Eur. J. Entomol. **93**: 419–433, 1996
ISSN 1210-5759

Tracking the profile of a specific antifreeze protein and its contribution to the thermal hysteresis activity in cold hardy insects

KATHLEEN L. HORWATH*, CHRISTOPHER M. EASTON, GEORGE J. POGGIOLI JR., KEVIN MYERS and INGRID L. SCHNORR

Department of Biological Sciences, Binghamton University, Binghamton, New York 13902-6000, USA

Antifreeze proteins, thermal hysteresis, Tm 12.86, activators, cold hardiness, *Tenebrio molitor*

Abstract. This study summarizes some important new directions in research on antifreeze protein biosynthesis and regulation. It describes the recent development and availability of essential biochemical and cellular tools that make possible more direct cellular investigations, and an assessment of the relationship between thermal hysteresis protein (THP) levels and antifreeze activity (both thermal hysteresis and recrystallization inhibition [RI]). These tools include: 1) the isolation of a specific THP of high activity (designated Tm 12.86), and an additional endogenous activating factor of this antifreeze protein; 2) the ability to track the cellular and secretory patterns of Tm 12.86 immunologically; 3) the use of an in vitro fat body cell culture system for direct investigation of cellular events, and, 4) a means of quantifying RI behavior of purified Tm 12.86, and samples of unknown concentrations of THPs, to provide a more sensitive detection method for antifreeze activity at scaled down values associated with the in vitro system. In combination, these studies indicate that the adaptation mechanisms contributing to the overall antifreeze protein response in a cold hardy insect involves a complex interaction between antifreeze proteins and endogenous activators of these proteins. With the availability of these key tools, the details of a precise and seasonal regulation of these antifreeze protein/activator interactions, which ultimately generate an efficient cold hardy response, now have the potential to be worked out.

INTRODUCTION

The presence of antifreeze proteins (AFPs) is an important overwintering adaptation of many insects (Duman & Horwath, 1983; Zachariassen, 1985; Duman et al., 1991, 1993). These proteins are secreted into the hemolymph and depress the freezing point non-colligatively, presumably by hydrogen bonding to the ice lattice and inhibiting crystal growth, thereby creating a thermal hysteresis (difference between the melting and freezing points) (Raymond & DeVries, 1977; DeVries, 1984; Raymond et al., 1989). Because of this unusual property, attributed only to AFPs, they are also known as thermal hysteresis proteins (THPs) and are capable of providing substantial protection against freezing, without the large increase in osmotic pressure which would accompany a similar level of protection from accumulated polyols.

The list of overwintering insects identified that produce THPs as part of their cold hardening response has grown substantially over the past 15 years (Duman, 1979; Duman et al., 1982, 1991, 1993) indicating that this is a phylogenetically diverse adaptation among insect and non-insect arthropods. Moreover, within this same time frame numerous studies have contributed to our increasing knowledge of THP structure and function in insects,

* To whom correspondence should be addressed.

and in the identification of major environmental and physiological factors controlling the seasonal pattern of antifreeze activity (see Duman et al., 1991, 1993 for recent detailed reviews). However, in spite of many diverse studies this remains a relatively young field of investigation. For example, we know little about the cellular mechanisms regulating hemolymph THP levels, or even how insect THPs are acting to generate the substantial thermal hysteresis activities seen among cold hardy species.

Recently, our laboratory has begun to address these important issues. The goals of this paper are two-fold. First, it provides a consolidation of the outcome of our laboratory's considerable effort over the past few years to develop four key tools essential in addressing these issues. This includes: 1) isolating a specific THP of high activity; 2) obtaining a very sensitive and specific antisera generated against this THP; 3) developing a reliable in vitro system for fat body cells to more directly investigate cellular events, and 4) characterizing a more sensitive means of detecting antifreeze protein activity via recrystallization inhibition (RI), to allow for the detection of THPs at the scaled down levels associated with an in vitro system. Second, given these essential tools, we can now for the first time track the endogenous profile of a specific insect antifreeze protein and its contribution to the overall thermal hysteresis activity in a cold hardy insect.

MATERIAL AND METHODS

Animals

Tenebrio molitor larvae, originally purchased from Carolina Biological Supply were maintained in the laboratory on wheat bran, with potato halves for moisture, at 26°C, under long daylength (16 h light; 8 h dark). Unless otherwise stated, acclimation of larvae to winter hardening conditions consisted of a 21 day stepwise cold-acclimation (weekly steps at 15°C, 10°C and 5°C) while being maintained under short daylength (8 h light; 16 h dark). These conditions have previously been shown to cause significant elevation of hemolymph thermal hysteresis activity (Patterson & Duman, 1978). Most studies used final instar larvae typically weighing > 150 mg (Connat et al., 1984, 1991).

Purification of thermal hysteresis proteins

THPs were purified from winter acclimated larvae using methods modified from Wu et al. (1991a), including the use of ethanol extraction of whole larvae followed by ion exchange column chromatography, gel filtration chromatography, preparatory non-denaturing polyacrylamide gel electrophoresis and reverse phase HPLC. For complete details of these purification procedures see Poggioli (1996).

Thermal hysteresis determination

The presence and relative activity of THPs were determined by an assessment of non-colligative freezing point depression using the microcapillary method of Ramsay & Brown (1955) as detailed by Duman et al. (1991). Briefly, 5 ml samples of hemolymph or purified fractions were sealed in 10 ml capillary tubes with mineral oil. These received small (0.25 mm) ice crystals spray frozen in the samples (Cytocool, Stephens Scientific, Riverdale, NJ), and placed into a refrigerated alcohol bath equipped with a Plexiglas viewing port through which the crystals could be observed with a stereomicroscope. The temperature was raised and lowered at a rate of 0.02°C/2.5 min to determine the melting and freezing points of the crystals. In solutions void of proteinaceous antifreezes the melting and freezing temperatures are virtually identical (as theory predicts) and are based on the colligative properties of the solution. When THPs are present, the freezing point is depressed below the melting point, and the extent of this depression is related to the concentration and type of THP present (Patterson & Duman, 1979; Tomchaney et al., 1982).

Generation of an antibody against purified Tm 12.86

Following the purification of a specific THP referred to as Tm 12.86, Bethyl Laboratories (Montgomery, Texas) was contracted to receive sufficient purified antigen for a series of injections into two rabbits over a period of fourteen weeks. After which, terminal bleeds were conducted to obtain antisera. Antisera was returned to our laboratory for evaluation of antibody specificity and sensitivity. This anti-Tm 12.86

was then used for immunolocalization studies and immunoblots (Western blots) of electrophoresis gels (Towbin et al., 1979). For complete details of antibody generation and characterization, and Western analysis protocols see Poggioli (1996).

Establishment of fat body primary cultures

Long-term culturing of fat body cells from *T. molitor* were conducted according to the procedures detailed in Easton & Horwath (1994, 1996). Briefly, this involved dissection of fat body under sterile conditions in *Tenebrio* modified saline (Easton & Horwath, 1996). Cells were then dissociated and plated in 24 well cell culture plates (Cell wells, Corning) containing *Tenebrio* modified Grace's solution and fed according to an experimentally determined optimized regime (Easton & Horwath, 1996). All cultures were maintained in a humidified chamber at 26°C and 90% RH. Under these culture conditions, we have established that the in vitro fat body cells from *T. molitor* appear to retain the morphological, histochemical and functional features characteristic of their in vivo counterparts (Easton & Horwath, 1994).

Immunolocalization of Tm 12.86

Intact fat body and primary cell cultures derived from fat body were immunologically screened as detailed in Easton & Horwath (1994) for the presence of Tm 12.86 using the above generated antiserum. Briefly, fat body was dissected under cold formalin, fixed for 48 h, embedded, blocked, and sectioned at 5 mm. Sections were deparaffinized, treated with 15% formic acid and stained with anti-Tm 12.86. The secondary antibody was peroxidase-linked goat anti-rabbit antiserum. Detection was by DAB. Cultured cells were fixed in 4% formaldehyde for 2 h, then treated similarly to the tissue sections, but without the formic acid treatment, and using a second antibody conjugated to fluorescein isothiocyanate (FITC) for 2 h. Cells were visualized under epifluorescent microscopy (455 BP EX/520 LP EM).

Pulse-chase experiments

To assess biosynthesis of Tm 12.86 in vitro, groups of cultured cells were used in pulse-chase experiments (Horwath & Riddiford, 1988). This involved exposing cells to a 4 h pulse of ^{35}S-methionine and a 20 min chase in non-labeled methionine. Cells and medium were then harvested, and volumes of samples equivalent to 200,000 cpm were evaluated on 15% SDS-PAGE, electroblotted to nitrocellulose, and immunologically stained for Tm 12.86 (i.e. Western analysis). Additionally, an autoradiogram was then obtained for the same nitrocellulose membrane by placing Kodak X-OMAT film in contact with the dried membrane for 10 days at –80°C. The film was developed with Kodak D-76 developer and Kodak Rapid Fix in the dark at 20°C. Pre-stained molecular weight standards were co-electrophoresed to confirm successful electroblotting.

Recrystallization inhibition assay

We examined the possibility that Tm 12.86 could inhibit the process of recrystallization, perhaps even at concentrations too low to cause a thermal hysteretic effect. If so, this would be useful for detecting THPs at the scaled down levels associated with an in vitro system. Purified Tm 12.86, *T. molitor* hemolymph, and fat body primary cultures and media were screened for recrystallization inhibition (RI) activity using the "splat" cooling technique as detailed by Knight et al. (1988). Briefly, this involves obtaining a very thin frozen wafer from a 5 ml sample dropped down a 3 meter tube onto a polished aluminum surface sitting on dry ice. The sample is frozen almost instantaneously and then transferred to a refrigerated microscope stage which has been preset at a particular subzero annealing temperature (e.g. –6°C). The ice wafers are photographed initially and after a certain period of elapsed time (annealing time) at 30× magnification between crossed Polaroids. For solutions of pure water a 2 h annealing time at –6°C is sufficient to observe a significant recrystallization effect, i.e. an increase in size and a decrease in number of ice crystals observed. However, antifreeze proteins inhibit ice crystal growth, and as such may be expected to inhibit or retard this process of recrystallization (Knight et al., 1984, 1988, 1995).

Following a detailed analysis of RI behavior of purified Tm 12.86 we developed an RI assay and determined the conditions and selective criteria (e.g. annealing time and temperature, effects of solutes, limits of detectability, etc.) by which the assay is both quantitative and specific for antifreeze protein activity. This includes the calculation of an "RI factor," the absolute value of the dilution at which a sample loses observable RI activity, i.e. is comparable to pure water or saline. This "RI factor" is derived from regression analysis of measures of mean largest grain size obtained from a dilution series of 7 replicate samples. Since the regression analysis takes into account the variability associated with individual measures of

grain size, the extrapolated RI factor, therefore, becomes the basis for comparison of unknown samples to purified Tm 12.86. Also, evaluation of RI factors obtained from multiple sampling of solutions within a treatment group will allow for statistical comparison of samples from different experimental groups. Details of these characterizations and quantification protocols are described in Myers (1996).

Identification and purification of an "Activator"

Fractions from the original ion exchange column were screened for the presence of any factor capable of enhancing the thermal hysteresis activity of purified Tm 12.86 (at a concentration of 6 mg/ml). One particular fraction displaying such activator activity was subjected to further gel filtration chromatography. Fractions from this gel filtration column were then tested for thermal hysteresis activity (at 25 mg/ml). Elution peaks that did not exhibit thermal hysteresis activity were then screened for activator activity in the manner described above. This allowed for the screening of potential activators capable of enhancing or augmenting thermal hysteresis activity of Tm 12.86, while displaying no thermal hysteresis activity by themselves. Further procedural details on the identification and purification of activators is described in Poggioli (1996).

RESULTS AND DISCUSSION

THERMAL HYSTERESIS ACTIVITY

Ramsay (1964) in his classic studies describing the cryptonephridial rectal complex of the beetle *Tenebrio molitor* was the first to note thermal hysteresis activity in the hemolymph of this species. Later, Patterson & Duman (1978) demonstrated that larvae of *T. molitor* displayed a seasonal pattern of hemolymph thermal hysteresis activity showing relatively low antifreeze activity under summer-like conditions, while exhibiting a three fold increase in antifreeze activity following acclimation to winter-like conditions. This is a highly reproducible response as seen with our own studies (Table 1). Extended exposure to short photoperiod, low temperatures or low relative humidity are all capable of stimulating elevated antifreeze protein activity in *T. molitor* (Table 1; also Patterson & Duman, 1978). But perhaps the most detailed studies on the environmental triggers that control hemolymph thermal hysteresis activity have been conducted with the cold hardy beetle, *Dendroides canadensis* (Horwath & Duman, 1982, 1983a,b, 1984, 1986). These studies established that the seasonal profile of thermal hysteresis was precisely regulated by the interaction of photoperiod, thermoperiod and low temperature. Moreover, the photoperiodic response was shown to involve the participation of the circadian timing system and its entrainment by the light cycle (Horwath & Duman, 1982, 1984). In related studies with *T. molitor*, the photoperiodic control of thermal hysteresis was also found to be under the control of the circadian system (Horwath, unpublished).

A few studies have attempted to identify the physiological effector systems (e.g. neuroendocrine or endocrine) which are then initiated by the circadian system under the

TABLE 1. Amount of hemolymph thermal hysteresis (indicative of antifreeze protein activity) in larvae of *Tenebrio molitor* acclimated to various environmental conditions.

	N	Thermal hysteresis °C ± SEM
Summer conditions		
16L/8D, 20°C	7	0.68 ± 0.14
Winter conditions		
8L/16D, 20°C	9	1.76 ± 0.27*
8L/16D, 10°C	8	2.13 ± 0.18*

* Winter acclimation conditions significantly ($P < 0.01$) elevate hemolymph thermal hysteresis.

appropriate inductive conditions. Horwath & Duman (1983c) found evidence from in vivo studies with *D. canadensis*, that elevated juvenile hormone (JH) may play an important role in mediating the seasonal pattern of hemolymph THPs. Later, Xu et al. (1992) found similar results for *T. molitor*. More recently, we have been successful in using our fat body primary culture system to demonstrate a significant increase in thermal hysteresis of the culture media bathing the cells in response to JH (Easton & Horwath, 1995).

It is important to point out that two major underlying premises are behind all of the studies mentioned above. The first is that thermal hysteresis activity reflects THP levels. The second is that thermal hysteresis activity/THP levels reflect THP production. Since by definition thermal hysteresis is caused only by THPs, and studies using purified THP have shown that increasing amounts of pure protein cause a proportional increase in thermal hysteresis (Patterson & Duman, 1979; Tomchaney et al., 1982), the first premise is entirely reasonable. Moreover, in the case of the second premise, given that THPs are secreted into the hemolymph, hemolymph activity would be dependent upon THP production minus any degradation. Consequently, until now, one has generally accepted these premises without concern. Yet, we will show below that recent efforts to examine the cellular mechanisms regulating hemolymph THP levels and to track the endogenous profile of a specific insect THP and its contribution to overall hemolymph thermal hysteresis activity, have indicated that these premises must be used with reservation. In fact, there are several degrees of complexity in the regulation of hemolymph thermal hysteresis activity which we are only now beginning to realize and recognize.

Tracking a specific THP

Characterization of Tm 12.86

We have purified a single species of THP from *T. molitor* and have determined by mass spectrometry that it has a molecular mass of 12.86 kDa. The elution profile for this protein, designated Tm 12.86 (for *Tenebrio molitor*, 12.86 kDa) is described elsewhere, along with the documentation of its purity (Poggioli, 1996). Several lines of evidence including amino acid composition, molecular weight, migration behavior on SDS-PAGE, and thermal hysteresis activity indicate that Tm 12.86 is unique among antifreeze proteins previously purified from *T. molitor* (Table 2; Poggioli, 1996). In fact, thermal hysteresis determinations for Tm 12.86 indicate that it is the most potent THP purified from this species to date (Table 2). The complete thermal hysteresis activity curve for various concentrations of purified protein is shown in Fig 1.

Surprisingly, the contribution of specific THPs to hemolymph antifreeze activity has never been addressed. In the first attempt to do so, we used our antibody (anti-Tm 12.86) to perform western blot analysis comparing winter-acclimated *T. molitor* hemolymph to a serial dilution of purified Tm 12.86 (detailed in Poggioli, 1996). The physiological concentration of Tm 12.86 in winter hemolymph was found to be 2–3 mg/ml (0.15 mM–0.23 mM), making up 5 to 7.5% of total hemolymph protein, and accounting for approximately 45% of hemolymph thermal hysteresis activity, i.e. contributing about 1.0°C of thermal hysteresis to the 2.2°C observed in winter hemolymph (Fig. 1). It is also interesting to note that the physiological concentration of Tm 12.86 corresponds to the steepest part of its thermal hysteresis activity curve, suggesting that even modest change in endogenous titers may significantly influence the level of thermal hysteresis activity. Just how significant

TABLE 2. Comparison of previously purified *T. molitor* antifreeze proteins to Tm 12.86. 1) Patterson & Duman, 1979; 2) Schneppenheim & Theede, 1980; 3) Patterson & Duman, 1982; 4) Tomchaney et al., 1982; 5) Tang, 1994 (AFP 1); 6) Poggioli, 1996, Tm 12.86.

Study	1	2	3	4	5	6
MW (kDa)	ND	9.0	ND	17.0	13.4	12.86
TH °C (at 30 mg/ml)	1.5	0.4*	0.5	0.7	ND	2.5
Amino acid			% mole			
Cis	0.0	3.0	28.0	0.0	1.3	3.2
Pro	5.9	6.0	0.0	5.9	3.4	3.0
Phe	1.5	3.0	0.0	3.9	2.8	3.4
Ile	3.3	3.0	1.0	7.1	5.3	4.4
Val	7.2	7.0	2.3	11.5	9.6	8.5
Met	0.0	0.0	0.0	4.8	0.1	2.0
Leu	3.9	5.0	2.2	0.0	5.9	4.4
% Most hydrophobic	21.8	27.0	33.5	33.2	28.4	28.9
Gly	7.6	9.0	11.4	8.3	4.7	3.1
Ala	9.6	7.0	5.0	14.3	5.4	3.9
Tyr	1.2	3.0	0.0	2.3	2.9	3.8
His	1.5	3.0	3.1	1.9	1.7	3.2
% Hydrophilic	19.9	22.0	19.5	26.8	14.7	14.0
Asx	11.3	13.0	5.3	7.3	12.3	10.7
Glx	15.3	11.0	12.4	8.9	14.6	15.0
Arg	1.1	5.0	5.0	2.6	2.5	3.6
Lys	4.8	7.0	15.4	6.8	15.3	14.9
Ser	14.8	9.0	11.1	7.4	5.9	6.8
Thr	11.0	9.0	2.3	6.6	6.2	6.3
% Most hydrophilic	58.3	54.0	46.5	39.6	56.8	57.3

* Thermal hysteresis was conducted at 50 mg/ml.

the contributions of the other, less potent THPs purified from *T. molitor*, are to hemolymph antifreeze activity, remains unknown.

Cellular localization of Tm 12.86

Fat body has been identified as a key source of THPs (Xu & Duman, 1991; Xu et al., 1992). Thus, it was particularly interesting to discover that fat body from *T. molitor* displayed an unusual feature when compared to most previously studies insects, in that it provides a reservoir of stored protein in numerous discrete granules throughout larval development, regardless of developmental age, or stage relative to molt. (Easton & Horwath, 1994). Fig. 2A shows a histological section of fat body taken from a summer acclimated final instar larva and stained for protein. Note the heavy protein load in discrete granules. Furthermore, immunological screening of fat body with anti-Tm 12.86 (Fig. 2B) shows that Tm 12.86 is strongly associated with these protein containing granules (Easton & Horwath, 1994). Recently, granules from the fat body have been isolated and found to be highly immunoreactive to anti-Tm 12.86, and capable of displaying thermal hysteresis activity. Thus, we now have substantial evidence to indicate that there is intracellular storage of Tm 12.86.

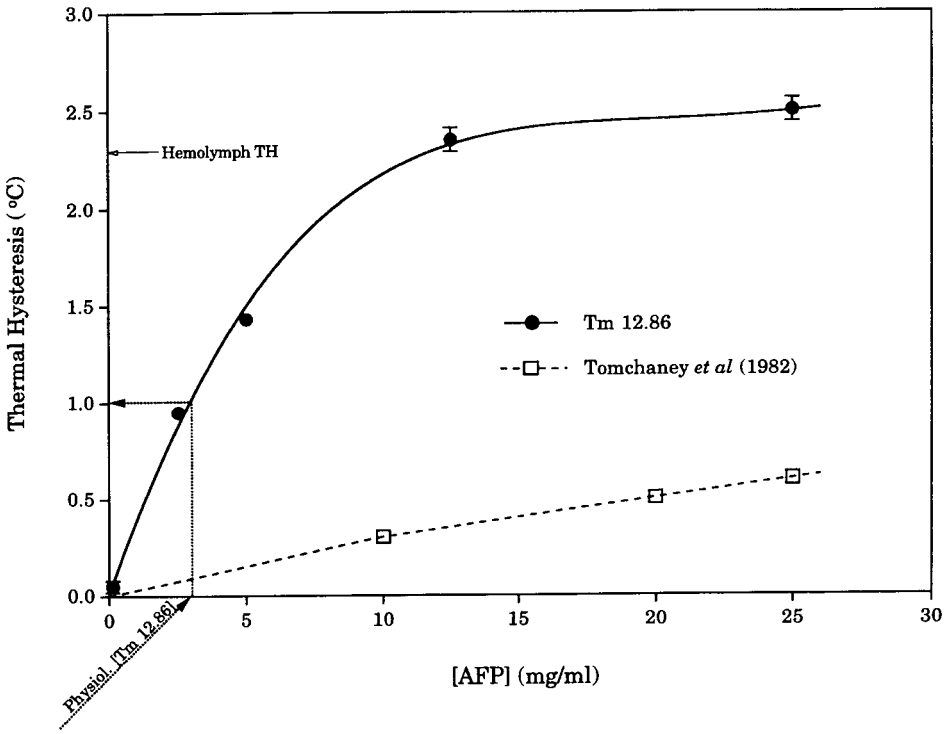

Fig 1. Thermal hysteresis activity curve for Tm 12.86. Included for comparison is the activity profile of a previously purified *T. molitor* antifreeze protein (Tomchaney et al., 1982). Top arrow indicates the average thermal hysteresis of hemolymph from winter-acclimated larvae. Also labeled is the endogenous titer of Tm 12.86 measured from winter hemolymph (see text for explanation). Adapted from Poggioli (1996).

The packaging of THPs into protein granules is likely to have some important ramifications in terms of seasonal elevation of hemolymph thermal hysteresis activity, and perhaps other adaptational benefits for overwintering insects. First, intracellular stores of THPs, if they were accessible to stop ice crystal growth and/or stabilize the supercooled state, would provide a means to protect against intracellular freezing. Second, intracellular stores of THPs are potentially available for mobilization and secretion into the hemolymph. This would mean on a day to day basis, that a steady state hemolymph concentration of THPs could be maintained without concurrent biosynthesis. Moreover seasonally, it would allow a means for production and storage of THPs in protein granules, at times when environmental conditions are still conducive to such, i.e. during the autumn prior to the onset of low temperatures. Larvae require approx. 2–3 weeks of exposure to stimulatory photoperiods before significant elevation of hemolymph thermal hysteresis activity can be detected (Horwath & Duman, 1983a; Xu & Duman, 1991). Perhaps the larvae are using this time to accumulate sufficient cellular stores of THPs, which can then be mobilized, as needed, throughout the winter. Results depicted in Fig. 2C showing

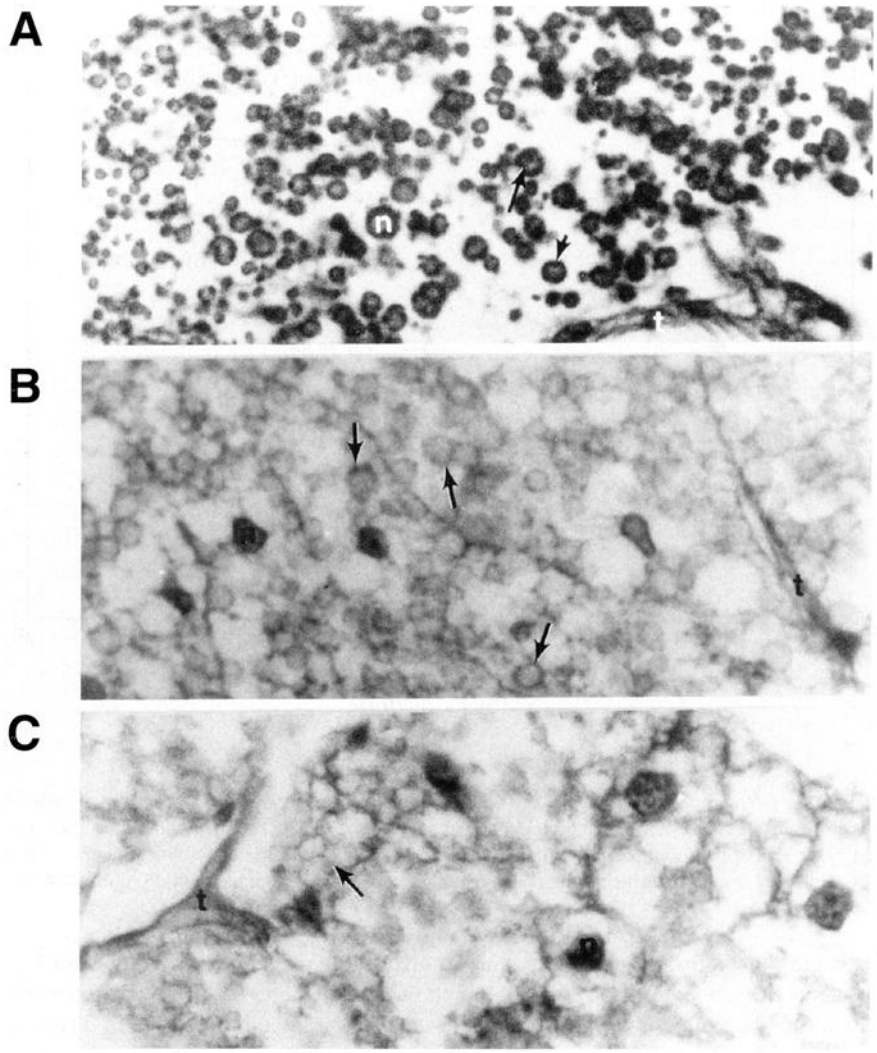

Fig. 2. Light microscopy of *T. molitor* fat body. (A) tissue stained for protein with hematoxylin and eosin and showing numerous protein granules (arrows). (B) and (C) are immunolocalizations of Tm 12.86. (B) fat body from a summer-acclimated larva showing positive immunoreactivity for Tm 12.86, which is strongly associated with the protein granules as indicated by a blackening of their edges (arrows). Note also the tracheoles (t) showing immunoreactivity. (C) fat body from a winter-acclimated larva. Note the general depletion of protein granules, but those which are visible (arrow) still display immunoreactivity for Tm 12.86. (n) nuclei. Negative controls (not included) show no darkening of the protein granules or tracheoles. (Easton & Horwath, 1994.)

immunolocalization of Tm 12.86 in fat body from a winter-acclimated larva are consistent with this hypothesis. There is a general depletion in abundance of protein granules, and a correlated reduction in the intensity of the intracellular stores of Tm 12.86.

Use of an in vitro fat body culture system

By focusing at the cellular level, we have now identified another degree of complexity in regulation of hemolymph THP activity and levels, specifically, intracellular storage of THPs before secretion by the fat body, thus challenging the premise that hemolymph levels of THPs reflect THP production. Therefore, any explanation of environmental and hormonal mechanisms influencing the seasonal pattern of thermal hysteresis, will have to consider their impact on biosynthesis, storage, mobilization from storage, and secretion. To accomplish this an in vitro system is essential. Our laboratory has developed such a system (Easton & Horwath, 1994, 1996). As shown in Fig. 3A, fat body primary cultures from *T. molitor* display strong positive immunoreactivity for Tm 12.86, with the brightest fluorescence associated with discrete granules and small vesicles, much like the situation described in vivo. Also, although thermal hysteresis activity in the culture media is not usually observed among newly established (< 10 day old cultures), at 30 days after plating cultures routinely show detectable low level activity, with even higher activity in longer term cultures (2–7 months) (Easton & Horwath, 1996). This suggests that the cells in vitro may be constitutively synthesizing some THPs without hormonal induction.

To examine de novo biosynthesis of Tm 12.86 in vitro, we have begun pulse-labeling experiments (Fig. 3B). Primary cultures were exposed during a 4 h interval to ^{35}S-methionine in their culture media. Therefore, any proteins synthesized during this 4 h would incorporate label and be detectable on X-ray film (Fig 3B lane AR). Results show numerous proteins being synthesized by these cells in vitro, most notably the high molecular weight bands consistent with those of storage proteins. To concurrently track the presence of accumulated Tm 12.86 in these same cells western analysis was also conducted. A modest radioactive band (lane AR) was then identified which co-migrates with the band identified as Tm 12.86 through western analysis (lane WB). From this study we cannot yet state definitively that the co-migrating, radioactive band is Tm 12.86. However, it illustrates the first step in a progression of experiments that can directly probe biosynthesis of Tm 12.86 in vitro, its secretion, and the nature of hormonal and environmental control of a specific THP.

Many of these studies can also be coupled with an examination of thermal hysteresis activity in vitro to assess any concurrent impact. However, one limitation is that the lowest limits of detectability for thermal hysteresis due to Tm 12.86 is 0.125 mg/ml (10^{-5}M) (Fig. 1). Given the relatively small numbers of cells in an in vitro system compared to in vivo, it would be useful to explore a potentially more sensitive assay for antifreeze activity, i.e. the ability of Tm 12.86 to cause RI. We have therefore conducted a detailed analysis of RI behavior of purified Tm 12.86, as described in Myers (1996), with a representative example shown here in Fig. 4 (solid line). These results confirm that Tm 12.86 significantly inhibits ice crystal growth of frozen solutions, producing a statistically significant RI effect, and in a concentration-dependent fashion with lower limits of detectability at 1.0 mg/ml (10^{-7}M) (Myers, 1996). Thus, detection of RI effect of Tm 12.86 is 100 fold more sensitive than screening for thermal hysteresis. We also detailed the development of an "RI" activity factor for samples of unknown concentrations of THPs (e.g. dotted & dashed lines in

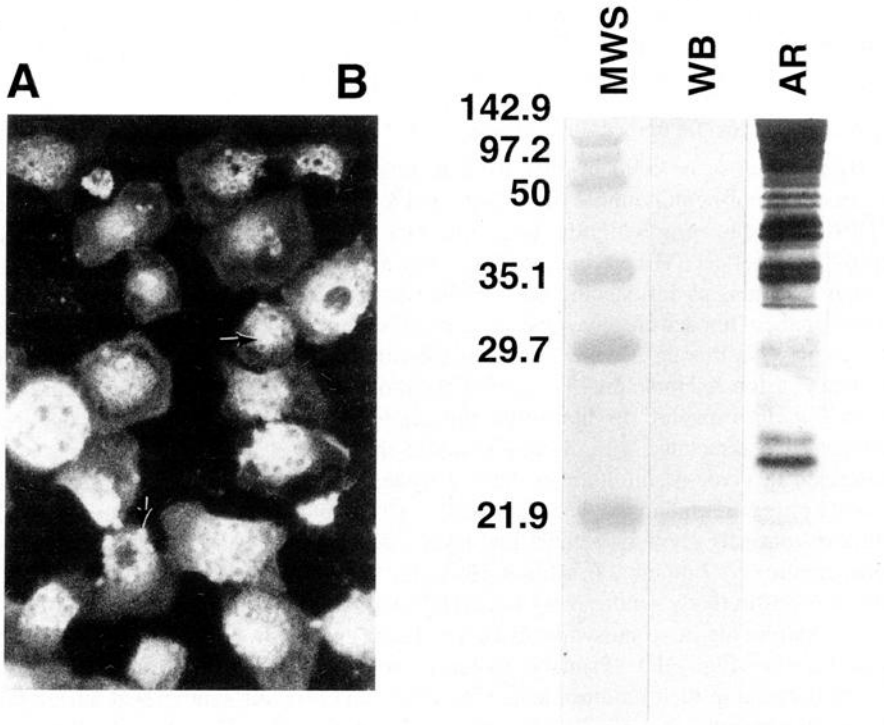

Fig. 3. Immunolocalization of Tm 12.86 in fat body primary cultures of *T. molitor*. (A) immunofluorescence for Tm 12.86 of fat body cells (derived from a prepupa). Fluorescence is strongest in granules and smaller vesicles (arrows). Adapted from Easton & Horwath (1994). (B) To examine biosynthesis of Tm 12.86 in vitro, primary cell cultures were harvested following pulse-chase experiments with labeled ^{35}S-methionine. The samples were evaluated by Western blot (lane WB) of 15% SDS-PAGE. The blot was then exposed to X-ray film for autoradiography (lane AR). Note that Tm 12.86 as detected in lane WB reflects an accumulation of Tm 12.86 by the cells in culture. Lane AR (indicative of biosynthesis during the 4 h pulse time) shows a modest band that co-migrates with Tm 12.86 seen in lane WR. Pre-stained molecular weight standards (lane MWS) were co-electrophoresed to confirm successful blotting, but are not accurate for molecular weight determination.

Fig. 4) that are based on the sample's dilution profile (see Fig. 4 legend for further explanation). This approach makes available a quantitative means of comparing RI activities of different samples, in an analogous fashion to comparing thermal hysteresis of different samples. Additionally, this RI assay is ideally suited to detecting very low antifreeze activity in our cell culture system.

Other contributions to thermal hysteresis

Perhaps one of the most interesting new concepts in the study of insect antifreeze proteins comes from the recent work of Duman and coworkers (Wu & Duman, 1991; Wu et al., 1991b; Duman et al., 1993). Their studies suggest that there are proteinaceous factors which can enhance thermal hysteresis activity of purified THP from *D. canadensis*. This

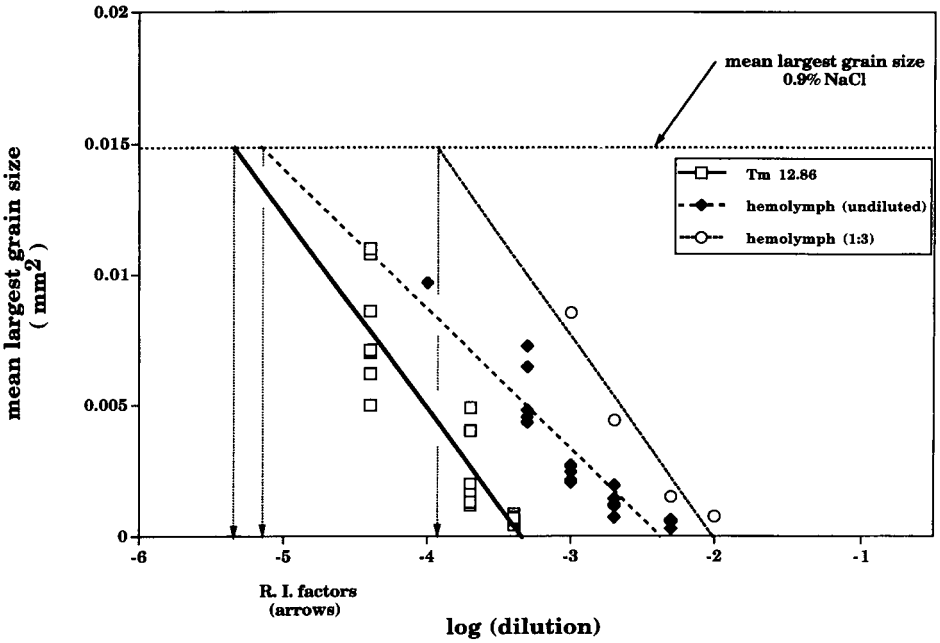

Fig. 4. Recrystallization inhibition activity of purified Tm 12.86, and of hemolymph obtained from winter-acclimated *T. molitor* larvae. Samples were subject to a series of dilutions and then tested for their ability to inhibit ice crystal grain growth (annealing time 30 min at –6°C). Pure saline shows a significant increase in grain size under these conditions (dashed horizontal line). In contrast, purified Tm 12.86 (25 mg/ml) (solid line, open squares) requires substantial dilution before grain size reaches that of saline. This purified sample displays an "RI factor", the absolute value of the dilution at which the sample loses observable RI activity, of 5.3 (left most arrow). Unknown samples with greater initial levels of THPs will display larger RI factors than samples with less THPs. This is illustrated by regression profiles from undiluted hemolymph (larger dashed line, solid triangles) in comparison to those from diluted hemolymph (smaller dashed line, open circles), and by their respective RI factors (5.1 middle arrow; 3.9 right most arrow). Moreover, this method of evaluation also allows for differences in potency between types of THPs or in the presence of activating factors, to be reflective in the steepness of the slopes, and thus, the RI factors. Adapted from Myers (1996).

was first shown with the addition of a rabbit polyclonal antibody to *D. canadensis* THP (Wu et al., 1991b). Rather than titrating out antifreeze activity, there was a substantial increase. Their proposed explanation, consistent with findings for the activity of fish AFPs differing in size (DeVries, 1984), is that the large additional molecular weight of the antigen-antibody complex protects a larger surface area of the seed ice crystal from the onslaught of liquid water molecules, resulting in an increase in thermal hysteresis (Wu et al., 1991b). This sparked the search for and led to the purification of a 70 kDa endogenous activator protein in *D. canadensis* (Wu & Duman, 1991). Some other proteins, particularly ice nucleating proteins from other species, all having large molecular weights, have also been found to display activator activity (Wu & Duman, 1991; Duman et al., 1993).

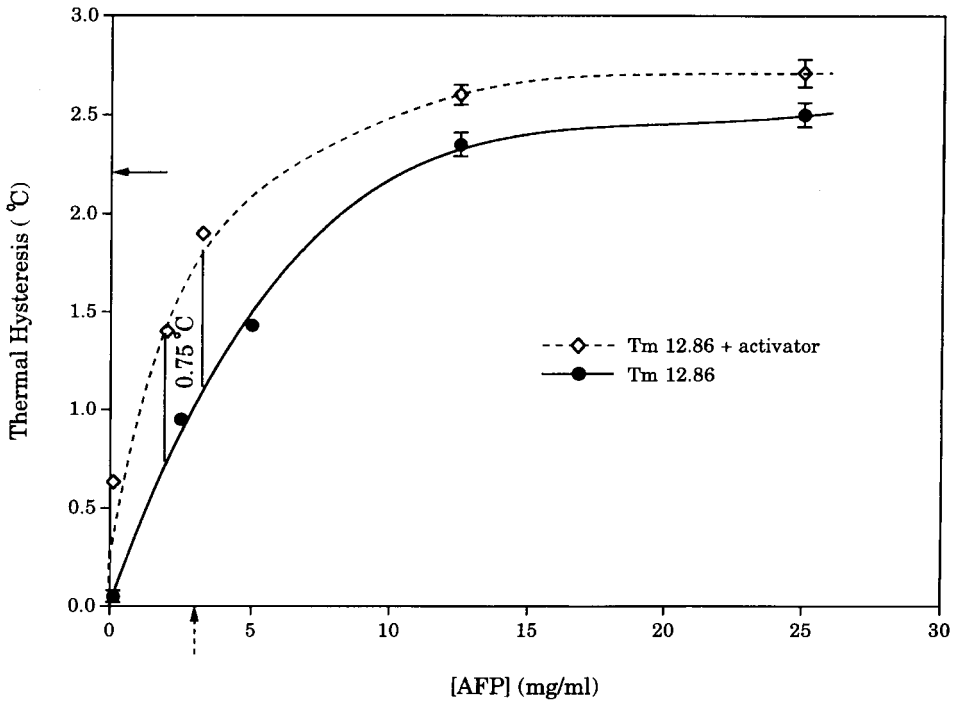

Fig 5. Thermal hysteresis activity curve showing the enhancement of activity to Tm 12.86 by addition of an activating factor (at 12.5 mg/ml). Note that the highest degree of enhancement (0.75°C) occurs around the physiological range of Tm 12.86 (bottom arrow). Top arrow indicates the average thermal hysteresis of hemolymph from winter-acclimated larvae. Adapted from Poggioli (1996).

Until now, endogenous activating factor(s) have not been documented for any other thermal hysteresis producing insect. Recently, we found that *T. molitor* produces an endogenous activating factor with respect to Tm 12.86. (Poggioli, 1996). This activator has been partially purified and the details of its elution profile and characterization are described in Poggioli (1996). The *T. molitor* activator displays no thermal hysteresis activity by itself, but is capable of significantly augmenting thermal hysteresis of Tm 12.86. Interestingly, in comparison to previously described activators (Wu & Duman, 1991), that of *T. molitor* is unusual in that it appears to be very small (smaller than Tm 12.86). Activation of Tm 12.86 by this activator occurs over all antifreeze protein concentrations (Fig. 5). At the lower level of antifreeze protein detectability (0.05°C of thermal hysteresis at 0.01 mM of Tm 12.86) activation is nearly 10 fold (0.6°C of thermal hysteresis). Furthermore, activation is most pronounced at non-saturated concentrations of antifreeze protein (0.125–12.5 mg/ml of Tm 12.86) and reaches a maximum level around 2–3 mg/ml of antifreeze protein concentration, i.e. at the endogenous hemolymph concentration of Tm 12.86 in winter-acclimated larvae. Therefore, Tm 12.86 at physiologically relevant concentrations, together with its activator, account for nearly 75% of total hemolymph thermal hysteresis (Fig. 5). So despite the presence of multiple antifreeze protein species in *T. molitor*

(Table 2), Tm 12.86 and its activator are clearly playing a dominant role in influencing thermal hysteresis levels. Such antifreeze protein – activator interaction suggests that by precisely regulating Tm 12.86 and/or the activator, *T. molitor* can generate an efficient cold hardy response.

Clearly, these studies challenge the premise that thermal hysteresis activity mostly reflects THP levels. Thermal hysteresis, indicative of total antifreeze protein activity, is dependent upon the different contributions and interactions of Tm 12.86 and its activator, other THPs (and potentially other activators), even simple solutes like salts and polyols, or pH, which also show some thermal hysteresis enhancement effects (Duman et al., 1993).

Likewise, recrystallization inhibition associated with antifreeze protein activity presumably would also be dependent upon those same factors contributing to thermal hysteresis. Consistent with this hypothesis are the studies described in Fig. 4 regarding RI activity of undiluted verses diluted hemolymph from winter larvae. Full strength hemolymph displays an RI factor rather close to that of purified Tm 12.86 (at 25 mg/ml). Given that winter hemolymph contains about 3 mg/ml Tm 12.86, this would not be sufficient by itself (i.e. without activator or other THPs) to account for the high RI factor described. In fact, it is probably the presence of the activator in full strength hemolymph that effectively enhances the potency of antifreeze protein activity, as reflected by a lesser slope. However, the diluted hemolymph sample shows the reversed trend, perhaps due to sufficient dilution of the activator.

Certainly, the concept of an activator raises some interesting questions regarding structure-function mechanisms of antifreeze protein-activator interaction. And, given the large disparity in activator size of *T. molitor* and *D. canadensis*, their mode of action may differ. Mostly however, the existence of specific activators adds a whole new dimension to any explanation of regulatory mechanisms influencing the seasonal pattern of thermal hysteresis.

CONCLUSION

Given the recent development and availability of key biochemical and cellular tools, it is now possible to probe in a much more direct fashion, the various physiological and cellular mechanisms contributing to the involvement of antifreeze proteins in the overwintering response of insects. As such, it has become clear that the nature of this cold hardy response is quite sophisticated. Thermal hysteresis activity is the product of a combination of factors and their interactions (i.e. specific THPs, multiple THPs, activators). Moreover, tracking of a major component (Tm 12.86) has revealed that hemolymph levels reflect complex cellular regulation of it's biosynthesis, storage, mobilization and secretion; any of which may experience seasonal control from environmental and endocrine triggers. Presumably, these regulatory mechanisms of Tm 12.86 are then coordinated in some fashion with the regulation of the activator, for which no information is yet available, to generate a very efficient cold hardening response. Clearly, the insect's adaptational mechanisms contributing to the precise regulation of THP-activator interaction, and the impact of environmental and hormonal control, provide tremendous opportunities for further study.

ACKNOWLEDGMENTS. We thank Y. Zhou and M. Bang for some technical assistance. This work was supported by NSF No. IBN-9207044 to KLH and from HHMI Undergraduate Biological Sciences Education Program to Binghamton University.

REFERENCES

CONNAT J.L., DELBECQUE J.P. & DELACHAMBRE J. 1984: The onset of metamorphosis in Tenebrio molitor L: Effects of a juvenile hormone analogue and of 20-hydroxyecdysone. *J. Insect Physiol.* **30**: 413–419.

CONNAT J.L., DELBECQUE J.P., GLITHO I. & DELACHAMBRE J. 1991: The onset of metamorphosis in Tenebrio molitor larvae (Insecta, Coleoptera) under grouped, isolated and starved conditions. *J. Insect Physiol.* **37**: 653–662.

DEVRIES A.L. 1984: Role of glycopeptides and peptides in inhibition of crystallization of water in polar fishes. *Phil. Trans. R. Soc. Lond. (B)* **304**: 575–588.

DUMAN J.G. 1979: Thermal-hysteresis-factors in overwintering insects. *J. Insect Physiol.* **25**: 805–810.

DUMAN J.G. & HORWATH K.L. 1983: The role of hemolymph proteins in the cold tolerance of insects. *Annu. Rev. Physiol.* **46**: 261–270.

DUMAN J.G., HORWATH K.L., TOMCHANEY A. & PATTERSON J.L. 1982: Antifreezes of terrestrial arthropods. *Comp. Biochem. Physiol. (A)* **73**: 543–555.

DUMAN J.G., XU L., NEVEN L.G., TURSMAN D. & WU D.W. 1991: Hemolymph proteins involved in insect subzero-temperature tolerance, ice nucleators and antifreeze proteins. In Lee R.E. & Denlinger D.L. (eds): *Insects at Low Temperature*. Chapman and Hall, New York, pp. 94–127.

DUMAN J.G., WU D.W., OLSEN T.M., URRUTIA M. & TURSMAN D. 1993: Thermal-hysteresis proteins. *Adv. Low-Temp. Biol.* **2**: 131–182.

EASTON C.M. & HORWATH K.L. 1994: Characterization of primary cell cultures derived from fat body of the beetle, Tenebrio molitor, and the immunolocalization of the thermal hysteresis protein in vitro. *J. Insect Physiol.* **40**: 537–547.

EASTON C.M. & HORWATH K.L. 1996: Establishment of long term primary cell cultures derived from the fat body of Tenebrio molitor L. (Coleoptera: Tenebrionidae). *Comp Biochem. Physiol (B)* (in press).

HORWATH K.L. & DUMAN J.G. 1982: Involvement of the circadian system in the photoperiodic regulation of insect antifreeze proteins. *J. Exp. Zool.* **219**: 267–270.

HORWATH K.L. & DUMAN J.G. 1983a: Preparatory adaptations for winter survival in the cold hardy beetles, Dendroides canadensis and Dendroides concolor. *J. Comp. Physiol.* **131**: 225–232.

HORWATH K.L. & DUMAN J.G. 1983b: Photoperiodic and thermal regulation of antifreeze protein levels in the beetle Dendroides canadensis. *J. Insect Physiol.* **29**: 907–917.

HORWATH K.L. & DUMAN J.G. 1983c: Induction of antifreeze protein production by juvenile hormone in larvae of the cold hardy beetle Dendroides canadensis. *J. Comp. Physiol.* **151**: 233–240.

HORWATH K.L. & DUMAN J.G. 1984: Further studies on the involvement of the circadian system in photoperiodic control of antifreeze protein production in the cold hardy beetle Dendroides canadensis. *J. Insect Physiol.* **30**: 947–955.

HORWATH K.L. & DUMAN J.G. 1986: Thermoperiodic involvement in antifreeze protein production in the cold hardy beetle Dendroides canadensis: Implications for photoperiodic time measurement. *J. Insect Physiol.* **32**: 799–806.

HORWATH K.L. & RIDDIFORD L.M. 1988: Stage and segment specificity of the secretory cell of the dermal glands of the tobacco hornworm, Manduca sexta. *Dev. Biol.* **130**: 365–373.

KNIGHT C.A., DEVRIES A.L. & OOLMAN L.D. 1984: Fish antifreeze protein and the freezing and recrystallization of ice. *Nature* **308**: 295–296.

KNIGHT C.A., HALLETT J. & DEVRIES A.L. 1988: Solute effects on ice recrystallization; as assessment technique. *Cryobiology* **2**: 55–60.

KNIGHT C.A., WEN D. & LAURSEN R.A. 1995: Nonequilibrium antifreeze peptides and the recrystallization of ice. *Cryobiology* **32**: 23–34.

MYERS K. 1996: Recrystallization inhibition behavior of an insect thermal hysteresis protein. Master Thesis, University of Binghamton.

PATTERSON J.L. & DUMAN J.G. 1978: The role of the thermal hysteresis factor in Tenebrio molitor larvae. *J. Exp. Biol.* **74**: 37–45.

PATTERSON J.L. & DUMAN J.G. 1979: Composition of a protein antifreeze from larvae of the beetle, Tenebrio molitor. *J. Exp. Zool.* **210**: 361–367.

PATTERSON J.L. & DUMAN J.G. 1982: Purification and composition of protein antifreezes with high cysteine contents from larvae of the beetle, Tenebrio molitor. *J. Exp. Zool.* **219**: 381–384.

Poggioli G.J. 1996: Characterization of an antifreeze protein and its associated activator from Tenebrio molitor. Honors Thesis, University of Binghamton.

Ramsay J.A. 1964: The rectal complex of the mealworm Tenebrio molitor L. (Coleoptera, Tenebrionidae). *Phil. Trans. R. Soc. Lond. (B)* **248**: 279–314.

Ramsay J.A. & Brown R.A. 1955: Simplified apparatus and procedure for freezing point determinations upon small volumes of fluid. *J. Sci. Instrum.* **32**: 372–375.

Raymond J.A. & DeVries A.L. 1977: Adsorption inhibition as a mechanism of freezing resistance in polar fishes. *Proc. Natl. Acad. Sci. USA* **74**: 2589–2593.

Raymond J.A., Wilson P. & DeVries A.L. 1989: Inhibition of growth of nonbasal planes in ice by fish antifreezes. *Proc. Natl. Acad. Sci. USA* **86**: 881–886.

Schneppenheim R. & Theede H. 1980: Isolation and characterization of freezing point depression peptides from larvae of Tenebrio molitor. *Comp. Biochem. Physiol (B)* **67**: 561–568.

Tang W. 1994: *Biochemical and Molecular Biological Studies of Antifreeze Proteins from the Insect Tenebrio molitor.* Ph.D. Dissertation, Department of Biological Sciences, Binghamton University, New York, 45 pp.

Tomchaney A.L., Morris J.P., Kang S.H. & Duman J.G. 1982: Purification, composition, and physical properties of a thermal hysteresis "antifreeze" protein from larvae of the beetle, Tenebrio molitor. *Biochemistry* **21**: 716–721.

Towbin H., Staehelin T. & Gordon J. 1979: Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: prcedures and some applications. *Proc. Natl. Acad. Sci. USA* **76**: 43–50.

Xu L. & Duman J.G. 1991: Involvement of juvenile hormone in the induction of antifreeze protein production by the fat body of larvae of the beetle Dendroides canadensis. *J. Exp. Zool.* **258**: 288–293.

Xu L., Duman J.G., Goodman W.G. & Wu D.W. 1992: A role for juvenile hormone in the induction of antifreeze protein production by the fat body in the beetle Tenebrio molitor. *Comp. Biochem. Physiol. (B)* **101**: 105–109.

Wu D.W. & Duman J.G. 1991: Amplification of insect antifreeze protein from the beetle Dendroides canadensis. *J. Comp. Physiol. (B)* **161**: 279–283.

Wu D.W., Duman J.G., Cheng C. & Castellino F.J. 1991a: Purification and characterization of antifreeze protein from larvae of the beetle Dendroides canadensis. *J. Comp. Physiol. (B)* **161**: 271–278.

Wu D.W., Duman J.G. & Xu L. 1991b: Enhancement of insect antifreeze protein activity by antibodies. *Biochem. Biophys. Acta* **1076**: 416–420.

Zachariassen K.E. 1985: Physiology of cold tolerance in insects. *Physiol. Rev.* **64**: 799–832.