There are at least 26 species of flagellate protozoans representing at least 2 orders and 9 families, in the hindgut of C. punctulatus (Cleveland et. al., 1934; Yamin, 1979). In addition, there appears to be a close association between rod-shaped bacteria and many of the protozoans. These bacteria may provide the protozoans with the ability to digest cellulose or fix nitrogen (Bloodgood and Fitzharris, 1976). The ability of C. punctulatus to utilize cellulose as a food source has been demonstrated in 2 ways: 1) the insect can survive on a diet of pure cellulose, and, 2) the gut fluids contain cellulase activity (Cleveland et. al., 1934). Grave (1980) described an additional 31 species of protozoans in the gut of C. punctulatus which do not digest cellulose, therefore they must be considered commensals.

2.2 Ice Formation and it's Effects on Insects

Aqueous solutions respond to the lowering of temperatures by either freezing, or supercooling below the actual freezing point of the solution. In the absence of ice nucleators (materials which provide sites for ice crystal formation and growth), small volumes of water can

be supercooled to below -40 ^{o}C (Salt, 1961). The freezing point of a solution is the temperature at which it changes from the liquid state to a solid. Supercooling is defined as maintaining a solution in the liquid state below the actual freezing point of the solution (Block, 1981). The initiation of ice crystal nucleation is a random, statistical event which occurs after certain specific conditions have been met. As temperature decreases, the activity of molecules is lowered, and the probability that an appropriate orientation of a group of water molecules (aggregate) into a crystal-like structure increases. Freezing occurs when this aggregate reaches a critical size (Salt, 1961). The freezing point of a solution is affected by the concentration of dissolved solutes. When 1 mole of non-ionic solute is added to a solution, certain changes occur in the physical properties of that solution. These are referred to as the colligative properties and include: 1) a freezing point depression of 1.86 ^{o}C, 2) a boiling point increase of 0.52 ^{o}C, and, 3) an osmotic pressure increase of 1,700 mm Hg. In many insects, the freezing point of their hemolymph is around -12 ^{o}C which is below the freezing point of water. This is because the dissolved solutes contained in the hemolymph (sugars, salts, proteins, and many other compounds) which act to lower the hemolymph freezing point in a "normal" colligative manner.

Since insects are poikilothermic, they exhibit very little control over their body temperature. In winter, internal ice formation may occur and result in detrimental effects on the integrity of the organism. Ice formation within an insect's body can cause considerable damage and may be lethal. In insects, damage due to intracellular ice formation includes: denaturation of proteins, disruption of cellular membranes, and osmotic stress (Baust, 1973, 1982; Storey and Storey, 1983). As extracellular water or hemolymph freezes, solute concentrations rise in the remaining unfrozen liquid surrounding and within the cells. This very high concentration of solutes can result in collapsed cell membranes and protein denaturation. Nervous tissue appears to be the most sensitive to freezing damage of all insect tissues (Zachariassen et al, 1979).

2.3 Insect Overwintering Strategies

Insects inhabiting temperate zones may deal with the approach of winter in a number of ways. They may: 1) migrate to a warmer climate, 2) find an appropriate or well insulated harborage, 3) alter their physiology/biochemistry to tolerate cold conditions or, 4) combine changes in their physiology/biochemistry with the selection of a suitable environment.

2.4 Overwintering Site

As winter approaches, insects inhabiting temperate zones may migrate to a more favorable environment (climate). For example, the monarch butterfly (*Danus plexippus*), and the green darner dragonfly (*Anax junius*) migrate South as winter approaches (Brower, 1977; Corbet, 1980). The selection of an appropriate overwintering stage and hibernaculum or overwintering site is of extreme importance to insects which do not migrate to warm regions for winter. As a result, each insect species usually overwinters during a particular life stage, (Baust and Morrissey, 1976), and may choose a well protected and thermally stable environment in which to overwinter.

2.4.1 Insulated Hibernacula

Many insects choose a well protected and thermally "buffered" site in which to overwinter. Rotting wood and snow cover are two examples of insulators which insects use to minimize their exposure to harsh winter conditions. The arctic ground beetle (*Pterostichus brevicornis*) overwinters in the center of decayed spruce stumps (Baust, 1982). Decayed, wet stumps and logs are good insulators against fluctuations in ambient temperatures (Baust, 1976). Insects overwintering within these logs are not exposed to the full severity of winter temperatures due to the thermal inertia of the moist wood. In addition, moist, rotting

wood provides an environment in which desiccation rates are reduced. Solar warming of the wood provides frequent mid-winter thaws within the hibernaculum (Baust, 1982). Thawing within the stumps was reported to occur twice as fast as freezing (Baust, 1976). This is an important consideration in the spring; since thawing occurs rapidly, insects can quickly resume their normal activities.

Snow cover also may serve to insulate insect hibernacula from extreme fluctuations in ambient temperature. Danks (1978) has provided a review on the thermal buffering capacity of both powdered and packed snow. Powdered snow is the better insulator because it is less dense and able to entrap more air than packed snow. Holmquist (1931) reported that sheltered sites (ie. stumps and logs) that are covered with snow remain near 0 °C throughout the winter, even when temperatures drop below 0 °C for a month or longer.

2.4.2 Exposed Sites

Insects which overwinter above the snowline in more exposed sites (ie. under bark, in galls, or within twigs) encounter much greater fluctuations, durations, and extremes in temperature. These exposed sites provide very little protection from ambient conditions. As a result, insects which overwinter in exposed sites essentially encounter ambient air temperatures (Miller, 1982). The

freeze-tolerant beetle, Upis ceramboides overwinters under loose bark of dead standing trees in Alaska and can withstand temperatures of -60 °C (Miller, 1978; 1982). Most insects overwintering under bark, orient to the south side of the tree (Dennys, 1927) where temperatures can be considerably warmer than ambient, and conditions are generally less severe, due to frequent solar warming (Danks, 1978).

2.5 Physiological Mechanisms of Insects Which are Exposed to Subzero Temperatures

Insects appear to have developed two main physiological/biochemical means for dealing with subzero temperatures. These are: 1) the avoidance of freezing by extending the tissue and hemolymph supercooling point by accumulating antifreeze compounds in their hemolymph (freeze-susceptible insects), and, 2) the utilization of Ice Nucleating Factors (INF's) to initiate the formation of extracellular ice in their hemolymph and body fluids; extracellular freezing removes water from cells, concentrating intracellular fluids which in turn protects the cellular contents from freezing (freeze-tolerant insects). Details regarding these two strategies will be discussed in the following sections.

2.5.1 Freeze-Susceptible Insects

During winter, freeze-susceptible insects produce and

concentrate high levels of antifreeze(s) within their hemolymph which lower the hemolymph supercooling point and prevents freezing. Supercooling is, in itself, harmless to insects; although a practical lower limit is set at about -30 °C (Ring and Tesar, 1980; Salt, 1961). If temperatures much below this level are to be experienced, the freeze-tolerance protection mechanisms become appropriate (Salt, 1961). If temperatures drop below the insects capacity to supercool, or if temperatures remain low for long periods of time, spontaneous ice nucleation may occur and death will follow as a result of significant ice crystal formation within their bodies.

The ability of insects to supercool is affected by many factors, these may include the presence or absence of ice nucleating sites, carbohydrates (polyols), Thermal Hysteresis Proteins (THP's), Ice Nucleating Factors (INF's), and body water content. These factors are discussed below.

2.5.1.1 Particulate Matter

Food particles contained in the insect gut may provide a site for ice crystal formation (nucleation sites). Initiation of ice crystallizaticn within the insect gut may cause the rest of the body to freeze since the crystallization front may penetrate membranes and tissues (innoculative freezing). Salt (1953) examined the effects

of food in the gut of larval insects feeding on plants, insects, and very dry whole wheat flour (7.8 % water), as well as the effects due to starvation, on the supercooling points of the insects. Due to the very low water content, the dry flour was not expected to freeze solid, or result in a low level of nucleation activity within the insect gut. However, it was found that larvae containing any particulate type of food or foreign matter in their gut (dry or wet) had greatly elevated supercooling points (approximately 10 ^{o}C higher) when compared to non-feeding, pre-molt stages or freshly molted, unfed larvae. In addition, Salt (1953) reported that the supercooling points of starving mid-instar larvae were no different than those of feeding larvae. In discussing this information, Salt (1953) suggested that perhaps the starved larvae had not completely evacuated their gut contents and contained small bits of food and liquid which served as nucleation sites. Further support for this hypothesis is derived from the observation that collembolans with full guts have significantly higher ($P<0.001$) supercooling points than ones with empty guts (Block and Zettel, 1980).

In summary, it appears that food particles contained in the gut influences the supercooling point of freeze-susceptible insects. Because of this, evacuation of the gut prior to winter appears to be an important cold-hardening

step in those insects which rely upon extending their supercooling points for winter survival.

2.5.1.2 Water Content

There is some controversy over the role that desiccation plays in insect cold-hardiness. Some of the earlier literature indicates that water loss is an important cold-hardening step. However, more recent information indicates that body water content and cold-hardening are independent.

In support of the "dependence" theory, Payne (1926) found that supercooling points in artifically desiccated larval Synchroa punctata were considerably lower than non-treated larvae. The larvae of the viceroy butterfly (Limenitis archippus) accumulates glycerol and total body water is reduced from 80% to 55% as it enters winter diapause. When diapause is broken, body water returns to 80%. These fluctuations in water content are apparently under active control (Frankos and Platt, 1976). This information was reported as being associated with winter diapause and no attempt was made to correlate body water content to cold-hardiness in the viceroy larvae.

The independence of body water content with respect to increased cold-hardening capabilities is well documented (Baust and Morrissey, 1975, 1976; Salt, 1953, 1961). In order for supercooling points to be lowered by a

substantial amount, dehydration would be extreme and toxicity would result due to increased osmotic pressure and increased fluid viscosity. When 33.3% of the total body water is lost in cold-hardy Diplolepis radicum prepupa, the supercooling point is lowered about 3 °C. This drop is probably due to a concentration increase of glycerol resulting from the loss of body water. The pupae contain no glycerol, and identical treatment of them yields no shift in supercooling point (Somme, 1964). The ladybird beetle, Coleomegilla maculata, changes its supercooling point throughout the year (-5.5 to -18.4 °C) while maintaining body water near 55% (Baust and Morrissey, 1975).

It appears that slight seasonal changes in body water content may occur in insects. These changes may even have a limited effect on supercooling due to the concentration of hemolymph solutes. They do not, however, appear to play a major role in the cold-hardening process. Supercooling is most affected by seasonal accumulation of other compounds (sugars, polyols, and thermal hysteresis factors) in the hemolymph.

2.5.1.3 Polyols

Chino (1957) discovered the occurrence of two polyols, or sugar alcohols, (glycerol and sorbitol) in overwintering eggs of the silkworm moth, Bombyx mori. Salt (1957)

suggested that certain polyols provide protection by acting as antifreezes, in the insect cold-hardening process. Since these early reports, many studies have focused on the production, accumulation, and role which polyols play in insect cold-hardening. It is known that polyols lower the supercooling point of solutions due to their "normal" colligative properites. However, more importantly, due to their extreme hydrophilic nature, they also lower the supercooling points by keeping a large percentage of water "bound" and out of solution. As a result, other solutes are concentrated which act to lower supercooling points (Ring, 1980). The addition of glycerol to a solution lowers the supercooling point twice as much as the freezing point (Duman, 1982; Miller, 1982; Somme, 1982). This is important, since supercooling points, being the lowest temperature achieved before spontaneous ice nucleation occurs, are biologically more meaningful than the actual freezing point of body fluids. Therefore, the addition of glycerol to hemolymph will lower it's supercooling point while having little effect on the freezing point.

Polyol concentrations in insects have been reported to reach levels as high as 5 M (Salt, 1959), resulting in supercooling points as low as -47 ^{o}C. Polyols are accumulated in the hemolymph of overwintering insects in response to shortened photoperiod and/or lower temperatures

(see Section 2.6). However, their disappearance from hemolymph is temperature dependant and may be quite rapid. For example, upon exposure to "warm" temperatures, polyols can be lost in a matter of hours (Baust and Morrissey, 1976) or days (Ring and Tesar, 1980; Zachariassen, 1977). Careful laboratory handling of cold-acclimated insects is extremely important if reliable polyol levels are to be determined (Baust and Morrissey, 1976).

Five polyols ranging from 3 to 6 carbons have been described in insect hemolymph. Although glycerol is the most common polyol, sorbitol, mannitol, threitol, and erythritol have also been reported to act as antifreezes in insects (Block, 1981; Baust and Lee, 1983; Miller and Smith, 1975). In addition, Block and Zettel (1980) have reported the occurrence of an unidentified 5-carbon polyol, possibly either ribitol or arabitol in whole body extracts of winter-acclimated collembolans.

Many insects utilize a combination of polyols and/or sugars to increase cold-hardiness. For example, overwintering eggs of Bombyx mori contain both glycerol and sorbitol (Chino, 1957). Also, the arctic midge (Belgica antarctica) produces a 3-phase cryoprotectant system comprised of glucose, trehalose, and erythritol (Baust and Lee, 1983). The role of sugars (glucose, trehalose) in cold-hardening appears to be due solely to their colligative properties and not to their "water binding"

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properties. The purpose of a multi-component cryoprotectant system has been attributed to reducing the potential toxic effects resulting from the accumulation of very high concentrations of one compound (Baust, 1976; Morrissey and Baust, 1976; Ring, 1980).

2.5.1.4 Thermal-Hysteresis Proteins

Some insects overwinter in a supercooled state but do not contain carbohydrate antifreezes which provide for their freeze-protection (Baust and Morrissey, 1975; Duman, 1977a, 1977b, 1979; Ring and Tesar, 1981; Zachariassen and Husby, 1982). Instead, these insects concentrate specific hemolymph proteins which cause a thermal-hysteresis (produce a difference between freezing and melting point). These Thermal-Hysteresis Proteins (THP's) lower the supercooling point of hemolymph in a non-colligative manner: their concentration does not greatly affect hemolymph osmolality. In fish, and presumably insects, THP's bind or adsorb to the surface of small developing ice crystals (seed crystals) and prevent or retard their development. In this way, THP's may greatly lower the supercooling point of insect hemolymph without affecting its osmolality or requiring the buildup of high concentrations of carbohydrates (Duman, 1982; Duman et. al., 1982). In the beetle _Mercantha contracta_, THP's are present only in winter and are formed in response to low

temperatures and/or short photoperiods. Their loss, however, requires both long photoperiod and warm temperatures. This presumably ensures that antifreeze activity will not be lost prematurely (Duman, 1977a, 1977b). Thermal-hysteresis proteins from *Tenebrio molitor* have been partially purified from hemolymph of cold-acclimated larvae. In this species, 6 proteins and 3 glycoproteins have been found which are capable of producing thermal hysteresis activity (Patterson and Duman, 1979).

2.5.2 Freeze-Tolerant Insects

Most insects which are freeze-tolerant contain Ice Nucleating Factors (INF's) in their hemolymph. These INF's appear to be special proteins which initiate or "seed" ice crystal formation in the insect's body. Ring (1982) reported on an alpine beetle (*Pytho deplanatus*) that is freeze-tolerant, but does not contain INF's in the hemolymph. Presumably, this insect relies on external nucleating factors (such as food in the gut) to seed ice crystals. This insect has a very low supercooling point (-54 ^{o}C), due to glycerol and trehalose accumulation within the hemolymph, but can withstand internal ice formation when and if it occurs.

2.5.2.1 Ice Nucleating Factors

In 1969, freeze-tolerance was first documented in an adult insect. The arctic ground beetle (Pterostichus brevicornis) was reported to spend the winter in a frozen state, and could withstand temperatures below -80 °C (Miller, 1969). Since then, INF's have been reported to occur in the hemolymph of Hymenoptera, Diptera, and Coleoptera (Lee et. al., 1981; Storey and Storey, 1982; Zachariassen, 1977, 1982; Zachariassen and Hammel, 1976). Zachariassen and Hammel (1976) described "nucleating agents" in the hemolymph of a different freeze-tolerant beetle (Eleodes blanchardi). In this species, hemolymph ice crystal formation occurred around -6 °C. These factors were found to be heat-labile (100 °C for 5 min), able to override the antifreeze effects of glycerol, and remain active at a 1:20 dilutions. It should be noted that hemolymph dilution has become a standard test in the evaluating of nucleating capacity. For example, nucleating capacity is considered to be present in hemolymph if a 1:20 dilution of a sample freezes betweem -5 to -10 °C (Zachariassen and Hammel, 1976; Zachariassen et. al., 1982).

Since hemolymph containing INF's freezes at relatively high subzero temperatures, it is presumed that the nucleators "seed" or initiate ice crystal formation. It is thought that this produces a controlled freezing of

extracellular fluids. Cells surrounded by extracellular ice, release intracellular water (cellular dehydration) as it travels down a concentration gradient established by the extracellular freezing. The resulting cellular dehydration is thought to reduce the possibility of intracellular ice formation, which would cause extensive tissue damage (Duman et al, 1984; Storey and Storey, 1983; Zachariassen, 1980, 1982; Zachariassen and Hammel, 1976).

Recently, nucleating "factors" have been isolated, purified, and characterized in 2 freeze-tolerant insect species. The overwintering queen of the bald-faced hornet (Vespula maculata) contains a very hydrophilic protein nucleator with a molecular weight of 74,000 daltons (Duman et. al., 1984). It is postulated that the hydrophilic nature of this protein is involved in its ice nucleating ability. The function of this protein may well be to attract and orient water molecules in an "ice-like" configuration so that freezing proceeds at a higher temperature than would occur in it's absence. Protein and lipoprotein ice nucleators have recently been purified from a freeze-tolerant terrestrial crane fly (Tipula trivittata), which overwinters as a larva in decomposing logs (Duman et. al., 1985). The fact that more than one nucleating compound has been found in this insect raises the question of possible dual functions of these "ice

nucleating" compounds. They may also function as storage or transport proteins/ lipoproteins as well as providing for ice nucleation. Since these proteinaceous materials are active at such low concentrations (1:20 dilutions initiate nucleation as well as undiluted hemolymph) it may be that high levels are necessary for their other (more important?) role(s) in the insect's biochemistry (Duman et. al., 1985).

Nucleating agents are normally present in freeze-tolerant insects only during winter (Zachariassen, 1982). There are, however, exceptions where nucleating activity has been found in the hemolymph of insects during the summer. There is no clear explanation for the occurrence of INF's in summer-acclimated insects, but they may be present under circumstances where summer night temperatures drop below freezing (Van der Laak, 1982; Zachariassen, 1982). Other explanations may include: the result of nitrogen economy (ie storage proteins which act as an amino acid pool for protein needed for growth, reproduction, etc) or dual functions as discussed above.

2.5.2.2 Polyols and INF's in Freeze-Tolerant Insects

Polyols have been reported in the hemolymph of freeze-tolerant insects which also contain INF's. However, INF's apparently override the antifreeze effects of polyols. Baust and Morrissey (1976) suggested that polyols are a

necessary component of the freeze-tolerance mechanism. In these circumstances polyols may act as cryoprotectants, which may help in: 1) stabilizing protein structure, 2) protecting membrane integrity, 3) altering ice crystal growth, and, 4) preventing extreme osmotic fluctuations (Baust, 1973, 1982; Ring, 1980; Zachariassen, 1977, 1979, 1980).

2.5.2.3 Advantages of the Freeze-Tolerance Mechanism

If an insect is to be exposed to subzero temperatures for an extended period of time, it is energetically more favorable to be frozen than to remain in a supercooled state (Miller, 1982; Ring, 1980; Ring and Tesar, 1980; Storey and Storey, 1983). In the frozen state, metabolism slows considerably, and oxygen consumption is reduced. Comparisons of theoretical calculations indicate that nutritional reserves sufficient for an unfrozen insect (0 °C) for 10 days would meet the needs of a frozen insect (-23 °C) for 1,000 years (Ring, 1980).

There are two reports in the literature where insect populations were found to change their overwintering strategy. In both cases, the insects (<u>Cucujus clavipes</u>, and <u>Dendroides canadensis</u>) were found to be freeze-tolerant, and a few years later, freeze-susceptible. It is interesting that lower lethal temperatures remained constant in both species throughout the study period

(Duman, 1984; Horwath and Duman, 1984).

2.6 Environmental Cues to Cold Hardening

Insects use various environmental cues to determine and prepare for the onset of winter. These include changes in photoperiod, temperature, and a combination of both temperature and photoperiod.

Photoperiod undoubtedly plays a large role in winter acclimation. Shortened photoperiod is a very reliable environmental cue which allows insects to "anticipate" the approach of winter (Duman and Horwath, 1983). A critical photoperiod of between 10 and 11 hrs of light per day is necessary to induce production of protein antifreezes (THP's) in certain beetles, even at room temperature. In these insects, it is believed that photoperiod induces antifreeze production, and continued exposure to low temperatures maintain the necessary levels (Duman and Horwath, 1983).

Insects which overwinter in protected sites (deep in soil, beneath or within rotting logs, fruits, or stumps) do not receive strong photoperiod cues (Baust, 1982). It is believed that these insects must rely on decreased temperature alone as an indicator of the approaching winter. Young and Block (1980) found that decreased temperature induces glycerol accumulation in laboratory maintained mites (*Alaskazoides antarcticus*). Freeze-

tolerance was induced in _Chymomyza amoena_ larvae kept in apples at 4 °C for 2 wks in the refrigerator (Band and Band, 1982). The arctic ground beetle (_Pterostichus brevicornis_) hibernates within spruce stumps and has little or no access to photo-cues (Baust, 1982). Glycerol production can be induced by maintaining the insects in total darkness and gradually exposing them to lower temperatures. Day length independence has also been demonstrated in cold-acclimation of the midge, _Belgica antarctica_ (Baust, 1982).

Both shortened photoperiods and low temperatures which occur as winter approaches, may induce the development of cold-tolerance mechanisms in some insects. It seems logical to assume that the combined effect of shortened photoperiod and low temperature would enhance the cold-hardening process in insects. Unfortunately, there is no recent, conclusive evidence that shortening photoperiod combined with the lowering of temperatures induces any significantly greater level of cold-tolerance than either could produce individually. Duman (1982) states that photoperiod, as well as temperature, is a critical environmental cue in the production of protein antifreezes in insects. His data show a slight (6%) decrease in the supercooling point (increased cold-hardiness) of _Tenebrio molitor_ larvae when held under conditions of short photoperiod and low temperature.

3 MATERIALS AND METHODS

3.1 Collection of Insects

Cryptocercus punctulatus nymphs and adults were collected from logs in various stages of decay in the Jefferson National Forest, near Mt. Lake, Giles Co., Va. Upon return to the laboratory, insects were held in moist wood litter for no more than 1 day in a refrigerator before experimentation. Usually, insects were utilized immediately upon return to the laboratory.

3.2 Live Insects

3.2.1 Subzero Storage of Adult Insects

Insects which were brought to the laboratory were used for various purposes. These included studies on cold-hardiness, water content, supercooling points, and composition and properties of their hemolymph. Immediately upon return to the laboratory, freshly collected adult *C. punctulatus* were placed in subzero storage. Ten to twenty randomly selected individuals were placed in small round wax ice cream containers (8.5 cm diameter x 8.5 cm height) along with moist wood and leaf debris and held in laboratory freezers at either -7 to -10 °C or -25 °C. Survival was evaluated at various intervals. "Survival" was determined on the basis of insect's ability to behave in a coordinated, "normal" manner upon thawing. If, after thawing, individuals moved and behaved in a normal, or

only slightly impaired manner, they were judged as having survived. All other insects were considered to be dead. Initially, insects were evaluated at 24 hrs. post thaw, but, later studies indicated that observations at 72 hrs. post thaw was more indicative of true "survival" rates. This will be discussed in more detail in the results section.

3.2.2 Body Water Determination

Freshly collected insects were used for dry weight determinations. Individuals were weighed on a Mettler analytical balance before and after drying in a laboratory drying oven at 80 ^{o}C (24-48 hrs).

$$\text{Percent body water} = 1 - \left(\frac{\text{dry weight}}{\text{wet weight}}\right) \quad \text{X } 100$$

3.2.3 Supercooling Point

Supercooling points were determined on live, freshly collected insects by the method described under supercooling in the "Hemolymph" section (Section 3.3.2.1).

3.3 Studies on the Hemolymph

3.3.1 Hemolymph Collection

Two methods were employed to obtain C. punctulatus hemolymph samples. In both situations, insects were at room temperature when bled, but once the hemolymph was collected, it was held at 0-4 ^{o}C to reduce the effects of coagulation and temperature related degradation. In early

experiments, insects were bled by puncturing the membrane surrounding the metathoracic coxa followed by collection of the exuded hemolymph into a chilled microcapillary (10 ul microcap). In later experiments, a less cumbersome method which yielded greater hemolymph volumes was utilized. This preferred method of hemolymph collection involved clipping one of the insects antennae near the pedicle, gently squeezing the insect to express hemolymph out of the cut end, followed by collection of the hemolymph in a cooled capillary tube. Using these procedures, it was possible to collect an average of 5-10 ul of hemolymph per insect.

3.3.1.1 Treatment of Hemolymph Samples

Since such small quantities of hemolymph were obtained from individual insects, samples were pooled for experimentation and analysis. Hemolymph samples were routinely pooled from 50-100 adult insects, regardless of sex. In later collections, male and female hemolymph was kept separate in order to determine if there were any detectable qualitative differences between the sexes (ie. proteins, cryoprotectants, etc.).

Hemocytes were separated from the hemolymph samples by centrifugation in a refrigerated (4 °C) centrifuge at 5,000 rpm (12,000 x g) for 10 min. The supernatant (serum) was removed and frozen at -25 °C in individual 10 ul capillary tubes. The ends of the glass capillaries were sealed with

Seal-ease compound (Preiser Scientific). Samples stored this manner appeared stable for the duration of the study (ie. no notable precipitation or loss of nucleating activity).

3.3.2 Mean Supercooling Point Determination

In order to accurately determine individual supercooling points (SCP) of both whole insects and 2 ul serum samples, an apparatus was designed and assembled (Figure 1). This apparatus consisted of a thermoelectric cooling plate (Thermoelectrics Unlimited Stirkool Model SK-12), a constant temperature circulating water bath (Brinkman Instruments, Lauda K2/R), and a dual channel recording potentiometer (Leeds and Northrup, Speedomax 250). Fourty gauge type "T" (copper-constantan) thermocouples (Omega Engineering) were connected to the recorder and used to monitor temperatures.

Cooling rates of -0.3 ^{o}C per minute were obtained using the variable cooling level thermoelectric cooling plate coupled with the constant temperature water bath (0 ^{o}C; coolant - ethanol:water 1:1). The water bath was used to supply a 0 ^{o}C cooling mixture to the cooling plate which acted as a heat sink for the generation of subzero temperatures. In order to slow the cooling rate of the plate, a 2 mm piece of cardboard was sandwiched between two, 5 mm aluminum blocks and placed on the cooling stage.

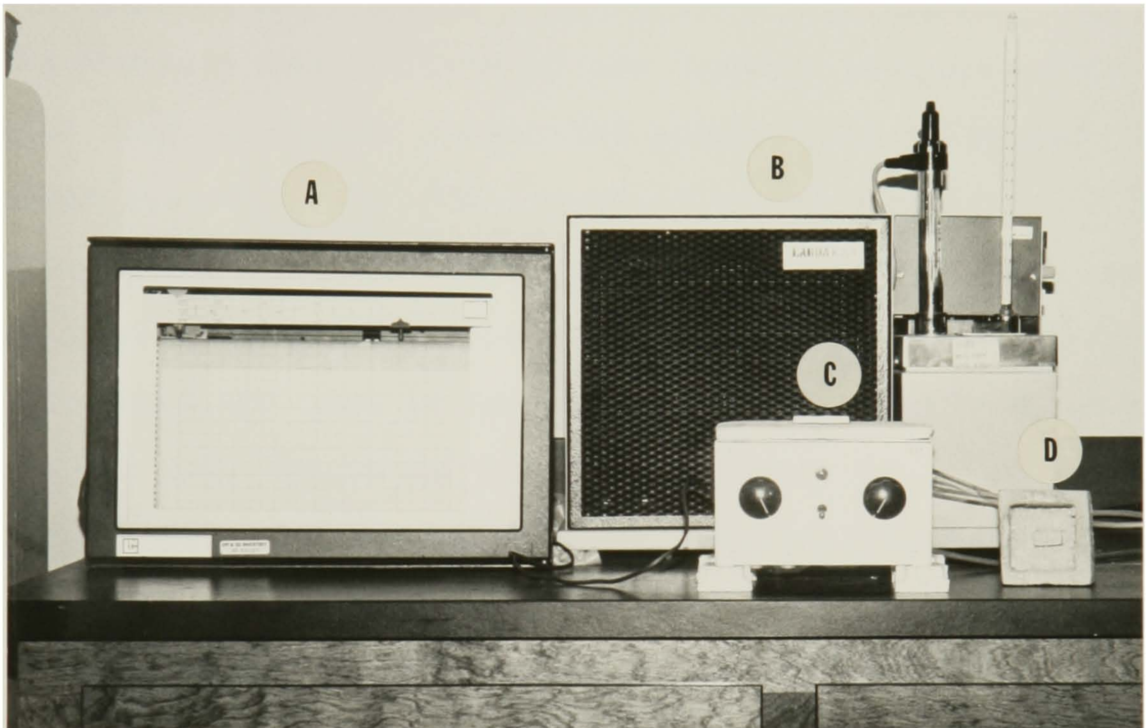


Figure 1. Supercooling point apparatus

A= Recording potentiometer
B= Circulating water bath
C= Thermoelectric cooling plate
D= Styrofoam insulating chamber

For specific details see Section 3.2.2

The cooling stage and aluminum block assembly was enclosed within a removable styrofoam chamber which insulated the assembly from room temperatures during the supercooling point determinations.

3.3.2.1 Determination of Whole Insect Supercooling Points

Insects were placed on the cooling "stage" and a thermocouple was affixed to the insects abdomen using a small drop of thermal conductive grease (zinc oxide and immersion oil 1:1). The temperature was slowly (-0.3 ^{o}C /min) lowered until the supercooling point (SCP) was observed as a sudden rise in temperature. This rise in temperature was due to the latent heat of fusion released from the body fluids as they froze (Figure 2).

3.3.2.2 Determination of Solution Supercooling Points

A sample holder was designed and constructed to determine SCP's on 2 ul samples of hemolymph. A small groove was cut in a 25x50x5 mm piece of styrofoam, and a thermocouple positioned such that when a 2 ul glass microcap was inserted into the groove, direct contact was made between the glass and thermocouple (Figure 3). This styrofoam sample holder was placed over a microcap containing hemolymph serum, secured in place with an aluminum block (50x50x5 mm), and covered with the styrofoam insulating chamber. The sample was then subjected to a controlled cooling rate of -0.3 ^{o}C per min. until the

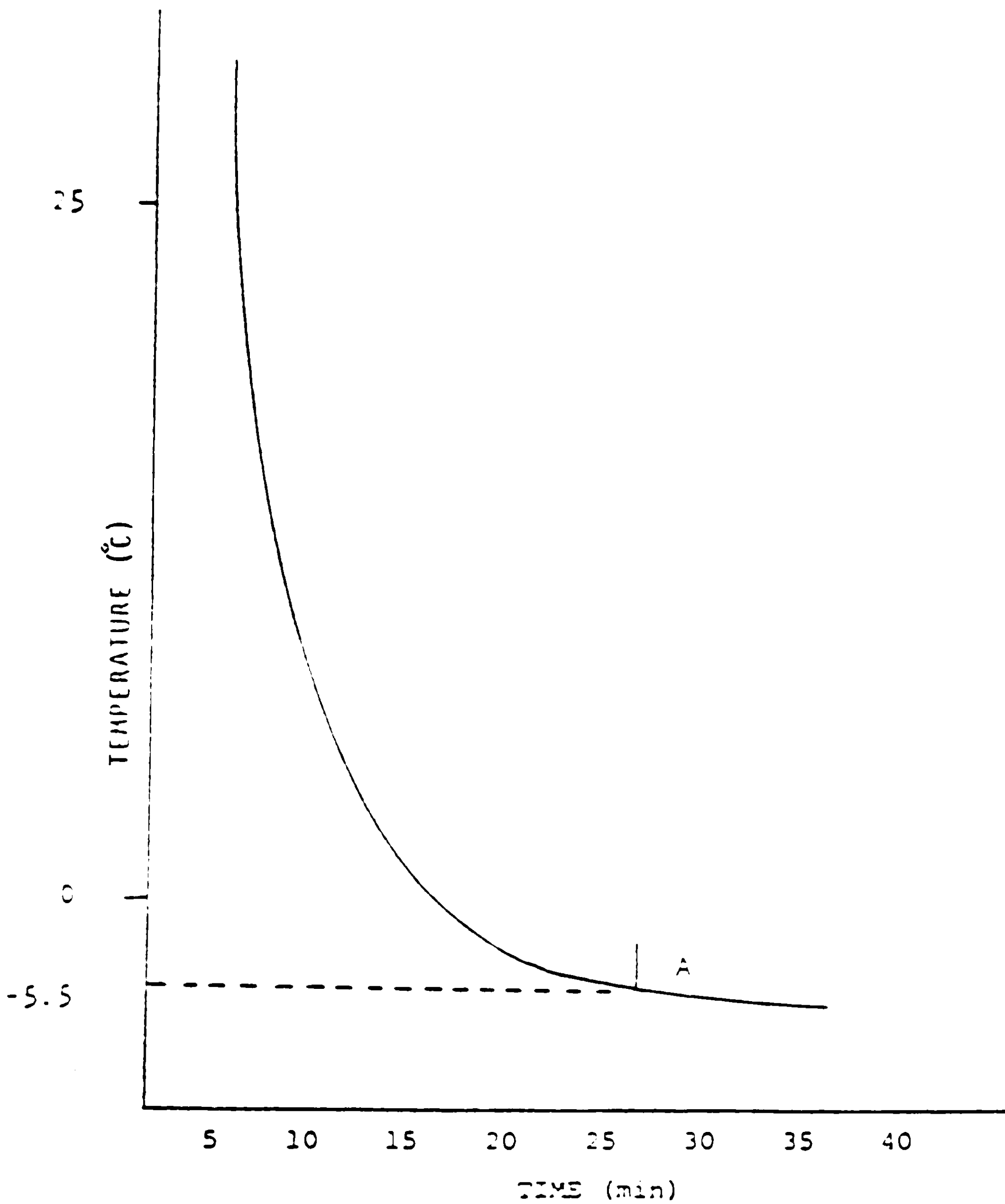
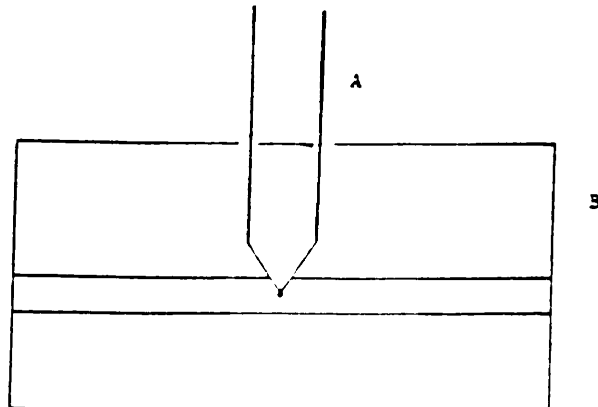


Figure 2. Typical supercooling curve

A= Rapid rise in temperature due to the release of latent heat of fusion of the solution

For specific details see Section 3.3.2.1

Top View



Side View

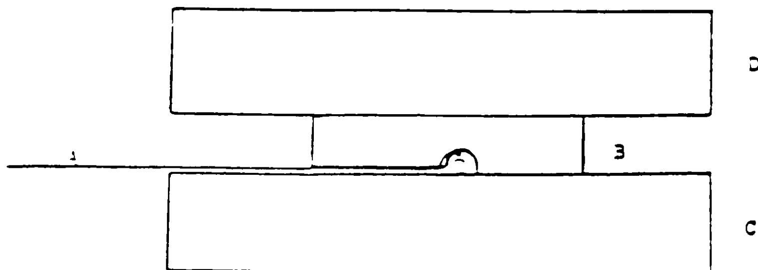


Figure 3 Apparatus for determining supercooling points in solution filled capillary tubes

A= 40 gauge type "T" thermocouple
B= Styrofoam block
C= Aluminum cooling stage
D= Aluminum block for weight

For specific details see Section 3.3.2.2

latent heat of fusion was released from the sample. This was taken as the supercooling point and was replicated 5 times per sample.

3.3.3 High Performance Thin Layer Chromatography

Identification of hemolymph polyols was initially done using High Performance Thin Layer Chromatography (HPTLC). The procedure involved the use of prepared 10x10 cm Silica gel plates (Kieselgel 60 F 254, Merck), a Camag Nanomat spotter (Camag), and polyol and sugar standards (Sigma Chemicals). Standards were mixed to an appropriate concentration (5 mg/ml) with distilled water, and various organic solvents were used for chromatogram development. One-half ul samples were applied to the plates, using the automated Nanomat spotting system, allowed to dry, and the plates developed in an ascending manner until the solvent front had moved to a distance of 6 cm from the origin. After development, the plates were dried, sprayed with a detection agent, and heated to visualize the polyol and sugar standards. Two detection reagents were used. The most sensitive, but non-specific reagent was a 1% (1 gm per 100 ml of water) aqueous potassium periodate ($KMNO_4$) solution. This reagent reacted equally well with all reducing carbohydrates. The other reagent used was a bromphenyl blue-boric acid reagent (40 mg bromphenyl blue in 100 ml ethanol containing 100 mg boric acid and 7.5 ml

1% (1gm per 100 ml water) aqueous sodium tetraborate). This reagent proved not to be as sensitive, but appeared to be specific for polyols.

Using the HPTLC systems as described above, a preliminary identification of ribitol was obtained for the polyol present in cold-acclimated C. punctulatus hemolymph. Gas chromatography was then used for confirmation and quantitation.

3.3.4 Gas-Liquid Chromatography

Two different gas-liquid chromatography systems were used in the identification and confirmation of the hemolymph polyol. The first system involved preparations of hemolymph and polyol standards as trimethylsilyl derivatives (equal volumes of Sylon T P (Supelco) and either lyophilized serum or polyol standard). This mixture was allowed to stand for 10 min. at room temperature before 4 ul volumes were injected into the gas chromatograph (Bendix Model 2600). A 3 foot (4 mm I D) 3% SE-30 (80/100 mesh, Supelco) column was used. Chromatographic conditions were: oven temperature = 160 ^{o}C (isothermal), inlet = 250 ^{o}C, Flame Ionization Detector (FID) = 240 ^{o}C, and nitrogen carrier gas flow = 36 ml/min. This system was used in initial experiments and confirmed that the hemolymph polyol was either ribitol or arabitol, however further separation of these isomers was not possible using this system.

The second gas chromatography system used provided good separation between ribitol and arabitol (Figure 4), and was used for subsequent quantitation of the ribitol levels in the hemolymph serum. This system employed the use of 4 ul volumes of acetylated derivatives on a 6 ft. (2 mm I D) column in a Bendix (Model 2500) chromatograph. The derivatives were prepared by mixing 1 part hemolymph sample to 24 parts of a mixture of pyridine and acetic anhydride (1:1) and heating at 100 ^{o}C for 4 hrs. in a tightly sealed container. Chromatographic conditions were: oven temperature = 210 ^{o}C (isothermal), Inlet = 230 ^{o}C, FID = 220 ^{o}C. nitrogen carrier gas flow =30 ml per min. Under these conditions, the retention times for ribitol and arabitol were 5.2 and 5.6 min., respectively.

5) Polyacrylamide Gel Electrophoresis (PAGE)

Polyacrylamide gel electrophoresis was performed on hemolymph serum samples to determine if any qualitative differences in hemolymph serum protein banding patterns were occurring throughout the year. That is, if a nucleating protein was produced with the onset of winter, it would result in a new protein band with a different mobility than any seen in the summer-collected hemolymph, or a drastic increase in the concentration of an existing protein band. Davis (1964) provides a general reference on electrophoreses. Small electrophoresis tubes (5X75 mm)

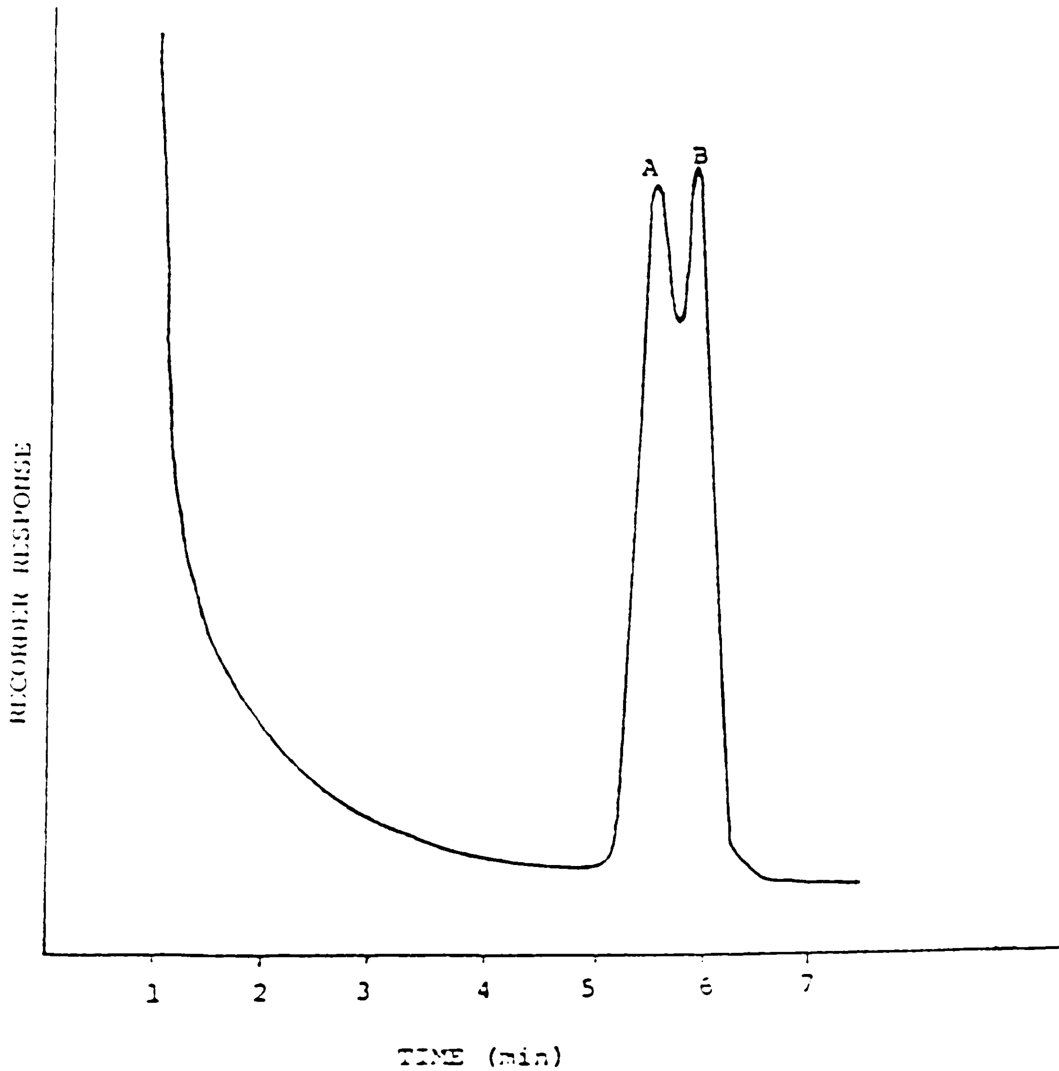


Figure 4 Separation of ribitol and arabitol using acetate derivatives on an OV-225 column

A= Ribitol Acetate Retention time = 5.2 min.
B= Arabitol Acetate Retention time = 5.6 min.

For specific details see Section 3.3.4

were dipped in 1% (1 ml per 100 ml of water) column coat solution and were air dried. The bottoms of the tubes were sealed with Parafilm and the tubes were set up vertically. Stock solutions were made as follows: 7% gel solution (28.0 gm acrylamide, 0.7 gm bis (N,N'-methylenebisacrylamide), and distilled water to 100ml then filtered), gel buffer (48 ml 1 N HCl, 36.6 gm THAM (tris (hydroxymethyl) amino methane) 0.23 ml TEMED (N,N,N',n'-tetramethylethylenediamine), distilled water to 100 ml and filtered), ammonium persulfate (0.14 gm ammonium persulfate, 100 ml water and filtered). A mixture of 2 parts 7% gel solution were mixed with 1 part distilled water, 1 part gel buffer and 4 parts ammonium persulfate. This solution was mixed well and 1 ml was placed in each gel tube. Water was quickly, but gently layered onto the top of the gel tubes to allow polymerization to occur. The finished tube contained 55 mm of acrylamide under 20 mm of distilled, deionized water. Gels were usually left overnight before use to allow for complete polymerization.

Cold chamber buffer was placed in the bottom portion of the Canalco electrophoresis tank (Model 1200). The parafilm was removed from the bottom of the gel tubes, and the water shaken from the top of the tubes. They were then placed in the top portion of the electrophoresis tank with the open end up. Cold chamber buffer was then placed in

the top portion of the tank. Care was taken to ensure that there were no air bubbles in the top of the tube. Five ul of hemolymph were mixed with 15 ul of water and 3-4 grains of sucrose. Five ul of this sample were then carefully layered on top of the gel using an Eppendorf micropipet. Fifty to one hundred ul of tracking dye (0.5 gm bromphenyl blue in 100 ml 1% NaOH) solution was then mixed in the top buffer compartment. A constant voltage of 400 volts was applied (or 4 mA per tube, whichever was lowest), until the tracking dye had moved out of the gel at the ends of the tubes (about 2 hrs). The gels were removed from the tube by inserting a 24 gauge needle between the gel and the wall of the tube, forcing water in a manner such that the gels were loosened and would easily slide out of the tubes. Gels were stained for 20 minutes in protein stain (0.25 gm Coomassie Brilliant Blue R250 (CBB), 100 ml glacial acetic acid, and distilled water to 500 ml and filtered), rinsed with water and allowed to stand for two days with frequent changes of the destaining solution (200 ml glacial acetic acid, 800 ml water, filtered).

3.3.6 Lowry Protein Assay

A protein assay method was used to determine any seasonal fluctuations in total hemolymph protein. Presumably, if specific proteins were produced in response to winter, they might have contributed to an increased

total protein level which would have been detected by this procedure. A modification of the Lowry protein assay (Hess et al, 1978) was performed on hemolymph serum samples collected at various times of the year.

One microliter of hemolymph sample was mixed with 150 ul of distilled water. Two hundred microliters of 2 N NaOH (80 gm in 1000 ml water) were added to the sample and mixed well. Three hundred and fifty ul of solution A (0.2 gm sodium tartarate ($Na_2C_4H_4O_6.2H_2O$) and 10 gm sodium carbonate ($NaCO_2$) dissolved in 55 ml of 1 N NaOH, diluted with water to 100 ml) were added to the sample, vortexed, and allowed to stand for 30 minutes. Fifty ul of solution B (2 gm sodium tartarate and 1 gm of cupric sulfate (CU_2SO_2) dissolved in 90 ml of water and 10 ml of 1 N NaOH) was added, mixed, and left to stand for 20 minutes. One thousand two hundred ul of solution C (1 volume of phenol-folin reagent and 2 volumes of water) were added in two (500 ul) and one (200 ul) aliquots and mixed sequentially to ensure complete reaction of all binding sites, then allowed to stand for 30 minutes. The resulting solution absorbance was then measured using a double beam spectrophotometer at a wavelength of 650 nm. Using this procedure, protein concentrations of Bovine Serum Albumin (BSA) standards between 10 and 100 mg/ml give linear responses (r^2=0.999), and polyols concentrations of up to 20 mg/ml did not alter absorbance.

3.4 Log Temperatures

Since snow covered logs are "known" to be good insulators providing protection from short term extreme changes in ambient temperatures, an experiment was conducted in order to gain a better understanding of the actual temperature extremes to which *Cryptocercus punctualtus* may be exposed in winter. A Campbell Scientific CR-21 micrologger was used to collect temperature data from logs placed at the Horton Research Center near Mt. Lake Va. Five thermistor probes were used, one to monitor air tempratures, and 4 to monitor internal temperatures of 4 different sized log sections. These log sections were selected from a downed timber which represented a potential *Cryptocercus* habitat. A few galleries were noted, in some of these log sections, however, established colonies were not present. All logs were cut from the same downed tree, but were of different diameters [log 1= 56(l)x29(d) cm; log 2=56(l)x13(d) cm; log 3= 48(l)x18(d) cm; log 4= 71(l)x17(d) cm]. It was thought that by using the different sized logs the information obtained would allow for some understanding of the difference in thermal inertia of large vs. small logs, and the extremes which a population of *C. punctulatus* might encounter. The ends of the logs were covered with 4 mil plastic, in order to reduce desiccation of the log. Holes

were drilled to a depth of 5 cm in the center of each log, and a thermistor probe inserted, secured by packing tissue paper around the probe in the test hole. Temperatures from the five probes were collected every minute and averaged hourly by the micrologger. When the memory of the micrologger was exceeded, this information was automatically transfered to a 30 minute cassette tape. The tape provided enough memory capacity for at least 2 months of data collection. The tape was collected every 2 weeks and the data fed to an IBM Personal Computer with a Campbell C-20 interface module. These data were edited using the Wordstar (Sorcim) word processing package, and graphed using Supercalc 3 (Sorcim).

4. RESULTS

Cryptocercus punctulatus were collected at various times of the year in order to examine the seasonality of hemolymph factors which provide for cold-hardiness in this species. At the onset of this study, no information was avaliable on the overwintering strategy of this insect. Initial experiments were conducted in order to determine if the insects could tolerate internal ice formation (freeze-tolerant), or if they relied on extending their supercooling point (freeze-susceptible) for winter survival.

Cold-acclimated *Cryptocercus punctulatus* were found to tolerate subzero temperatures for considerable lengths of time. Insects were placed in small containers along with some moist wood and moss in the freezer at -7 to -10 ^{o}C and held at this temperature range for various time intervals to evaluate survival under subzero conditions. Table 1 contains data on the survival of *C. punctulatus* individuals collected on January 29, 1984, when held at -7 to -10 ^{o}C. It can be seen that exposure of up to 49 days resulted in 100% survival. Some mortality was observed as cold storage continued. To evaluate mortality, these insects were initially examined 24 hrs. post-thaw. However, after 205 days the thawed insects were observed at two intervals: 24 and 72 hrs. At 72 hrs. post-thaw there was a considerable increase in "survival" as compared to

Table 1

Percent survival of winter-acclimated (1/29/84) *C. punctulatus* adults after freezing at -7 to -10 °C for various time intervals.

Freezing interval (days)	Number of individuals	%Survival
8	7	100
23	7	100
49	7	100
98	7	71
112	6	33
178	21	33
205	94	52
205[1]	94	77

[1]observed 3 days post-thaw

the 24 hr. observation (77% vs 52%). Approximately one-half of the insects which appeared to be dead initially (24 hrs.), apparently had recovered from the freezing trauma and seemed normal after three days. This finding resulted in a change in observation time to 72 hrs. post thaw for subsequent experiments. The intermediate times of cold exposure (112 and 178 days, both showing 33% survival) would probably have resulted in better survival rates if they had been evaluated after a 72 hr. post thaw period. These preliminary experiments indicated that Cryptocercus tolerated subzero temperatures quite well. Since survival rates were high even after 200 days of subzero storage, this indicated that the insects might be freeze-tolerant. Further experiments were conducted to evaluate seasonality of subzero tolerance. Table 2 contains information on survival rates (72 hrs. post-thaw) of insects collected at various other times of the year (fall, summer, and spring). The summer-collected insects (July, 1984) did not appear to tolerate cold conditions well, since those insects tested did not withstand a moderate cold exposure of 4 hrs. at -7 ^{o}C, which is just 1.5 ^{o}C below their mean hemolymph supercooling point. The fall-collected insects (October, 1984) appear to have been moderately cold-tolerant. Eighty-five percent survived a temperature of -7 ^{o}C for 4 hrs., but not 6 days. Since the summer-collected insects could not withstand -7 ^{o}C for 4 hrs, and the fall-collected

Table 2

Percent survival of *C. punctulatus* adults after freezing at various temperatures and time intervals.[1]

Collection date	N	Freezing interval	Temperature (°C)	%Survival
Jan. 29, 1984	19	12 hrs	-25	68
July 24, 1984	90	4 hrs	-7	0
Oct. 1, 1984	13	4 hrs	-7	85
Oct. 1, 1984	59	6 days	-7	0
Oct. 1, 1984	11	4 hrs	-10	82
Oct. 1, 1984	24	4 hrs	-25	0
Nov. 12, 1984	10	3 days	-10	100
Nov. 12, 1984	12	14 days	-10	58
Nov. 12, 1984	15	28 days	-10	7
Nov. 12, 1984	10	3 days	-25	0
Dec. 13, 1984	12	4 hrs	-10	100
Dec. 13, 1984	12	6 days	-10	100
Dec. 13, 1984	12	65 days	-10	100

[1]observed 3 days post-thaw

insects could, some cold-hardening appears to have occurred between July 24, 1984, and October 1, 1984. As winter progressed, the ability to tolerate subzero temperatures increased. Insects collected in November (1984) did tolerate -10 °C for 3 days with 100% survival, and 14 days with 58% survival. Apparently, in November, C. punctulatus are just beginning to acclimate to harsh winter conditions. It appears that they may withstand mild subzero conditions for short periods of time, however, mortality may occur with any severe cold challenge (-10 °C for 14 days, or -25 °C for 3 days). December (1984) collected insects were apparently much better acclimated to subzero conditions and withstood -10 °C for 65 days with 100% survival.

As discussed in the literature review, (Section 2.5.1.2) there is some controversy over the role that seasonal water loss plays in insect cold-acclimation. In some insects, total body water is reported to drop with the onset of winter, others show no shift. This research project included seasonal determination of total body water content in Cryptocercus punctulatus (Table 3), in an attempt to obtain more information on this aspect of cold-acclimation. From these data it appears that there was little seasonal change in Cryptocercus punctulatus total body water content. Except for the first measurement taken (75.3% N=5), all body water contents were very similiar (64-68%). In October, males and females were examined

Table 3

Seasonality of water content in adult *Cryptocercus punctulatus*

Collection date	Number of insects	Percent body water[1]
Nov. 7, 1983	5	75.3 ±2.8 a
April 24, 1984	9	66.2 ±1.6 b
Oct. 17, 1984	9	68.2 ±2.9 b
Aug. 2, 1984	5	64.1 ±2.9 b
Oct. 1, 1984 (males)	10	67.3 ±2.0 b
Oct. 1, 1984 (females)	10	67.7 ±3.5 b
Dec. 13, 1984	10	67.2 ±1.9 b

[1]Means with the same letter are not significantly different (Duncans multiple range test: alpha= 0.05)

separately, and body water content was not statistically different (Duncans multiple range test; alpha=0.05).

These experiments on subzero survival rates of _C. punctulatus_ indicated that the insect is freeze-tolerant (because of high survival rates after extended subzero storage), but the actual "freezing point" (supercooling point) of the body fluids was unknown. Table 4 compares supercooling points of whole insects and 2 ul hemolymph serum samples. From this table, it can be seen that the mean SCP for summer- and winter-collected insects ranged from -5.6 to -5.8 ^{o}C. It can also be seen that isolated serum supercooled to essentially the same temperature (-5.2 to -5.5 ^{o}C). The SCP's of male and female hemolymph was identical (-5.0 ^{o}C), in the fall-collected (October 1, 1984) hemolymph pools, although the SCP is higher than previous determinations, it is indicative of the presence of INF's in the fall-collected insects. These data, combined with the subzero storage information, indicate that _C. punctulatus_ is freeze-tolerant, and that hemolymph ice nucleating factors are present throughout the year, providing relatively constant supercooling points. Heat treatment (2 min. at 100 ^{o}C) destroys some of the nucleating activity in both summer- (-5.2 $\pm$0.8 vs -9.5 $\pm$0.7) and winter- (-5.5 $\pm$0 vs -8.4 $\pm$2.1) collected pooled hemolymph (heat treatment yielded significant effects in both cases $p<0.05$; Students T-test). It is interesting

Table 4

Supercooling point comparisons of whole body and 2 ul pooled hemolymph samples of *C. punctulatus*

Sample	Supercooling point (°C ± S.D.)[1]
Winter-Collected[2]	
Whole insect (N=5)	-5.6 ± 0.3
Hemolymph (50 individuals)	-5.5 ± 0.0[a]
1:10 dilution	-5.2 ± 0.7
1:100 dilution	-6.2 ± 0.6
1:1000 dilution	-10.2 ± 2.6
Heated hemolymph	-8.4 ± 2.1[a]
Summer-Collected[3]	
Whole insect (N=5)	-5.8 ± 0.4
Hemolymph (50 individuals)	-5.2 ± 0.8[b]
1:10 dilution	-5.5 ± 0.4
1:100 dilution	-6.6 ± 0.4
1:1000 dilution	-11.4 ± 1.8
Heated hemolymph	-9.5 ± 0.7[b]
Fall-Collected Hemolymph[4]	
Pooled adults (50 individuals)	-4.8 ± 0.1
Nymphs (50 individuals)	-5.7 ± 0.2
Males (25 individuals)	-5.0 ± 0.3
Females (25 individuals)	-5.0 ± 0.3
Distilled water	-12.8 ± 0.5
Periplaneta americana	-12.1 ± 2.7
P. americana + 0.5M glycerol	-14.4 ± 3.8
C. punctulatus[2] + 0.5M glycerol	-5.8 ± 0.6
1M glycerol	Unfrozen at -17 for 5 min.

[1]Based on 5 different determinations

[2]Collected November 29, 1983

[3]Collected July 24, 1984

[4]Collected October 1, 1984 hemolymph pools consisted of 5 individuals.

[a]significantly different $p<0.05$ (Students T-test)
[b]significantly different $p<0.05$ (Students T-test)

that although statistically significant, not all nucleating activity is removed when the samples are heated (distilled water SCP= -12.8 °C, heat-treated hemolymph = -8 to -9 °C).

In his initial description of "ice nucleating factors" in the hemolymph of cold-acclimated beetles, Zachariassen (1976), indicated that nucleating activity (ie. SCP above -7 °C) should remain when the hemolymph is diluted as much as 20 fold, or polyols are added to the sample. Thus, dilution, and/or addition of glycerol has become a standard assay for determining nucleating activity in hemolymph samples (Zachariassen et al, 1982). It can be seen from the data in Table 4 that nucleating activity remains when samples are diluted as much as 100 fold (winter SCP -6.2 ±0.6 °C). However, further dilution results in loss of nucleating activity and an increased variability around the mean SCP (1/1000 dilution was found to be -10.2 ± 2.6 °C).

Addition of glycerol to samples of C. punctulatus and Periplaneta americana hemolymph provided some interesting results. Table 4 shows that when equal volumes of 1 M glycerol and hemolymph are mixed, the supercooling point of pooled C. punctulatus hemolymph was unaffected (-5.8 ±0.6 °C), whereas the supercooling point of the pooled P. americana hemolymph was decreased by 2 °C (-12 to -14 °C). This is not surprising, since P. americana is a lab-reared, non-cold-acclimated insect, it would not be expected to contain ice nucleating factors in the hemolymph, thus,

glycerol would be expected to lower the mean hemolymph supercooling point.

Since it has been reported that freeze-tolerant insects may also contain high levels of polyol(s) in the hemolymph, experiments were conducted to detect hemolymph polyol(s) in C. punctulatus. When hemolymph was examined for the presence of the 5 polyols reported to occur in insect hemolymph (using HPTLC), none of the "traditional" compounds were detected. However, a polyol (ribitol), not yet confirmed as occurring in insects, was found. Table 5 compares the R_{-f} values of various sugar and polyol standards using 6 solvent systems. Although some of the R_{-f} values obtained were similar, separation was consistant, and a preliminary identification of ribitol was established using the HPTLC methodology. Confirmation was achieved by GLC of trimethylsilyl ether and acetate derivatives of ribitol. Identical retention times were obtained for both the derivatized ribitol and the hemolymph polyol. The procedure using trimethylsilyl ether derivatives (see Section 3.3.4 for details) was used initially, and confirmed that the hemolymph polyol was either ribitol or arabitol. Ribitol-spiked hemolymph samples provided one large, well defined peak. Using acetate derivatives and an OV-225 column at 210 °C, good separation between ribitol and its isomer arabitol was

Table 5

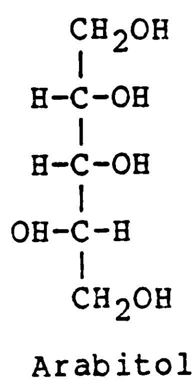
A comparison of R_f values of sugars, polyols, and components of *C. punctulatus* hemolymph serum on silica gel HPTLC plates using various solvent systems.

Compound	Solvent number 1	2	3	4	5	6
Glycerol	0.58	0.65	0.50	0.69	0.66	0.52
C. punctulatus	0.35	0.50	0.32	0.57	0.48	0.35
Ribitol	0.35	0.50	0.32	0.57	0.48	0.35
Arabitol	0.31	0.44	0.30	0.52	0.42	0.32
Xylitol	0.27	0.41	0.25	0.63	0.40	0.31
Sorbitol	0.19	0.28	0.19	0.38	0.33	0.24
Mannitol	0.21	0.35	0.20	0.41	0.35	0.24
Inositol	-	0.06	0.06	-	-	-
Threitol	-	0.56	0.35	-	-	-
Dulcitol	0.19	0.28	0.19	0.36	-	-

Solvent 1 Ethyl acetate:methanol:butanol:water (16:4:4:2)
Solvent 2 Butanol:acetone:water (5:4:1)
Solvent 3 Butanol:water (9:1)
Solvent 4 Acetone:butanol:water (5:3:1)
Solvent 5 Methanol:chloroform:acetic acid (5:4:2)
Solvent 6 Acetonitrile:water (85:15)

obtained (Figure 4 in Materials and Methods). The structures of these compounds are shown in Figure 5. These two compounds have identical molecular weight, but differ slightly in configuration; the positon of one hydroxyl group separates the two compounds (geometric isomers). Although these compounds behave quite similarly in chemical reactions and physical separations, it was possible to achieve adequate separation and determine that C. punctulatus accumulates ribitol in the hemolymph during the winter. This latter gas chromatography system (acetate derivatives) was then used for seasonal quantitation of ribitol.

Table 6 shows the ribitol concentration in serum from C. punctulatus collected at various times of the year. Hemolymph collected in January contained the highest level of ribitol (14.9 mg/ml), and none was detectable in the summer and early fall months (July 24, 1984 through October 17, 1984). It appears that ribitol levels fluctuate seasonally, and may be associated with acclimation to cold conditions. An experiment supporting this hypothesis involved maintaining C. punctulatus (Nov., 1984-collected) in the dark at 0 °C for 1 month. It was found that hemolymph ribitol levels were greatly elevated when compared to field collected individuals from the same time interval. One group (N=50)of the insects collected on November 12, 1984 were analyzed for ribitol content (1.7



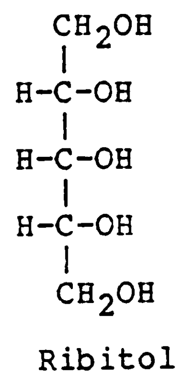


Figure 5

Structural comparison of ribitol and it's geometric isomer arabitol

Table 6

Seasonality of ribitol concentrations in pooled adult *C. punctulatus* hemolymph serum.

Collection date	Concentration mg/ml	mM
Nov. 29, 1983	13.9	91.3
Jan. 29, 1984	14.9	97.9
April 8, 1984	2.3	15.1
July 24, 1984	N.D.[1]	-
Sept. 29, 1984	N.D.	-
Oct. 1, 1984	N.D.	-
Oct. 17, 1984	1.0	6.6
Nov. 12, 1984	1.7	11.2
Dec. 13, 1984	0.4	2.6
Insects held in cold storage[2]	10.0	66.0
Log extract	0.0	0.0

[1]N.D. = not detectable

[2]Cold storage: *C. punctulatus* collected on Nov. 12, 1984 and held in the dark at 0 ^{o}C for 1 month.

mg/ml). Another group (N=12) collected on the same date were maintained in the dark at 0 °C until December 13, 1984. On December 13, additional insects were collected. Pooled hemolymph from these newly-collected insects and those maintained in the laboratory for one month was examined for ribitol content. In field collected insects, ribitol concentrations were lowered between November (1.7 mg/ml) and December (0.4 mg/ml). This difference was most likely due to an unusually warm early winter (from October to mid-December). The insects maintained in the dark at 0 °C, however, showed a considerable increase in hemolymph ribitol concentration (10 mg/ml). This information, although inconclusive, suggests that C. punctulatus, like other freeze-tolerant insects, is able to accumulate polyol(s) (ribitol) independantly of photoperiod.

Polyacrylamide Gel Electrophoresis (PAGE) hemolymph serum banding patterns were compared seasonally. This was done in an attempt to detect any quantitative and qualitative differences in hemolymph protein composition which might occur as these insects cycle through seasonal changes. Figure 6 provides infromation on hemolymph protein banding patterns from insects collected in summer, winter, and insects held in cold storage (4 °C) for one month. Based on staining intensity, protein band "a" appears, to have decreased in concentration from winter to summer. Proteins "b" and "c" appears to have increased in

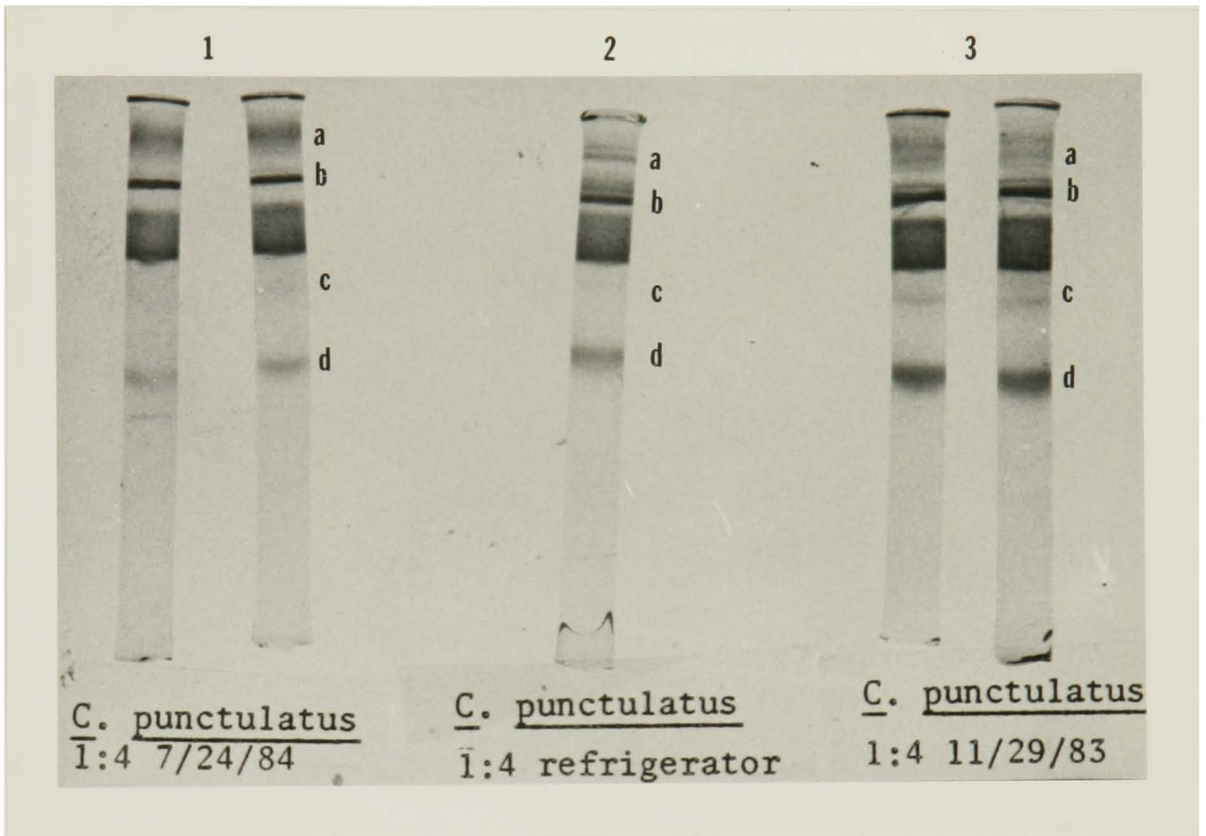


Figure 6 Acrylamide banding patterns of C. punctulatus hemolymph, diluted 1:4, from summer-, winter-acclimated insects, and insects held in cold storage.

1= summer-acclimated insects collected 7/24/84
2= cold storage- insects held at 4 °C for one month
3= winter-acclimated insects collected 11/29/83

a= protein band that showed a decreased concentration during cold-acclimation.

b, c, d= protein bands that showed an increased concentration during cold-acclimation.

concentration during cold-acclimation, therefore, may prove to have some significance in the nucleating capacity of *C. punctulatus*. Relative fluctuations in concentrations of these 3 protein bands were noted utilizing this one set of electrophoretic conditions. However, further experimentation (different electrophoresis systems/ techniques, or methods of protein separation) would provide better resolution/separation to determine actual changes in these protein levels. Protein band "d" appeared to fluctuate seasonally (Figure 6). Based on staining intensity, this protein band appears to be present in low amounts during the summer. It appears in moderate amounts when insects are subjected to cold storage (refrigerated at 4 ^{o}C), and is present in higher levels in winter. If this protein band is an ice nucleator, it's relative levels appear to correlate nicely with cold-acclimation. It should be noted that this fast migrating protein band indicates that it has a relatively low molecular weight. Attempts to isolate and demonstrate nucleating activity in this protein band were unsuccesful. One such experiment included: electrophoretic separation, slicing the gel into 4 sections, elution of protein from the gel matrix into buffer, dialysis, lyophylization and testing the four gel regions for nucleating activity. This procedure proved inconclusive, since all gel fractions showed nucleating activity. Discussion of this problem with Dr. Jack Duman

(personal communication) proved to be very enlightening. Dr. Duman claims that commercially prepared dialysis tubing (or cellulose-type membranes) contains an intrinsic nucleating substance which induces nucleation at -6 ^{o}C in any sample which it has contacted. Due to time constraints, further attempts to associate INF-activity with the PAGE-separated protein bands were not carried out. Total hemolymph protein levels were determined at various intervals throughout the year. This was done to determine whether total protein concentration fluctuated seasonally. This information, coupled with that of the protein banding patterns (PAGE), would help confirm the presence, and possibly aid in the identification of protein ice nucleator(s). Table 7 shows total protein levels of pooled C. punctulatus hemolymph. Protein levels were found to fluctuate seasonally, ranging from 38 mg/ml (July 24, 1985) to 115 mg/ml (Nov. 29, 1985). Male and female hemolymph protein levels were examined separately on two different occasions (Feb. 21, 1985; and March 21, 1985). They did not appear to differ (Students T test $p<0.01$) on the dates tested (range from 53-72 mg/ml).

Figure 7 provides a comparison of log temperatures from 3 of the 4 different-sized logs placed at the Horton Research Facility near Mt. Lake, Va. Only three logs are represented in this figure because one of the temperature

Table 7

Seasonality of total protein content in pooled adult *Cryptocercus punctulatus* hemolymph.

Collection date	Protein concentration mg/ml
Pooled Hemolymph	
September 23, 1983	50
November 29, 1983	115
April 8, 1984	55
July 24, 1984	38
October 1, 1984	61
November 12, 1984	90
December 13, 1984	93
Subsamples[1]	
February 21, 1984 (males)	67 ± 7
February 21, 1984 (females)	72 ±14
March 21, 1984 (males)	67 ±23
March 21, 1984 (females)	53 ±12

[1]Samples represent 3 separate pools of 5 individuals each. Population standard deviation based on duplicate analysis of the three pools (N=3).

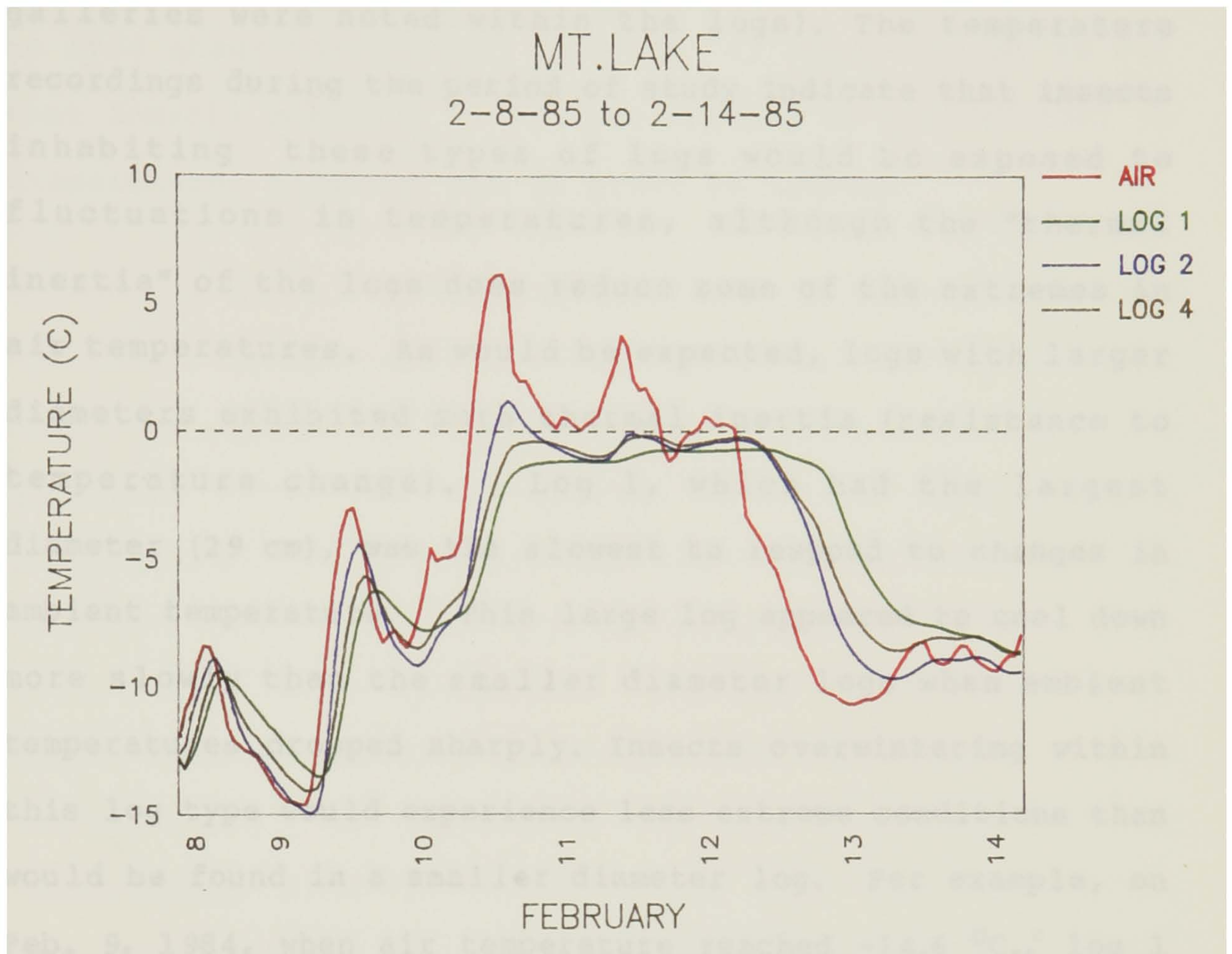


Figure 7 Comparison of air and log temperatures from Mt. Lake, Va. from February 8, 1985 to February 14, 1985.

Log 1=29 cm diameter, length 56 cm
Log 2=13 cm diameter, length 56 cm
Log 4=17 cm diameter, length 71 cm

For further details see Section 3.4

sensing probes became nonfunctional during this interval. These logs did not contain active colonies of C. punctulatus, but represented previous habitats (old galleries were noted within the logs). The temperature recordings during the period of study indicate that insects inhabiting these types of logs would be exposed to fluctuations in temperatures, although the "thermal inertia" of the logs does reduce some of the extremes in air temperatures. As would be expected, logs with larger diameters exhibited more thermal inertia (resistance to temperature change). Log 1, which had the largest diameter (29 cm), was the slowest to respond to changes in ambient temperature. This large log appeared to cool down more slowly than the smaller diameter logs when ambient temperatures dropped sharply. Insects overwintering within this log type would experience less extreme conditions than would be found in a smaller diameter log. For example, on Feb. 9, 1984, when air temperature reached -14.6 ^{o}C., log 1 reached an internal temperature of -13.5 ^{o}C. just 2 hrs later. However, on Feb. 12, 1984 when air temperatures dropped 10 ^{o}C in 15 hrs., the internal temperature of log 1 had dropped only 1 ^{o}C in that same time period. In this case, log temperatures appeared to lag about 16 hrs. behind ambient. Many parameters affect the thermal inertia of decaying logs. These include: log moisture, size, density, contact with the ground, and duration of temperature

changes. Since all logs were cut from the same tree, the only factor included in this experiment was size. Based on this brief experiment, no conclusive, general statement can be made about the thermal inertia of logs, except to say that insects dwelling within are exposed to fluctuations in temperature, although not as great as ambient.

5. DISCUSSION

It appears that the initiation of cold-acclimation in insects can be a very complex process. In some species, shortened photoperiod plays a major role in the anticipation of winter's onset (Duman and Horwath, 1983), while others rely solely upon decreasing temperature as a cue to winter's approach (Band and Band, 1982; Baust, 1982; Young and Block, 1980). Interestingly, in most reported cases freeze-tolerant insects overwinter within rotting wood (*Pterostichus brevicornis* Baust, 1982; *Vespula maculata* Duman and Patterson, 1978; and *Cryptocercus punctulatus*), or in situations where photoperiod cues are limited (ie. rotting fruit, or beneath bark). The observation that induction of polyol accumulation in freeze-tolerant insects is independant of photoperiod (see Section 2.6) is supported by the findings for *C. punctulatus*. Although inconclusive, the observation that low temperature alone may be sufficient to induce polyol accumulation in *Cryptocercus punctulatus* is consistant with information avaliable in the current literature, and supports the hypothesis that the cold-acclimation process may be independant of photoperiod in some freeze-tolerant insects.

There is some controversy as to whether insects utilize losses in total body water as a cold-hardening step (see Section 2.5.1.2). The controlled body-water loss would

increase solute concentrations (sugars, salts, etc) within the insects body, and would in turn, lower the insects supercooling point. This would appear to be most important to freeze-susceptible insects. Since ice-crystal formation is initiated at a high subzero temperature in freeze-tolerant insects, a loss of body water would seem to be of little advantage. Evidence obtained from this study indicated that the total body water content remained relatively constant over an annual cycle (Table 3). The first determination of body water content (Nov. 7, 1983) was high when compared to the other determinations. No explanation can be offered. However, it can be pointed out that throughout a similar period the next year (Oct. 1, 1984 to Dec. 13, 1984) body water contents were found to be 67%, which appears to be the average water content for adult C. punctulatus throughout a seasonal cycle.

The supercooling point of many insects exhibits seasonal variation. Freeze-susceptible insects demonstrate a significant lowering of their supercooling point, while many freeze-tolerant insects actually raise their SCP as winter approaches. Many factors affect the supercooling point of insects (see Sections 2.5.1 and 2.5.2.1). However, the most fascinating and apparently the least understood, are the "ice nucleating factors." Our present knowledge of INF's indicates that they are either proteins,

or a combination of proteins and lipoproteins. Although the chemical nature of INF's was not specifically examined in this study, some circumstantial evidence obtained suggests that C. punctulatus contains INF's which are most likely proteins. Heat treatment (100 ^{o}C for 2 min) resulted in a significant loss of nucleating activity, and as winter approached, the total hemolymph protein levels increased 2 to 3 fold. The observation that hemolymph protein levels increase while water content remained unchanged in winter indicates that either greater amounts of the existing protein (s) are present, or specific proteins are produced as a part of cold-acclimation. Early reports in the literature indicated that ice nucleators are present in hemolymph only during the winter months (Zachariassen and Hammel, 1976; Zachariassen, 1982), that is, INF's were thought to be produced only during the cold-acclimation process. Recently, some freeze-tolerant insects (C. punctulatus included) have been described which do not exhibit seasonal fluctuation in SCP (Duman et. al., 1985). Cryptocercus punctulatus serum exhibits INF activity throughout the year. Electrophoretic analysis of summer- and winter-collected hemolymph samples yielded some interesing comparisons. The migration of electrophoretically separated proteins is dependant not only on the electrical charge, but also on the relative size of the molecule. Protein bands "a" and "b" (Figure

6) both showed appeared to show seasonal fluctuation, and a slow migration rate in 7% acrylamide gel. This slow migration rate indicates a relatively large molecular weight and small electrical charge. A lower acrylamide percentage (ie 5-6%) would probably allow better resolution and closer examination actual seasonal changes which may occur. The protein band "d" in Figure 6 appears, from its seasonality, and electrophoretic mobility, to be a potential ice nucleating factor. Due to this proteins apparently high mobility in the 7% acrylamide gel, a fairly low molecular weight is indicated. In the one case where a protein nucleator has been purified, its molecular weight was determined to be 74,000 daltons, (Duman et. al., 1984) which is fairly low for proteins. Since the levels of protein "d" are very low in summer, intermediate when insects are held in cold storage, and very high in naturally cold-acclimated conditions this protein band appears to be a good candidate as an INF. The increase in total hemolymph protein content during the winter and the non-seasonality of ice nucleation could be explained if this protein is indeed an ice nucleator. However, initial attempts to separate and purify this protein by electrophoresis and chromatography have proven unsuccesful. Purification of the protein bands which showed seasonal fluctuation and the investigation of their quantitative and

qualitative differences is well worth future study. The presence of INF's in *C. punctulatus* serum throughout the year indicates either multiple roles in the insect's biochemistry / physiology (Duman et. al., 1985), or an attempt at nitrogen economy. Since this species feeds on a nitrogen-deficient diet (cellulose), the sustained presence of INF's may be a process designed to reduce the potential energy and amino acid losses which accompany repeated catabolism and anabolism of proteins. Therefore, *C. punctulatus* ice nucleators may act to initiate ice formation in the winter, and perform other function(s) (ie transport/carrier protein(s), enzyme(s), or storage proteins) during the other seasons. Support for this hypothesis may be derived from the observations that as winter approaches: 1) no new major protein band appears in the hemolymph, and, 2) total hemolymph protein levels increase dramatically. It may be that higher concentrations of these "factors" are necessary for increased physiological effectiveness in their INF role. Although these "INF's" are active in very low concentrations *in vitro*, higher concentrations may be required for their biological activity [ie. to ensure even nucleation throughout the body, prevent ice recrystallization and/or protection of certain types of tissue (ie. nervous tissue membranes)].

As discussed earlier, the seasonal accumulation of

sugar alcohols associated with the onset of winter conditions is an important step in the cold-acclimation process of freeze-tolerant insects (Baust, 1973). Circumstantial evidence from this research supports the relationship between the presence of polyol and freeze-tolerance. Insects collected in July were found to contain hemolymph INF's, but were not freeze-tolerant. Freezing occurred at -5.8 ^{o}C, but these insects did not recover when warmed to room temperature. Since they contained INF's, and hemolymph ribitol was undetectable, both materials may be needed to provide for the freeze-tolerance. The combined effects of INF's and polyols in freeze-tolerant insects may be to alter normal ice crystal growth. Ice nucleating factors induce nucleation at a high subzero temperature, and polyols act to alter the growth and structure of ice crystals. In the presence of polyols, ice crystals grow slowly, but more importantly, the resulting crystals have rounded, blunt edges (Baust, 1973). This alteration of the crystal structure is thought to be of major importance for freeze-tolerant insects, since tissues are less disrupted by the modified crystal structure.

The observation that C. punctulatus accumulates ribitol in the hemolymph, presumably as a cryoprotectant, is intriguing. This insect ingests wood and with the aid of the symbiotic organisms digests cellulose. Cellulose is a

polysaccharide composed simply of glucose molecules in the beta linkage. Presumably, the cellulose is digested by the symbionts to yield glucose (Cleveland et. al., 1934), a 6 carbon carbohydrate. The question as to why _C. punctulatus_ accumulates ribitol, a 5 carbon sugar alcohol, and not glycerol or one of the other "common" polyols as a cryoprotective substance is of interest. Glycerol (3 carbons) is a constituent of neutral fats. Free glycerol could be produced in the fat body and released into the hemolymph with little energetic investment. Both glycerol and sorbitol (6 carbons) are readily produced from glycogen during glycolysis and are easily reused as an energy source when they are no longer useful. The energetic cost of maintaining one of these polyols would seem to be very low. It would seem that maintaining high levels of ribitol in the hemolymph would be less energetically favorable. Insects are reported to have "little or no capacity to metabolize pentoses" (Bignell, 1982). Ribitol has not previously been reported to occur in insect hemolymph. Ribitol is, however, common in plants and bacteria. Most notably, it is found as a constituent of the cell wall of several gram positive bacteria. In mid-November, 1984, when ribitol was first detected in the hemolymph of _C. punctualtus_, extracts of the rotten wood in which they live as well as their feces were analysed for the presence of ribitol. Ribitol was not detected in either case.

Therefore, ribitol is probably not simply obtained directly and concentrated from their environment, but actively produced in response to cold. When C. punctulatus were maintained at 0 °C and held in the dark, an increase in serum ribitol levels was noted when compared to field populations. The source of ribitol then appears to be within the insect. It is possible that the gut symbionts including the rod-shaped "associated" bacteria and "commensal" protozoans (see Section 2.1) are involved in the production of ribitol. These organisms might produce ribitol in response to the cold conditions within the insects gut. Ribitol might then be absorbed through the gut wall and into the hemolymph. The actual source of ribitol and its possible production by a symbiotic relationship would be quite worthy of further study. Indeed, this system may represent symbiosis between the cockroach and gut-dwelling organisms on a higher order than simply for digestion of cellulose.

The use of the freeze-tolerance mechanism by an insect which inhabits such a "mild" environment deserves discussion. Freeze-tolerance is well documented in Arctic insects where temperatures routinely drop below -40 °C. Winters in the Arctic are extremely harsh and last for many months. In this type of environment, freeze-tolerance is energetically more favorable than supercooling. Mt.

Lake, Virginia does not experience these prolonged extremes in temperature and many freeze-susceptible species can be found there. Unusually cold temperatures did occur at Mt. Lake (-37 °C) during this study. However, it appears that the thermal inertia of rotted logs may protect *C. punctulatus* from exposure to these extremes in a short term cold spell. Freeze-susceptible ground beetles, carpenter ants and other insects have been collected from the same logs that *C. punctulatus* inhabits. These other insect species survive winter by extending their hemolymph supercooling point (personal observation). It is not clear why *C. punctulatus* has developed the freeze-tolerance mechanism of protection. These other insect species probably do not require a symbiotic relationship for the digestion of their food. During a mid-winter thaw, these insects could forage, feed, and digest their food. Cleveland et. al., (1934) state that the symbionts inhabiting the gut of *Cryptocercus* are extremely temperature sensitive (temperatures above 30 °C or "harsh" winters). These symbiotic organisms may be unable to function at temperatures near zero, so little or no digestion would occur after feeding by the cockroach. If *C. punctulatus* were to rely upon supercooling for winter survival, they may starve, due to the inactivity of the symbionts. Therefore, this insect may have evolved a protective mechanism to maintain itself frozen throughout

winter, when food is essentially unavaliable.

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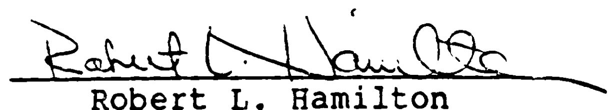
7. VITA

Robert L. Hamilton was born in Orleans, France on August 23, 1960, the third son of Col. Alexander M. and Helen M. Hamilton. He, his two brothers, and one sister were raised in an Army family, and consequently, lived in many different areas of the country. In 1968, his family settled in Fairfax, Virginia, which has remained his permanent residence. In 1978, he attended VPI&SU for two years as an undergraduate majoring in Biology. In 1980, he transferred to The George Washington University and received a B.S. in Zoology in 1982. In the fall of 1982, he entered graduate school at VPI&SU, in the department of Microbiology. In the spring of 1982 h transferred to the Entomology department to pursue an M.S. degree studying the cold-acclimation of *Cryptocercus punctulatus*. Mr. Hamilton is a member of the Entomological Society of America; and the honor societies Sigma Xi, and Gamma Sigma Delta.



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