SUPERCOOLING PHENOMENON AND WATER CONTENT INDEPENDENCE IN THE OVERWINTERING BEETLE, *COLEOMEGILLA MACULATA*

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Abstract—Insect supercooling phenomena observed during overwintering have been coupled with the seasonality of water and nucleator content and feeding behaviour. However, the strength of the conclusion has in most cases rested on presumption and model systems since nucleator content is not readily quantifiable. The ladybird, *Coleomegilla maculata* is an overwintering adult insect that fasts for weeks before hibernation, does not accumulate identifiable cryoprotectants during cold exposures, and is freezing intolerant. This species would be expected to overwinter with a constancy of ice nucleators subject to relative increases due to winter dehydration and decreases in supercooling ranges.

However, acclimatization and laboratory acclimation experiments have demonstrated both temporal and temperature independence of water content while supercooling levels varied substantially. Supercooling ranges were narrow during warm exposures with a unimodal peak at $-6.3°C$ (± 0.2 S.E.). Progressive temperature reductions yielded phasic shifts to multimodal ranges until a low temperature peak of $-18.4°C$ (± 0.5 S.E.) was attained. Induced feedings of controlled nucleator substances resulted in predictable supercooling variations while 'nucleator free' diets yielded results consistent with natural populations.

INTRODUCTION

SUPERCOOLING is defined as the extension of the liquid phase state below the *ideal* freezing point of a solution (liquid medium) and is of biological significance in determining the range and limits of an organism's motor activities and/or survival during subfreezing exposures (BAUST and MILLER, 1970). Supercooling points (SC_p) are a measure of the freezing ectotherm (latent heat of crystallization) position of a specimen on a controlled rate of cooling curve (SALT, 1966) and are of value in establishing the actual point at which freezing commences.

The seasonal extension of invertebrate supercooling ranges during overwintering is a commonly observed phenomenon (SØMME, 1964; ASAHINA, 1966; BAUST, 1972) generally considered due to random nucleation (SALT, 1970). Several factors affect nucleation temperatures thereby reducing the probabilistic nature and lending a degree of precision to predictions of a species SC_p. These factors include cooling rates, time, volume, biological activity, water content, and levels and types of cryoprotectants. However, an analysis of supercooling extensions, in particular insect populations, is often not feasible due to potentially multi-variable systems, aspects of which are not amenable to analysis (i.e. we cannot measure the types,

levels, and position of ice nucleating sites and compounds).

To date, studies of insect SC_p variations have led to the general conclusion that the reduction of SC_p is due to a diminution of nucleators in the gut and directly dependent on levels of hydration (SALT, 1968). Since dehydration has been observed to accompanying overwintering in all previous studies, therefore resulting in an elevation of SC_p to higher temperatures, the synthesis of cryoprotectants (i.e. glycerol and sorbitol) would appear necessary to buffer relative increased nucleator content. The strength of this conclusion is based on presumption and limited analysis of model systems since ice nucleator content is not readily quantifiable (SALT, 1970). The intuitive nature of this presumption is not questioned, but this study demonstrates that it is a generalization and possible oversimplification of natural systems.

MATERIALS AND METHODS

Coleomegilla maculata overwinter as non-feeding adults. During autumn aggregations, clusters may exceed 10^6 individuals while occupying a 30 cm^3 volume. Direct exposure to ambient temperatures is avoided from late autumn through early spring by maintaining a subterranean habitat. Adults were collected from various western New York hibernacula and maintained in suitable outdoor insectaries for acclimatization experiments or maintained in temperature regulated environmental chambers for acclimation experiments.

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Supercooling points were measured according to the method described by BAUST and MILLER (1970) while water content was determined after 24 hr of drying to a constant weight at 100°C. Water content was expressed as a percentage of weight change. Each specimen was analysed sequentially for both SC_p and per cent water loss. Attempts to identify and quantitate levels of cryoprotective compounds were made by procedures described by BAUST and MILLER (1970, 1972).

RESULTS

Throughout both outdoor acclimatization and laboratory acclimation studies, water content remained nearly constant while temperature exposures and SC_p ranges varied. Fig. 1 represents a
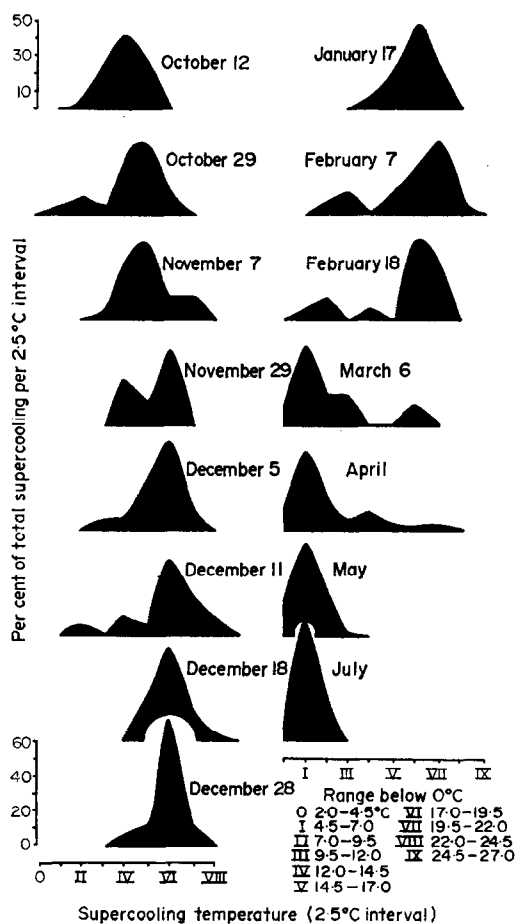

Fig. 1. Frequency distribution curves of supercooling (freezing) temperatures of C. *maculata* over a yearly cycle. Specimens were cooled at 1°C/min. Curves were smoothed by taking the running means of frequencies for each of five 0·5°C temperature intervals and correcting the midpoints of the tops of the histogram columns.

yearly distribution of freezing patterns. The summer transition to winter hardiness is accompanied by large-scale migrations to selected sites with a gradual reduction in freezing susceptibility. Summer populations demonstrate a narrow supercooling range indicative of high levels of ice nucleators (SALT, 1966, 1968). During autumn (September–October), fasting results in gut evacuation and an attendant reduction in nucleator levels. This is a period of transition from unimodal supercooling distribution to general bimodality. As winter progresses, a return to unimodality is observed but at lower temperature levels. Spring acclimatization is represented by a reversal of this trend.

Water content, however, unexpectedly maintains near constant levels within individual populations. C. *maculata* is known to attempt to maintain a constant humidity environment throughout its life cycle. This behavioural preference is reflected even in winter. Table 1 is a compilation of one

Table 1. Seasonal variations in mean supercooling points (SC_p) and water content of C. *maculata* *

Date	$\bar{x}\ SC_p$ (°C)	$\bar{x}$ (% H_2O)	Population†
12 October	−13·4±0·5	53·7±1·3	A‡
29 October	−13·9±0·7	50·3±1·9	A
7 November	−15·7±0·7	56·6±2·9	A
29 November	−16·7±0·6	56·8±1·0	A
5 December	−17·2±0·6	55·5±1·6	A
11 December	−17·9±0·8	55·0±0·8	A
18 December	−18·4±0·4	50·5±0·9	A
28 December	−18·1±0·4	53·9±2·3	A
17 January	−17·8±0·4	52·5±0·8	A
7 February	−17·0±1·1	62·3±2·5	B
18 February	−16·7±1·2	49·9±1·3	C
6 March	−8·5±1·0	59·3±1·6	D
April	−5·5±0·4	63·4	Mixed §
May	−6·0±0·2	62·7	Mixed
July	−6·3±0·2	53·2±1·6	E

* Values ± S.E.M.
† Population letters represent results data from separate hibernacula.
‡ Correlation coefficient for group A = 0·07.
§ Mixed represents a single monthly mean for non-overwintering groups.

mixed and five separate populations. This collection procedure was a function of the exhaustion of specimens from the initial group A hibernacula. Group A demonstrated a constancy of water content ranging between 50·3 and 56·8 per cent while groups B, C, and D varied significantly from A. Variation between separate populations is not unexpected due to the behavioural nature of the maintenance of hydration levels. Mean SC_p values and their functional relationship should be viewed cautiously for their use is valid only during summer

and mid-winter when SC_p distributions are uni-modal. However, the lack of interdependency between water content and SC_p is indicated by the low correlation coefficient of 0·07 (group A).

Laboratory acclimation experiments were conducted to further quantitate this response. One problem continuously encountered but too often overlooked is the rapidity of loss of cold hardiness in invertebrate groups (BAUST and MILLER, 1970, 1972). Even the most cold tolerant species resident in the Arctic are 'irreversibility' warm acclimated within hours at above freezing exposures.

Winter specimens were acutely warmed to 25°C and maintained on controlled diets. The shift in population supercooling distribution patterns is shown in Fig. 2. Within 12 hr after warming two
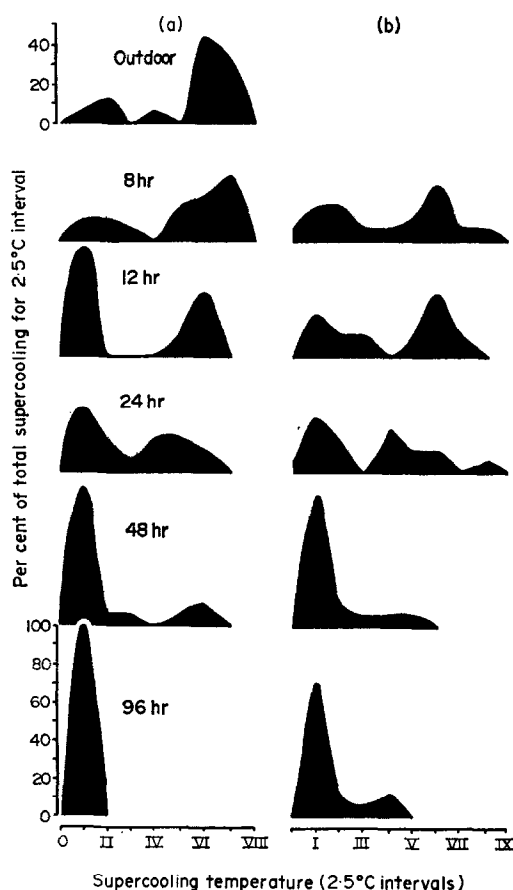

Fig. 2. Frequency distribution curves of supercooling temperatures of *C. maculata* during warm acclimation (25°C). (a) Distilled water diet. (b) Glucose (10%) diet. Refer to Fig. 1 for specifics of curve preparation.

distinct patterns were observed. Group A (distilled water diet) underwent an equivalent of a 6-month seasonal transition within 96 hr whereas group B (distilled water plus 10% glucose diet) demonstrated a more gradual transition to 'summer'

unimodality. More significant, however, is the rate of reacclimation to low temperatures illustrated in Fig. 3. After 96 hr at 25°C specimens from groups
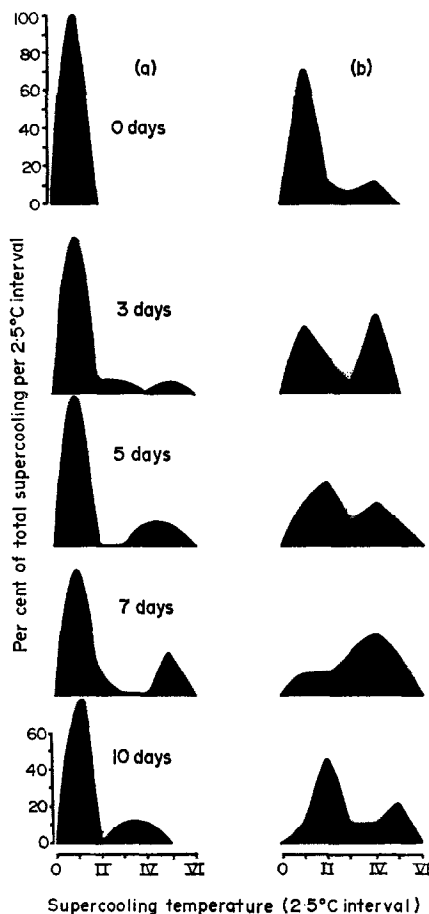

Fig. 3. Frequency distribution curves of supercooling temperatures of *C. maculata* during cold acclimation (5°C). (a) Distilled water diet. (b) Glucose (10%) diet. Refer to Fig. 1 for specifics of curve preparation.

A and B were cooled to 5°C. Low temperature reacclimation was not observed in group A after 10 days but group B demonstrated supercooling range shifts similar to autumn specimens. It is apparent from Figs. 2 and 3 that glucose, an efficient cryoprotectant, served as a nucleator 'mask' during the cyclical temperature fluctuations. In groups A and B water content maintained similar constancy with the natural populations and no statistical correlation between SC_p and water content could be drawn in any sample. Correlation coefficients varied between 0·01 and 0·33.

Cryoprotectant analysis

Attempts to correlate SC_p shifts with changing levels of polyhydric alcohols or cryoprotective

carbohydrates were unsuccessful. Identifiable levels of glycerol, erythritol, or sorbitol among a few were not found throughout a 12 month period indicating an absence of these SC_p modifying agents. Trace levels of carbohydrate (trehalose) were found but at levels well below those known to serve as efficient cryoprotectants (less than 50 mg %).

DISCUSSION

The basis of our understanding of insect over-wintering capabilities has rested on two observations. First, the low temperature induced extensions of supercooling ranges are a function of relative increases in ice nucleator content during winter due to dehydration (SALT, 1956, 1966; MILLER, 1969) and not feeding. Second, the influence of the nucleator materials is buffered by the accumulation of cryoprotectants (BAUST, 1973) which act to 'mask' nucleator action.

C. maculata differs from the accepted profile of overwintering response in that the non-feeding adults are observed to shift supercooling distribution patterns while maintaining a stable water content and without identifiable cryoprotectants. Attempts to lend further information to these observations through controlled feeding experiments provide interesting insights. The diets available were considered nucleator free following careful distillation and de-ionization procedures. Yet, nearly a full cycle of shifts in SC_p ranges was observed indicating that a complete compliment of nucleators remained within the insect. However, their effect was diminished over the autumn without the influence of polyhydric alcohols or significant levels of cryoprotective carbohydrates. Diet supplementation with glucose masked nucleator action. In a subsequent experiment the studies illustrated in Figs. 2 and 3 were repeated with the addition of kaolin, an efficient nucleator. The results did not vary from those presented in Figs. 2 and 3, offering a further indication of the presence of a nucleator masking (freezing inhibitor) substance possibly similar to that found in Antarctic fish (FEENEY, 1972).

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