

CHANGES IN PHOSPHOLIPID CLASS COMPOSITION WITH THE ONSET OF FREEZE TOLERANCE IN THE GOLDENROD GALL FLY, *EUROSTA SOLIDAGINIS*

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Abstract

Eurosta solidaginis is a unique insect that has developed the ability to withstand extra-cellular freezing. For this reason *E. solidaginis* has become a model organism for researching the freeze tolerance adaptations. The research presented in this paper considers membrane adaptations for freeze tolerance by looking closely at the changing phospholipid composition of the gall fly's membrane during cold acclimation and subsequent freeze tolerance. Phospholipids were extracted and separated from third instar larvae of the goldenrod gall fly. These phospholipids were quantified using methods of color development and UV spectrophotometry. It was found that there is a trend in the proportional phospholipid compositions based largely on headgroup shape. It is hypothesized that the increase in cylindrically shaped phospholipids headgroups such as PC in relation to the decrease in conically shaped phospholipids headgroups such as PE is an adaptation of *E. solidaginis* that is affective in preventing both lyotropic and thermotropic phase changes.

Introduction:

Many insects have developed various molecular and biochemical mechanisms that make it possible for them to withstand low temperatures (Lee and Denlinger 1991). The gall fly *E. solidaginis* has been a model for studies of freeze tolerance (Storey and Storey 1988). Adults emerge in the spring months and mate. Eggs are laid on the stem of the golden rod plant and the larvae live within the plant causing the plant to form a gall (giving the insect its common name). The larvae reach the third instar developmental stage in autumn and overwinter in this larval stage (Lee et al. 1992). In the months of September and October, environmental changes that occur during the transition from autumn to winter cues the larvae to acquire freeze tolerance (Morrissey and Baust 1976). These larvae have been studied closely in the third-instar stage as a model organism, due to the fact that this organism is easy to use in the lab, abundant in many environments and can withstand tissue freezing.

Most organisms that are exposed to cold temperatures have adapted in ways to tolerate these temperatures, but few are able to survive freezing within their body tissues. The ability to tolerate freezing is an adaptation that allows an organism to persist during cold winter months without the need to enlist other behavioral or biological methods of freeze avoidance, for example migration and supercooling (Lee 1989). In plants (like insects) cold acclimation can take several weeks and is often reliant upon the dropping

temperatures and photoperiod changes that are associated with the onset of winter (Steponkus et al. 1990). For insects, freeze avoidance is considered to be less favorable than developing the means to withstand freezing because there is a considerable amount of energy loss associated with freeze avoidance (Layne and Kuharsky 2000). Therefore the relatively few insect species that have developed methods of freeze tolerance have an advantage over those who undergo methods of freeze avoidance during cold winter months.

There are many cellular problems that are associated with ice formation. Tissue freezing is controlled by allowing ice to form in an organism's extracellular fluids. Ice-nucleating agents that are produced in freeze tolerant species help control ice formation within exposed tissues (Lee 1989). The most obvious obstacle that needs to be overcome is associated with the osmotic changes due to ice formation. When the water in an organism's tissues becomes ice, the extra-cellular fluid becomes more concentrated (Lee 1989). This concentration of solutes in the extra-cellular space creates an osmotic gradient across the cell membrane that causes cell shrinking. In unacclimated rye protoplasts of freeze tolerant species, this osmotic change causes a loss of membrane material due to endocytotic vesiculation (Steponkus et al. 1990). Cold acclimated protoplasts prevent this plasma membrane loss by forming finger like projections in order to satisfy the need for the cell to contract and maintain the membrane materials available for expansion during thawing (Steponkus et al. 1990).

Studies have shown that most of the freeze-thaw damage is caused by freeze induced desiccation (Steponkus and Webb 1992). As water is removed from the extracellular space the plasma membrane loses the stability that water provides the lipid-bilayer structure. This desiccation produces a breakdown of the plasma membrane's properties by ultimately lowering the lamellar-to-hexagonal phase transition temperature (Webb et al. 1994). These changes in the properties of the plasma membrane ultimately lead to the destructive lyotropic phase transitions. This phase transition occurs when water is removed from the lipid bilayer structure and causes the normal lipid bilayer of the plasma membrane to form a hexagonal-HII structure. The membrane folding in upon itself is directly related to the loss of water that helps maintain the membrane structure. It follows that as water levels decrease, the space occupied by fatty acids has to decrease to prevent HII. Since, saturated fatty acids occupy less space, an increased level of saturated fatty acids would be a beneficial adaptation. Furthermore, an increase in PC and other cylindrical headgroups would be expected as they do not require water to maintain a stable membrane structure (Uemura and Steponkus, 1994).

Cellular membranes also experience a change in dynamic properties due solely to an inherent decrease in temperature the organism is subjected to during cold winter months. A thermotropic phase transition occurs when the plasma membrane is exposed to decreased temperatures. With decreasing temperatures, the cell membranes become less fluid and enter into a stiff gel-like state. In this gel state the plasma membrane can not maintain fluidity and consequently any stressors break apart the membrane. In order to avoid a thermotropic phase transition, the membrane must introduce further unsaturation, ultimately aiding in maintaining the many dynamic properties of cellular membranes (Sinensky 1974). It has been found that many insects studied during cold acclimation have adapted by increasing the unsaturated fatty acid to saturated fatty acid ratio during cold acclimation (Bennett and Lee 1997). Unlike the lyotropic phase

transition, an increase in the conically shaped headgroup of PE would be favored during cold acclimation in order to avoid negative effects of the thermotropic phase transition (Uemura and Steponkus, 1994).

It is the goal of this research to resolve the fatty acid paradox that predicts an increase in saturated fatty acids which combats the lyotropic phase change in combination with a predicted increase in unsaturated fatty acid which helps keep the cell's fluidity and avoids a thermotropic phase change. In order to solve the paradox phospholipid head groups will be considered individually within the freeze tolerant model *E. Solidaginis*. By analyzing individual head groups with emphasis on the two most prevalent phospholipids head groups, phosphotidyl choline (PC) and phosphotidyl ethanolamine (PE), we predict that PC and PE will undergo saturation processes separately and will therefore change in proportion to total phospholipids throughout the cold acclimation and freeze tolerant process.

Materials and Methods:

Galls containing third instar larvae of *Eurosta solidaginis* were collected from golden rod plants in Madison County, NY on six dates in the fall of 2002. Galls collected on dates 8/30/02 and 9/12/02 were considered freeze susceptible. Collections from 9/22/02 and 10/4/02 were considered transitional while collections from 10/25/02 and 11/8/02 were considered freeze tolerant. Ten samples of ten larvae each were collected on each date.

Using a glass homogenizer and a solution of 2 mL of 100mM TrisCl buffered at pH 7, lipids were extracted from each sample on the date of collection by the method described by Bligh and Dyer (1959) including modifications made by Garbus et al. (1963). The samples were bubbled with N₂ and stored at -20 °C. Four samples containing extracted lipids from each collection date were used for to analyze phospholipids head group composition (six samples were set aside for later experimentation).

Thin layer chromatography (TLC) in a solvent system of hexane, diethyl ether and formic acid (ration 80:20:2) was performed as described by Christie (1982) to separate phospholipids from neutral lipids. Silica containing phospholipids were scraped from the site of origin and stored in 3 mL of a 2:1 solution of chloroform to methanol, bubbled with N₂ and stored at -20 °C.

Samples of phospholipids were transferred to a conical flask carefully avoiding any silica residues and then concentrated as before under a constant stream of N₂ and redissolved in 50 µL of a 2:1 solution of chloroform to methanol. This redissolved solution of phospholipids was then further separated and run against standards of phosphotidyl ethanolamine (PE) and phosphotidyl choline (PC) using the TLC methods described by Fine and Sprecher (1982). Briefly, Boric acid-impregnated plates were run in a solvent system of chloroform, methanol, H₂O and ammonium hydroxide (60, 32.5, 3 and 1 ml respectively). The different classes of lipids were visualized in I₂ vapor and scraped into perchloric acid. A quantitative lipid phosphorus assay was performed on the samples using Rouser et al.'s described methods (1970).

Absorbancies were obtained at 820 nm on a DU 640 spectrophotometer following color development described above (Beckman Dickson, Inc.). A one way analysis of variance (ANOVA) statistical test was used to determine significance between collection dates and headgroups.

Results:

With the onset of cold acclimation and transition to freeze-tolerance, a general trend for phospholipids headgroups in *Eurosta solidaginis* was observed. The proportion of PE to total phospholipids briefly increases until the transitional stage of cold acclimation begins; subsequently a decrease in percent composition occurs until the date at which half of the larvae have become freeze tolerant. Once freeze tolerance is achieved, the proportion of PE begins to rise to nearly the original proportion, at which PE represents 47.8% of the total phospholipids composition (Figure 1). Although there appeared to be differences between collection dates an analysis of variance test (ANOVA) did not show significance ($P = 0.1213$).

The opposite is true for the PC observed trend. The proportion of PC to total phospholipids is initially lower in comparison to PE. PC briefly decreases until the transitional stage begins followed by an increase until the point when half of the larvae have become freeze tolerant. The proportion of PE and PC at this critical date, October 4th in the case of this data, are nearly identical (0.4282 versus 0.4255 respectively). Once freeze tolerance is achieved the proportion of PC returns to nearly the original proportion observed on August 30th. An ANOVA test showed significance for the PC change across collection dates ($P = 0.0057$).

Figure 2 presents the changes in CL, PS and PI. These phospholipids were grouped with PE and PC on the basis of headgroup shape (conical: PE, CL, PI and cylindrical: PC, PI). Further comparison of the data between all conical and cylindrical shaped head group (Figure 3) found that the conical shaped head groups, as a whole, undergoes a similar trend to that of PE previously seen (Figure 1). The same is true for all cylindrically shaped headgroup phospholipids, which have a similar trend to that of PC. A test of ANOVA showed significance for both PC and PE change across collection dates (both $P = 0.0069$).

Discussion:

Based upon the results of this research, the phospholipids headgroups PE and PC undergo specific changes in the proportion of major phospholipids related to each headgroup as *E. solidaginis* undergoes cold acclimation. There is a clear trend represented by the data collected in this research (shown in figures 1 and 3). These figures suggest a change in PE that is oppositely mirrored by that of PC. The nearly opposite but equal changes in PC and PE represent adaptations in *E. solidaginis* that offset both the lyotropic and the thermotropic phase transitions. Unlike the findings of Uemura and Steponkus (1994) this research indicates that the proportions of PE actually

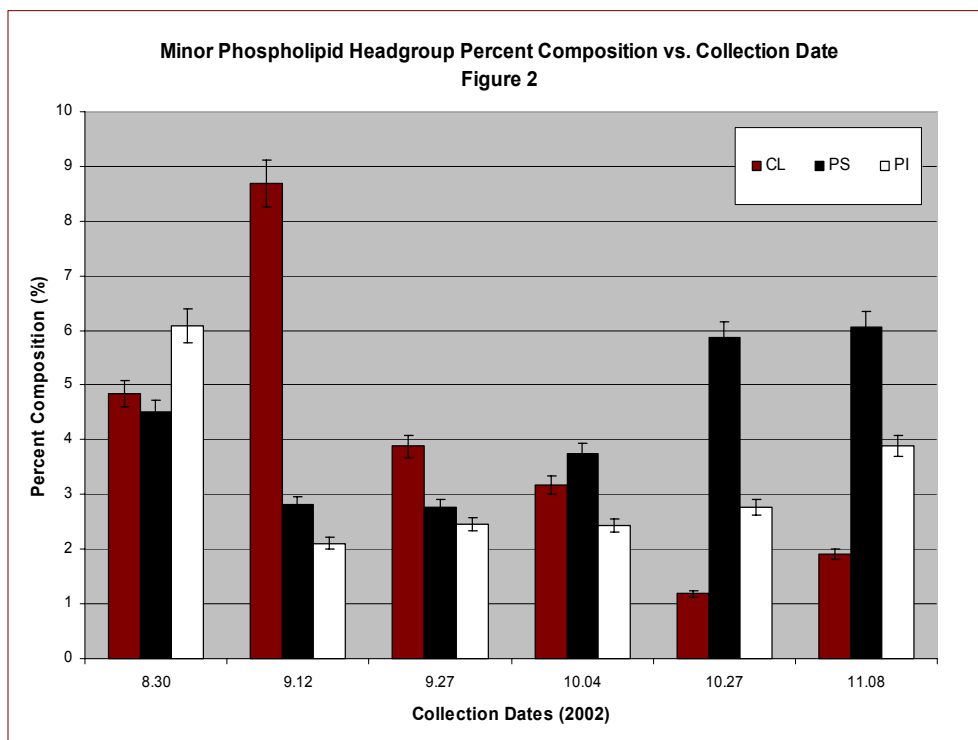
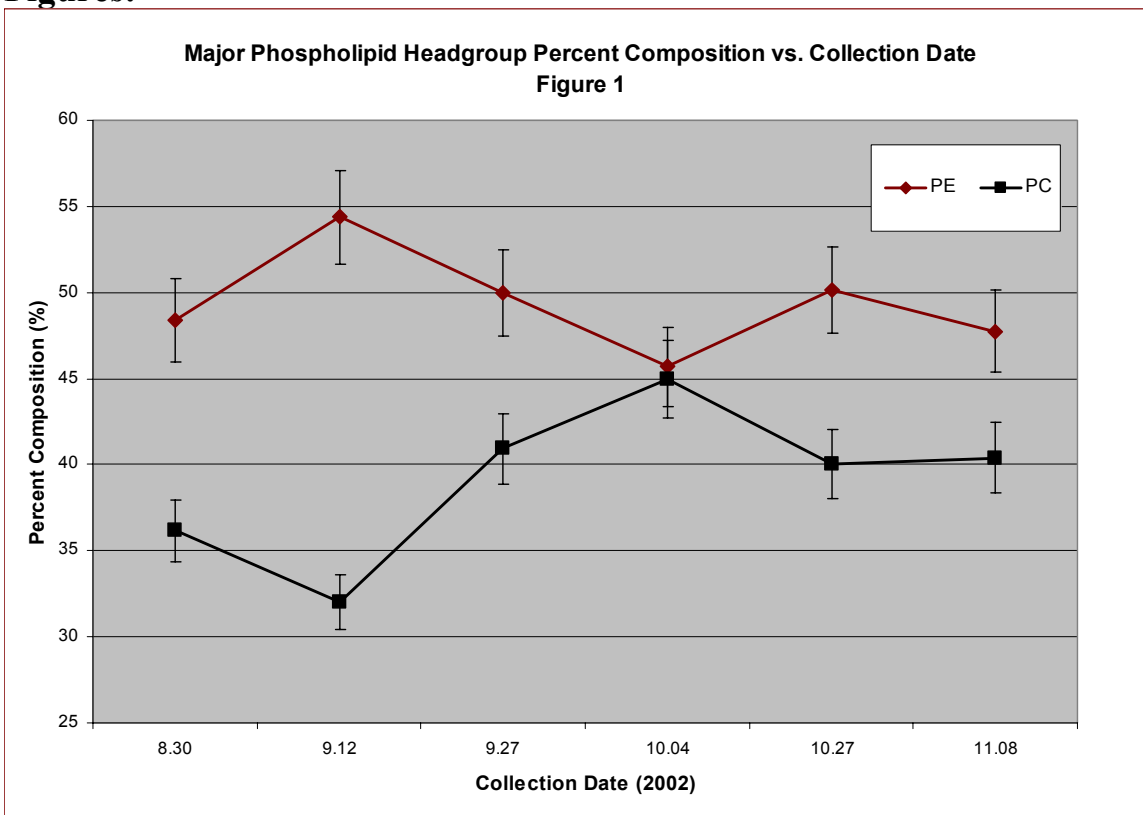
decrease as freeze tolerance is achieved. It is not until after the critical date of October 4th do levels of PE begin to rise again.

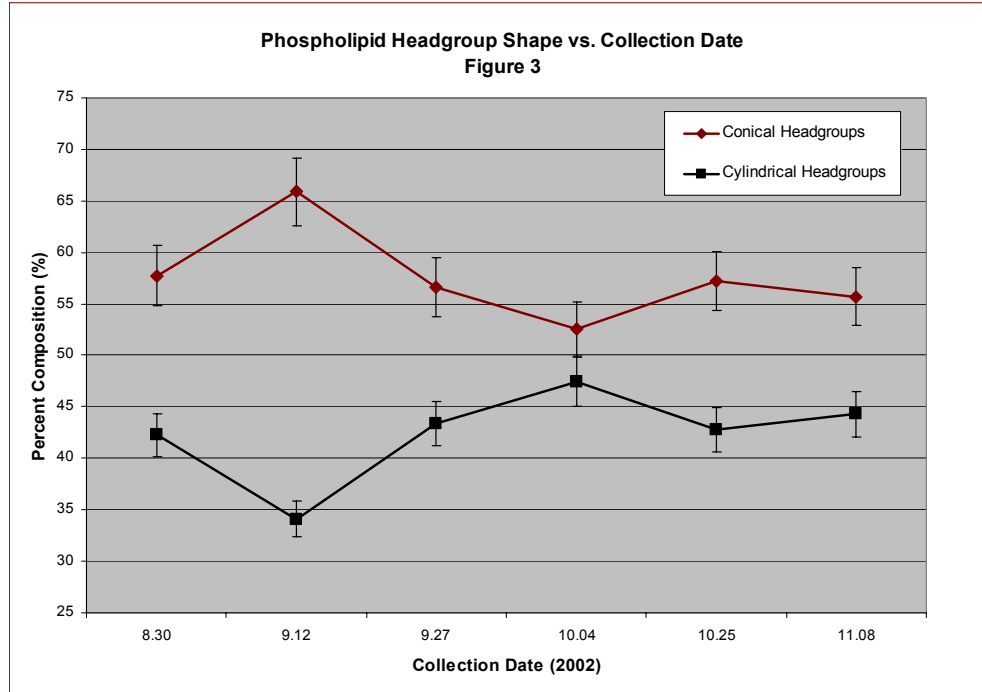
The first week in October is a critical time in *E. solidaginis*'s cold acclimation process. Not only does this time mark the point at which half of the larvae are found to be freeze tolerant, but it is also when production of cryoprotectant glycerols reach a maximum (Storey 1983). It is this increase in cryoprotectant glycerols that is hypothesized to be responsible for the trends this research demonstrated in PE and PC. Jensen et al. (2001) have shown that glycerol and water can compete for hydrogen bonding within the membrane structure. Once levels of glycerol production reach a peak in the first week of October, membrane stabilization can be achieved by the insertion of these small molecular weight sugars in the place of water. Due to the increased levels of stabilizing glycerols, levels of PE and PC are able to approach the proportions observed when there is no ice present in the organism.

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Figures:





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