STUDIES ON THE COLD HARDINESS OF THE PEACH-POTATO APHID *MYZUS PERSICAE* (SULZER)

A Thesis submitted to the University of Leeds
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Doctor of Philosophy

by

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Weep not for little greenflies who are orphaned in the morning:
They need no mother's tender care - by evening they'll be spawning

C.H. PERKINS
in The Countryman

Myzus persicae (Sulzer),
the peach-potato aphid

Adult apterous (wingless) virginopara, body length approximately 2 - 2.5mm. The dark spots within her abdomen are the eyes of her developing young. Born live, the first instar nymphs are virtually identical to their mother but are just over 0.5mm in length. They then develop to the parthenogenetic adult via four moults to second, third and fourth instar nymphs. Alate (winged) adults are slightly darker in colour.

Sexual reproduction (holocycly) commences in autumn with the production of sexual females (oviparae) and males and the eggs which are subsequently produced, overwinter on the woody winter (primary) host, peach.

Throughout Britain however, this species is mainly anholocyclic, surviving overwinter as active aphids on a wide range of herbaceous secondary hosts; it is essentially this overwintering strategy coupled with its ability to vector plant viruses which makes this aphid such an important agricultural pest.

To my parents, Sarah and Eamonn O'Doherty

whose understanding and love helped make

this thesis a reality

Rose O'Doherty

Studies on the cold hardiness of the peach-potato aphid Myzus persicae (Sulzer)

Abstract: Ph.D. Thesis, April 1985

A system incorporating a prototype automatic thermoelectric cooling method with computer-based recording of aphid supercooling points was developed and formed the basis of cold hardiness assessment.

Under laboratory culture all developmental stages of Myzus persicae had a mean inherent supercooling potential below -20°C, with first instar nymphs the most cold hardy. When maintained at 5°C, younger instars demonstrated acclimation ability unlike adult aphids, and in an insecticide resistant strain, adults lost cold hardiness. When in contact with surface moisture, the majority of aphids did not experience inoculative nucleation. Sexual morphs of M. persicae possessed supercooling ability comparable with laboratory maintained parthenogenetic morphs; eggs supercooled to below -30°C.

Seasonal studies of supercooling ability demonstrated that all aphid stages were most cold hardy in summer. Younger instars showed natural acclimatisation and were cold hardy throughout the year. Overall adults lost cold hardiness as winter progressed, exhibiting bimodal supercooling point distributions in two winters, with distinct high (HG) and low (LG) groups and mean supercooling points of approximately -20°C and -10°C respectively. Clonal differences and adult age did not account for this pattern. Following experimental starvation at 5°C, first instars of M. persicae maintained extensive supercooling potential but adults

exhibited losses of cold hardiness comparable with those in natural overwintering populations, suggesting that feeding may be necessary to maintain adult cold hardiness levels during winter. Subsequent starvation experiments did not reproduce the dramatic losses of cold hardiness implying that the feeding influence was more complex than the availability of food *per se*. In a series of host transfer experiments the mean supercooling point of *Aphis fabae* adults could be shifted by over 10°C, increasing when they fed on beans and reduced when transferred back to spindle; the LG (spindle/poor supercoolers) to HG (bean/good supercoolers) shift was more difficult to achieve and suggested a nucleating agent in spindle sap.

Trimethylsilyl derivatised carbohydrate extracts of *M. persicae* and *A. fabae* were analysed by capillary gas-liquid chromatography. Glucose, glycerol, fructose, mannitol, sucrose, and trehalose were detected in samples of both species, together with trace amounts of unidentified carbohydrates in *M. persicae* samples. Dulcitol was present in spindle-fed *A. fabae* only. There was no obvious correlation between carbohydrate content and supercooling ability but high total percentage body carbohydrate levels were revealed and may have a solute effect, enhancing inherent supercooling potential and dependent on carbohydrate-rich sap intake.

Laboratory cultured *A. fabae* were capable of extensive supercooling, as were individuals collected from summer herbaceous hosts; first instars were the most cold hardy. When associated with the primary host, spindle, all aphids showed poor supercooling potential, less than -15°C; overwintering eggs were capable of supercooling to below -30°C and acclimatised in winter. Eggs and oviparae were not subject to inoculative

nucleation. Preliminary experiments to relate supercooling ability to mortality at sub-zero temperatures proved inconclusive and were terminated when temperature shock and/or desiccation were thought to have induced premature mortality.

The results demonstrate that the cold hardiness characteristics of M. persicae are atypical of those observed in other freezing-susceptible arthropods. It is proposed that continued feeding during mild winters maintains cold hardiness levels in adult M. persicae and this influence may provide a possible explanation for the successful anholocyclic overwintering of this aphid during such winters. Avenues of research to further investigate this proposal are suggested.

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My S, R_1 and R_2 strains of Myzus persicae were supplied by Rothamsted Experimental Station via Adrian D Rice and the H clone by Dr Roger L Blackman of the British Museum (Natural History). Ken Redshaw and Alec Goodall supplied my Chinese cabbage plants and allowed me to search for aphids on field crops and spindle bushes at the University Experimental Garden (Ridgeway).

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True to the habit of leaving the most important things to the end, my fondest thanks go to Jacqui Lewis who typed this thesis, tolerated my excesses and made me realise how flat the world really is...

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CHAPTER ONE

GENERAL INTRODUCTION

Aphids (Aphidoidea) comprise over 3,700 species (Eastop and Hille Ris Lambers, 1976) of which the family Aphididae is the largest (over 3,600 species) and most studied. Biologically, they are of interest on account of their complex life cycles and highly developed polymorphism. As plant feeders aphids extract the phloem sap and often cause substantial 'direct damage', through the build up of dense colonies, weakening and perhaps causing the eventual death of host plants (Plate 1). Aphids are also the most important animal transmitters of phytopathogenic viruses; 5% of all aphid species account for the transmission of 57% of plant viruses with known vectors (Eastop, 1977), particularly in the temperate regions of the world in which aphid species are mainly distributed. Aphid transmitted viruses severely weaken and may kill the host plants to which they are vectored, and in the case of crop plant hosts may reduce harvest yields (Plate 2).

Myzus (Nectarosiphon) persicae (Sulzer), the peach-potato aphid (or green peach aphid) is known to transmit over 100 phytopathogenic viruses among 50 different plant families. Many of its hosts include major crops (e.g. sugar beet, beans, brassicas, potatoes, citrus) and on a world-wide scale this species is regarded as the most important aphid pest (Mackauer and Way, 1976).

Most Aphididae overwinter as sexually produced eggs, either on a woody winter (primary) host if they are a host-alternating species (heteroecious) e.g. Aphis fabae (black bean aphid), or on appropriate woody or herbaceous host plants in non-alternating species (autoecious) e.g. Drepanosiphum platanoides (sycamore aphid) and Acyrthosiphon pisum (pea aphid) respectively. In each case, the sexual forms which ultimately give rise to the eggs, are produced in

Plate 1 Colony of the black bean aphid *Aphis fabae* on a bean plant (attended by the ant *Lasius niger*).

Plate 2 The effects of potato leaf roll virus (PLRV), one of the viruses trasmitted by the peach-potato aphid *Myzus persicae*, on the potato plant.

autumn in response to decreasing photoperiod in combination with decreased temperature. Such species, which reproduce sexually each year are referred to as holocyclic. Some aphid species, however, continue to overwinter parthenogenetically, that is, as active aphids with no sexual phase. This is the anholocyclic life cycle. In Britain, Elatobium abietinum (green spruce aphid) survives overwinter without egg production. The annual sequence of events in each of these life cycle types is detailed in Figure 1.1. Other aphid species are capable of both holocyclic and anholocyclic overwintering; some of the population produce overwintering eggs while other individuals survive parthenogenetically, particularly under mild winter conditions (Eastop, 1983).

M. persicae belongs to this third category and in utilising to the full both overwintering strategies, has successfully colonised a wide range of habitats with very different winter conditions, including regions where the severity of winter climate can be variable from year to year (Fig. 1.2). Thus populations inhabiting areas with severe winters (e.g. North America, Central Europe), are almost entirely holocyclic, with anholocycly only occurring in sheltered sites e.g. glasshouses. In areas with more tropical-like climates the aphid has lost the capacity to produce sexuales and reproduces parthenogenetically throughout the year (van Emden, Eastop, Hughes and Way, 1969). In temperate regions, including Britain, both methods of overwintering are utilised and while the severity of winter weather will largely determine the main method of overwintering, in parts of Britain the scarcity of the primary host, peach (Prunus persica), restricts both the distribution and frequency of the holocyclic life

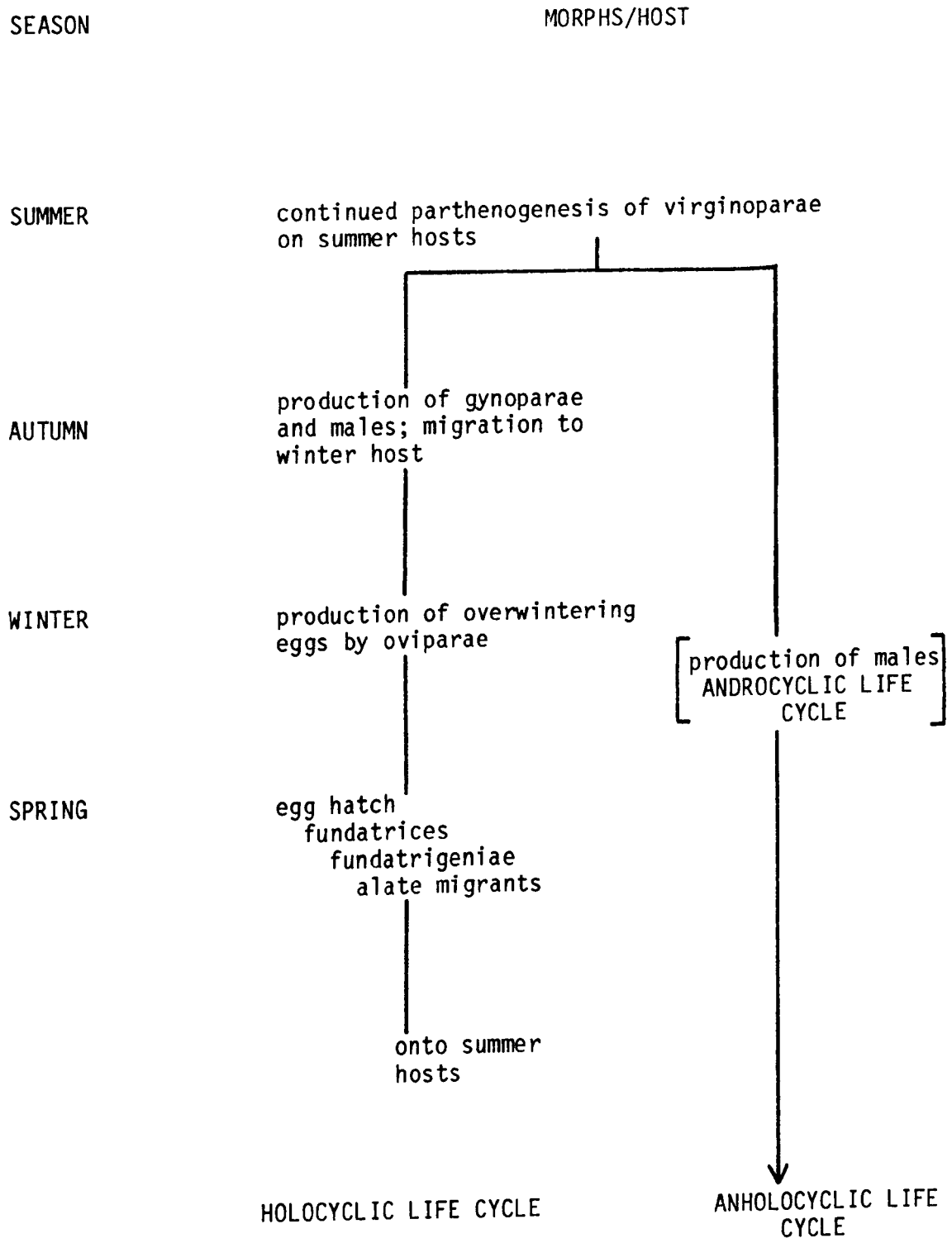


Figure 1.1 The annual sequence of events in aphid life cycles

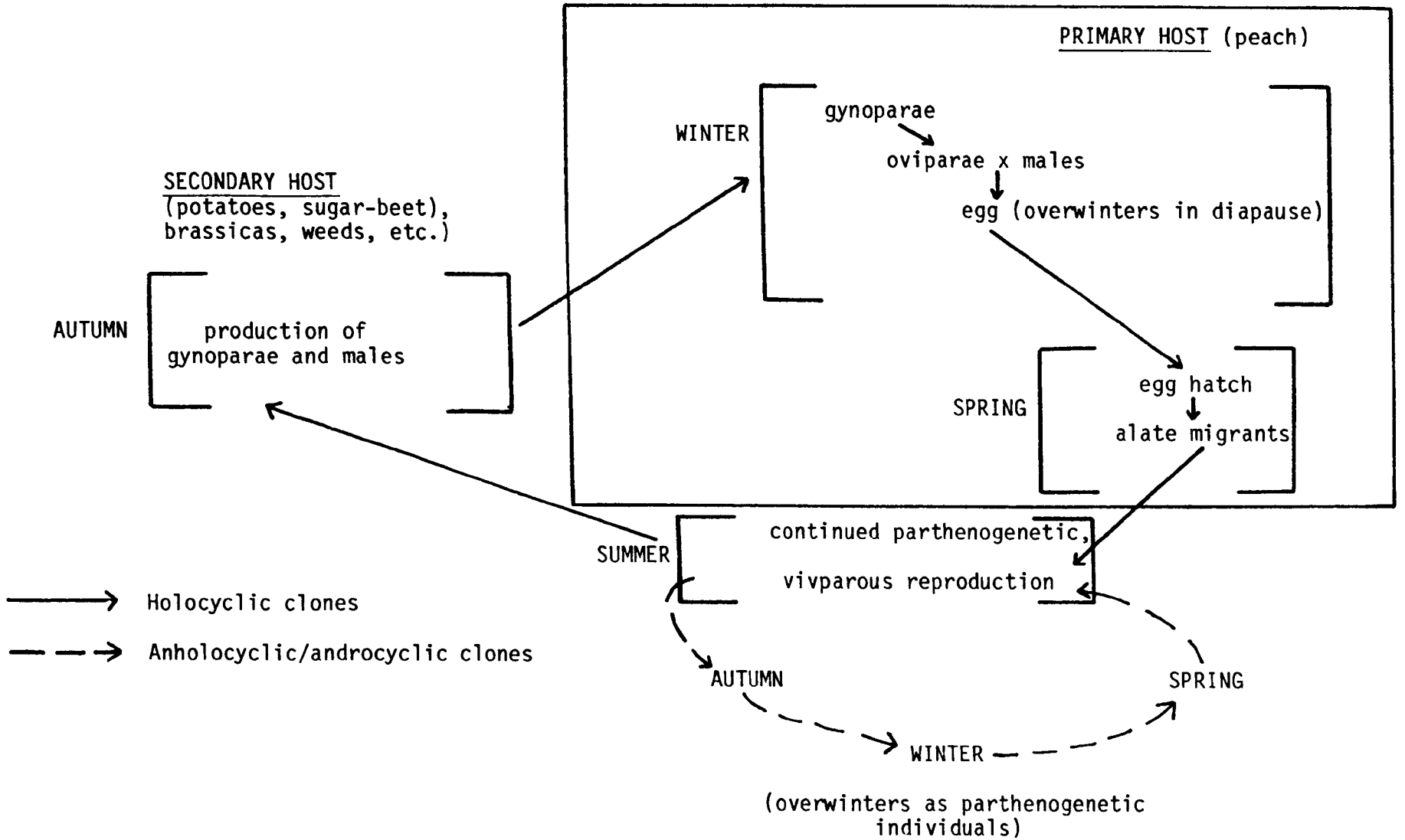


Figure 1.2 Life cycle of Myzus persicae

cycle (Broadbent and Heathcote, 1955; Blackman, 1971). In these regions (and probably worldwide) the life cycle is further complicated by the ability of most anholocyclic clones to produce a few males in autumn which may contribute to the sexual reproduction of a holocyclic clone through mating with the sexual females (oviparae). In this way the anholocyclic nature of the clone from which the males originated can be maintained via the sexual cycle to be expressed in later generations. This androcyclic life cycle type provides a mechanism whereby even in the most severe winters when parthenogenetic overwintering is not favoured, the potential for anholocycly/androcycly under more favourable conditions is maintained within the gene pool (Blackman, 1971, 1972, 1974b).

Since overwintering of M. persicae in Britain is mostly of the anholocyclic/androcyclic type, the success of overwintering in each year is largely determined by the severity of the winter climate, greater survival being associated with mild winter conditions (Turl, 1978; Eastop, 1983). Sheltered sites e.g. glasshouses, potato stores etc. will however provide for continued parthenogenesis in most winters. Virus epidemics in crop plant hosts in the following growing season are associated with increased aphid survival in such winters. Thus in the mid 1970s, the seed potato crop in Scotland experienced a devastating potato leaf roll virus (PLRV) epidemic as a result of increased levels of anholocycly in preceding mild winters (Howell, 1977; Turl, 1978). The same pattern has accounted for economically damaging levels of sugar-beet yellowing viruses in the English crop in past years (Watson, Heathcote, Lauckner and Sowray, 1975). Where anholocyclic strains of other aphid species are vectors transmitting

agriculturally important viruses epidemics again follow mild winters e.g. cereal aphids and barley yellow dwarf virus (BYDV) (Plumb, 1977). However because M. persicae has such a wide host range, including many agricultural crops, the effect can be much more important.

Aphids which overwinter in the active state migrate earlier in spring to the secondary summer hosts, including crop plants, than do the winged migrants from holocyclic lines, where egg incubation and aphid development delays the production of these winged morphs. This earlier spring migration by anholocyclic individuals is therefore to younger and thus more virus susceptible plants. In addition, incoming aphids may be carrying viruses acquired from their overwintering hosts including wild plants (Eastop, 1981) or if a virus source exists within the crop, spread of the virus will commence earlier in the growing season. For these reasons the probability of a virus epidemic is greatly increased after mild winters with increased aphid survival.

In recent years there has been an increasing interest in the ecology and overwintering biology of the aphid (Taylor, 1977; Turl, 1983) with a view towards 'predictive forecasting' of both the date and size of the spring migration from overwintering hosts to crops (Woodford, Shaw, McKinlay and Foster, 1977). Ideally this would allow earlier and more accurate advice to growers on the use of insecticides in spring as a control strategy. In years when overwintering success has been limited it would also permit a recommendation "not to spray" to be given (Foster et al, 1981). This is ecologically and economically desirable, particularly in those regions where M. persicae is showing signs of resistance to many of the available

chemical treatments (Woodford, Harrison, Aveyard and Gordon, 1983).

In Britain, such a forecasting system has been successfully developed for the black bean aphid, Aphis fabae (Way and Cammell, 1973). This species is mainly holocyclic on spindle (Euonymus europaeus) which is almost exclusively the primary host on which the eggs overwinter. This facilitates accurate egg counts in autumn and spring assessment of egg survival, followed by an estimation of migration date to bean crops and a recommendation to spray should the size of the subsequent population on beans threaten 'direct damage'. By comparison, attempts to do the same for M. persicae have been less successful since overwintering throughout Britain is primarily as anholocyclic individuals on a wide range of host plants, wild and cultivated, which makes aphid location and counting almost impossible (Turl, 1980).

Nevertheless a number of systems which aim to predict spring migration dates for M. persicae have been developed. A scheme to predict the likelihood of yellowing viruses in the sugar beet crop, using regression analyses based on meteorological factors found to influence aphid build up (and consequently virus incidence in earlier years), was described by Watson, Heathcote, Lauckner and Sowray (1975). 'Frost days' and April mean temperatures were the most significant factors with respect to virus incidence. In Scotland, very much as a result of the 1970s PLRV epidemic, there have been attempts to produce a predictive forecast of aphid spring migration in cereal as well as potato aphids, with limited success. The dates of first aphid catches in suction traps were originally used to indicate migration into crops, whereas a negative correlation between winter

temperatures and first catches of aphids in traps now forms the basis of most forecasts (Turl, 1978, 1980). The suction traps used in Scotland are also part of the Rothamsted Insect Survey; a national network of groups providing data which is used to model populations with the overall aim of predictive forecasting (Taylor, 1977, 1983). It is therefore surprising that despite these insights into the epidemiology of M. persicae and particularly the association between mild winters and increased aphid survival, that there has been little work to directly assess the cold hardiness of the aphid; the lack of such studies may be a limiting aspect in the predictive forecasting of spring migration.

In temperate zones winter climate is the main factor governing insect winter survival (Danks, 1978), with high mortality occurring in years with severe winters. A number of observations relating aphid winter survival to climate have been described, including correlations between minimum temperatures and reduced survival (Adams, 1962; Powell and Parry, 1976) and mean winter temperatures and aphid survival (Heinze, 1939; Heie and Petersen, 1961).

Other winter variables can however have an important influence on aphid overwintering as described by Smith (1981) in which wind and rain were found to influence mortality in cereal aphid populations. It was suggested that low temperatures were more subtle in effect, influencing reproduction and thus recruitment to the overwintering population.

Taylor (1977) reiterated that in Britain, while overall winter climate and particularly temperature is the main limiting factor in M. persicae winter survival there were also spatial considerations; in

the north, temperature was the more important while in the west a precipitation factor was often more influential. Therefore, the modifying effect of weather variables other than temperature cannot be totally ignored.

Cold or low temperature is the most significant adverse factor affecting overwintering organisms and therefore involving a number of physiological and biochemical adjustments in preparation for winter survival (Block, 1980); of these cold hardiness or cold tolerance is the most studied (Danks, 1978; Duman, Horwarth, Tomchaney and Patterson, 1982). Invertebrate poikilotherms, including arthropods, are classified into two main categories depending on the cold tolerance strategy involved; freezing-susceptible (or freezing-intolerant) and freezing-tolerant (Block, 1982b).

Freezing-tolerant species can survive freezing of the extracellular body tissues and fluids; intracellular freezing however is lethal (Asahina, 1969). To ensure the protection afforded at low temperature by freezing, these species synthesise nucleating agents, mainly proteins, which promote (extracellular) freezing at temperatures a few degrees below their true freezing point (Zachariassen and Hammel, 1976; Zachariassen, 1982). Among arthropods, freezing-tolerance is the least widespread of the two strategies (Block, 1982b); it predominates in animals inhabiting regions where long term low temperatures are encountered or where short term minimum temperatures are so low as to exclude survival by supercooling, such as in Arctic regions (Downes, 1965). Consequently, insects are the highest taxon within the animal kingdom to survive 'whole body freezing' (Asahina, 1966).

Insects are the most studied of all invertebrates with respect to cold tolerance and within this group, freezing-susceptibility is the most common of the two overwintering strategies (Block, 1982b). Freezing-susceptible species will die if their body tissues and fluids freeze and avoid this lethal condition by the process of supercooling. That is, as the ambient temperature falls below their true freezing point they remain unfrozen. It is not particularly unusual that insects supercool to some extent, since in nature, supercooling is the more normal condition compared with freezing (Salt, 1969). Many insects have, by physiological and other means, optimised this ability to supercool, to avoid freezing death and so enhance their winter survival (Ring and Tesar, 1980; Heinrich, 1981).

The supercooling potential of an organism can be determined experimentally by measurement of the supercooling point (SCP), which is the temperature at which the animal freezes spontaneously during gradual cooling. In freezing-susceptible species the supercooling point is also a direct measure of the instantaneous low-temperature death point (Salt, 1961). By standardising the supercooling point determination technique as suggested by Salt (1961), the pioneer of modern arthropod cold hardiness studies, it is possible to make comparisons of cold hardiness between different species and studies. However the supercooling point within a species is not a fixed measurement but is influenced by a number of inherent and environmental factors. Sømme (1982) reviewed the subject of supercooling in terrestrial arthropods and the factors which influence supercooling potential.

In freezing-susceptible species the main features of cold hardiness assume a general pattern. Many demonstrate seasonal changes in cold hardiness, undergoing a natural acclimatisation process in preparation for winter when supercooling potential is often maximised. This cold hardening response can be demonstrated by laboratory acclimation at low temperatures. Contact with surface moisture and the presence of food in the gut can both act to reduce inherent supercooling ability since they may induce freezing at high sub-zero temperatures which then results in freezing of the whole animal. Therefore while supercooling point studies provide an index of cold hardiness for freezing-susceptible species, such studies must also consider these potential influences which may increase or detract from the inherent supercooling potential and so influence overwintering survival of the species.

The aim of the work described in this thesis was to assess the cold hardiness of M. persicae, and to provide information on the overwintering survival of the economically important anholocyclic populations, by using supercooling point studies to investigate inherent cold hardiness and factors which might modify this overwinter.

Earlier studies on aphid cold hardiness had shown all species to be freezing-susceptible (Powell, 1974; Parry, 1978; Sømme, 1982) and therefore dependent on supercooling to avoid freezing death overwinter. This study was therefore mainly concerned with supercooling ability assessment as the main indicator of cold hardiness levels under laboratory and natural conditions. Part of the

research involved the development of an integrated system to record aphid supercooling points and this is described in Chapter Three. Chapter Four describes studies on inherent supercooling potential and factors influencing this in laboratory and field experiments while Chapter Five involved work to assess the influence of feeding on cold hardiness (supercooling). Potential biochemical correlates of cold hardiness were also studied and this is discussed in Chapter Six. Chapter Seven describes comparative studies with the black bean aphid, Aphis fabae, which in contrast to M. persicae is almost entirely holocyclic in this country, overwintering as sexually produced eggs.

It was hoped that such information would further understanding of the overwintering trends exhibited by M. persicae as outlined earlier; such knowledge may assist those working towards predictive forecasting of outbreaks of this aphid species. In addition the study provides a comparison of M. persicae and aphid cold hardiness characteristics with those demonstrated in other freezing-susceptible arthropods (Sømme, 1982). Many of these studies have been concerned with freezing-susceptible species which inhabit those parts of the world where low temperature is extreme and sometimes long term (e.g. Arctic and Antarctic regions) with comparatively fewer studies of supercooling ability conducted on temperate, pest freezing-susceptible species where low temperature tolerance is mainly a requirement of overwintering individuals and such a study would provide further data on this aspect.

A fuller introduction to the various aspects of the study as described above together with the associated techniques is given in the relevant chapters.

CHAPTER TWO

LABORATORY MAINTENANCE OF *MYZUS PERSICAE* AND ASSESSMENT OF BIOLOGICAL CHARACTERISTICS AND INSECTICIDE RESISTANCE STATUS

SUMMARY

Clonal cultures of Myzus persicae (S, R_1, R_2, OH, Savoy, OSR, Kale, H) were maintained in the laboratory at 20 ± 2°C with a 18h/6h photoperiod on Chinese cabbage.

Differences in the nymphal developmental periods of the S, R_1 and R_2 strains at 20°C and 5°C were observed. The fecundity and longevity of S and R_2 adults at 20°C was similar. Maintenance under short photoperiod conditions (10h/14h) indicated that the S strain was of the holocyclic life cycle type, while the insecticide resistant R_1 and R_2 strains were androcyclic.

Tile tests (a biochemical assay) were used to measure total levels of carboxylesterase in the M. persicae clones and allowed preliminary determination of insecticide resistance. Separation of the carboxylesterases was achieved by conventional polyacrylamide gel electrophoresis and by comparing directly the level of the 'resistance associated carboxylesterase' in individual aphids with that in the S (susceptible), R_1 (moderately resistant) and R_2 (highly resistant) clones, the OSR clone was determined as being moderately resistant and the remainder (OH, Savoy, Kale and H) as highly resistant.

INTRODUCTION

This chapter gives details of the maintenance techniques used to culture the various clones of Myzus persicae studied during the research. Establishing clonal material in a standard manner to reduce as far as possible background (environmental) variation is important and was particularly so during this work, where one of the aims was to compare the cold hardiness of a number of different M. persicae strains and clones. Intraclonal variation is thought to be the result of slight environmental changes (Blackman, 1971) rather than an 'endomeiosis' mechanism within clonal material as suggested by Cognetti (1961).

Information on field-originating and field-based cultures of other M. persicae populations and of the other aphid species studied during the research (Aphis fabae) is given in the appropriate chapters.

Certain biological (developmental and reproductive biology; short photoperiodic response) and physiological (insecticide resistant-type) characteristics of the M. persicae clones were studied to provide foundation data for the cold hardiness studies and the relevant techniques and results are given in this chapter.

ESTABLISHMENT AND CULTURE OF APHIDS

Aphids.

Three strains of M. persicae differing in their resistance to insecticides were obtained from Rothamsted Experimental Station (RES) in October 1981. They were classified according to the terminology of Sawicki et al (1978);

a) susceptible to organophosphorus (OP), carbamate and pyrethroid insecticides - S strain,

b) moderately resistant to organophosphorus and pyrethroid insecticides but susceptible to carbamates - R_1 strain,

c) highly resistant to organophosphorus and pyrethroid insecticides but moderately resistant to carbamates - R_2 strain

(Rice, Gibson and Stribley, 1983).

The data on inherent levels of cold hardiness in M. persicae was provided by these three strains and they were also used to investigate the possibility that the resistant-types (susceptible, moderately and highly resistant) might vary in their cold hardiness. This investigation was prompted following work by Baker (1977a, 1977b, 1978) which suggested that in M. persicae resistance to insecticides may also confer lowered fitness (ie. cold hardiness). However Devonshire and Needham (1975) and Baker (1977a) had also suggested that frost may select for OP resistance.

The Rothamsted strains were standard laboratory-reared clones (Sawicki and Rice, 1978), having been reared over a number of years on Chinese cabbage (Brassica pekinensis). The same host plant was used in this study and consequently the aphids experienced the minimum of cultural changes. Field-collected populations of the aphid were

subsequently established as clones in the laboratory (clones OH, Savoy, OSR and Kale) and used in field experiments in winter 1983-84. During laboratory maintenance they were kept in the same manner as the Rothamsted clones.

A holocyclic clone, obtained from the British Museum (Nat. Hist.), London (clone H) was used to provide sexual morphs and eggs for cold hardiness studies.

The field origins and details of previous culture of the aphids studied are given in Appendix 2.

Plants.

Chinese cabbage (Brassica pekinensis, var. Wong Bok) was used as the experimental host material. A weekly supply of six-week old plants was obtained from the University experimental garden (Ridgeway) and these were then maintained in a departmental glasshouse. Brassicas are among the most common overwintering hosts of anholocyclic M. persicae (van Emden, Eastop, Hughes and Way, 1969) and in the laboratory this aphid does particularly well when cultured on Chinese cabbage (Mackauer and Way, 1976).

Almost all the experimental work was carried out using leaf discs of Chinese cabbage which provide a uniform food source, minimising experimental and replicate variation (Adams and van Emden, 1972). Leaf discs require less culturing space than whole plants or excised leaves and overall are easier to handle and maintain. The discs (3.5 and 5 cm in diameter) were cut from the centre of the younger, more turgid leaves.

Clonal stocks of aphids were maintained on two-week old seedlings of Chinese cabbage in transparent perspex boxes ('Blackman boxes') as detailed by Blackman (1971). All aphid populations were established as clones from the progeny of a single apterous, parthenogenetic, viviparous female (virginopara), so that each clone constituted a pure line of inheritance (Blackman, 1971). The seedlings were changed every week and the clone maintained by collecting the progeny of the previous generation adults. All subcultures of aphid clones used in experiments were taken from these clonal stocks.

Where other host plants were used (eg. field experiments) details of their culture and use is included in the appropriate chapters.

Experimental conditions.

All aphids were maintained at 20 $\pm$ 2°C either in cooled, illuminated incubators or under 'aphid bays' within a controlled-temperature room (Plate 3). A long photoperiod (18h/6h) was supplied by timed cool-white fluorescent lights. Humidity was not controlled but was between 60-80% R.H.

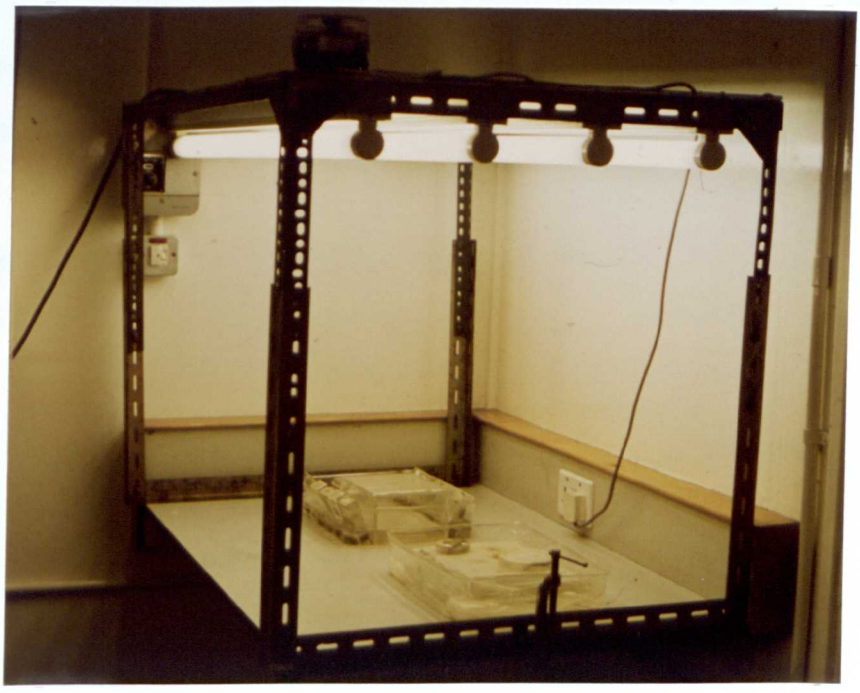
Standard culturing technique.

After cutting, the leaf discs were washed in water, inspected for any wild aphids, and dried on tissue.

The discs were placed underside up, on layers of white tissue stretched over a glass or perspex supporting block (Plate 4). The tissue prevented the discs from drying out and also formed a wick from a reservoir of water (tap) in a plastic dish in which the block rested. Petri dish bottoms with holes covered by fine mesh netting formed cages into which the leaf discs just fitted. When cultures

Plate 3 'Aphid bay' in which aphids were maintained within a controlled-temperature (20°C) room

Plate 4 Culturing technique used to maintain *Myzus persicae* in the laboratory



were maintained in incubators, a lid was placed over the plastic dish to prevent evaporation of water. Leaf discs were changed as necessary, usually every 3-4 days and the water reservoir topped up daily.

During transfers, aphids were handled with a moist, fine paintbrush.

BIOLOGICAL CHARACTERISTICS

1. Developmental and reproductive biology.

The nymphal development periods of the S, R_1 and R_2 strains and adult fecundity and longevity of the S and R_2 strains of M. persicae were determined at 20 ± 2°C. Development of the S and R_2 strains was also assessed at 5 ± 2°C. This information was required to assist in planning the synchronous production of nymphs and adult morphs for both the inherent cold hardiness studies and acclimation experiments.

Method. Newly born nymphs (first instar) were collected from adults (usually apterae) and set up individually on leaf discs of Chinese cabbage at the appropriate temperature in cooled incubators with a long photoperiod (18h/6h). Moults to the subsequent instars (second, third and fourth) were determined by direct observation of the cast exuviae. After the final moult the adults were retained to assess fecundity and longevity.

Results. The nymphal development periods at 20°C for the S, R_1 and R_2 strains are given in Table 2.1. Development to adult was slightly quicker in the S strain than in either the R_1 or R_2. Table 2.2 shows the longevity periods and fecundity of the S and R_2 aphids at 20°C. While fecundity was almost identical, S adults usually lived longer than their R_2 counterparts. Nymphal developmental periods at 5°C for the S and R_2 strains are given in Table 2.3; S strain individuals showed faster development than R_2 aphids.

Table 2.1 Nymphal development of S, R_1 and R_2 strains of *M. persicae* at 20 ± 2°C on Chinese cabbage

Strain	Developmental period (h) (mean ± S.E.; n=20)				
	First instar	Second instar	Third instar	Fourth instar	Total developmental period (h)
S	42.7 ± 0.82	27.0 ± 1.06	33.1 ± 1.23	37.9 ± 0.79	140.9 ± 0.79
R_1	34.4 ± 0.81	32.0 ± 2.41	37.9 ± 1.27	43.4 ± 1.79	147.8 ± 2.70
R_2	33.0 ± 1.27	34.6 ± 1.65	34.0 ± 2.13	49.9 ± 1.42	151.6 ± 1.28

Table 2.2 Longevity and fecundity of S and R_2 strains of *M. persicae* at 20 ± 2°C on Chinese cabbage

Strain	Reproductive period (including pre-reproductive)	Post-reproductive period	Total longevity	Fecundity mean ± S.E.(n=20) nymphs per adult
		mean ± S.E. (n=20) (days)		
S	18.2 ± 0.29	13.0 ± 0.93	31.4 ± 0.80	90 ± 3.01
R_2	20.1 ± 0.51	9.7 ± 0.64	29.9 ± 0.58	90.8 ± 1.48

Table 2.3 Nymphal development of S and R_2 strains of M. persicae at 5 ± 2°C on Chinese cabbage

Strain	Developmental period (h) (mean ± S.E.)				
	First instar	Second instar	Third instar	Fourth instar	Total developmental period (h)
S	279.8 ± 13.7 (n=18)	284.4 ± 8.7 (n=15)	227.2 ± 16.9 (n=10)	327.8 ± 18.9 (n=12)	1062.2 ± 13.5 (n=15)
R_2	243.7 ± 14.1 (n=16)	296.7 ± 6.25 (n=12)	307.7 ± 7.63 (n=10)	371.6 ± 23.8 (n=11)	1195.5 ± 19.7 (n=15)

Full details of the data on which the above are based are given in Appendix 3.

2. Photoperiodic response (life cycle type)

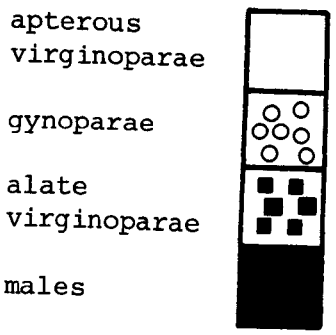
M. persicae populations show varying responses to decreasing photoperiod in autumn; while some respond by producing sexual forms and ultimately overwintering eggs (holocyclic life cycle type), others show no response but continue to overwinter as parthenogenetic individuals (anholocyclic life cycle type) (Fig. 1.2). As the photoperiodic response of the S, R_1 and R_2 strains was unknown when received (A.D. Rice, personal communication), this was determined experimentally by exposing the three strains to a short photoperiod. It was hoped to carry out comparative studies on the cold hardiness of anholocyclic and holocyclic clones of this aphid species, as well as assessing the supercooling ability of sexual forms and eggs. (Attempts to locate field holocyclic M. persicae proved unsuccessful).

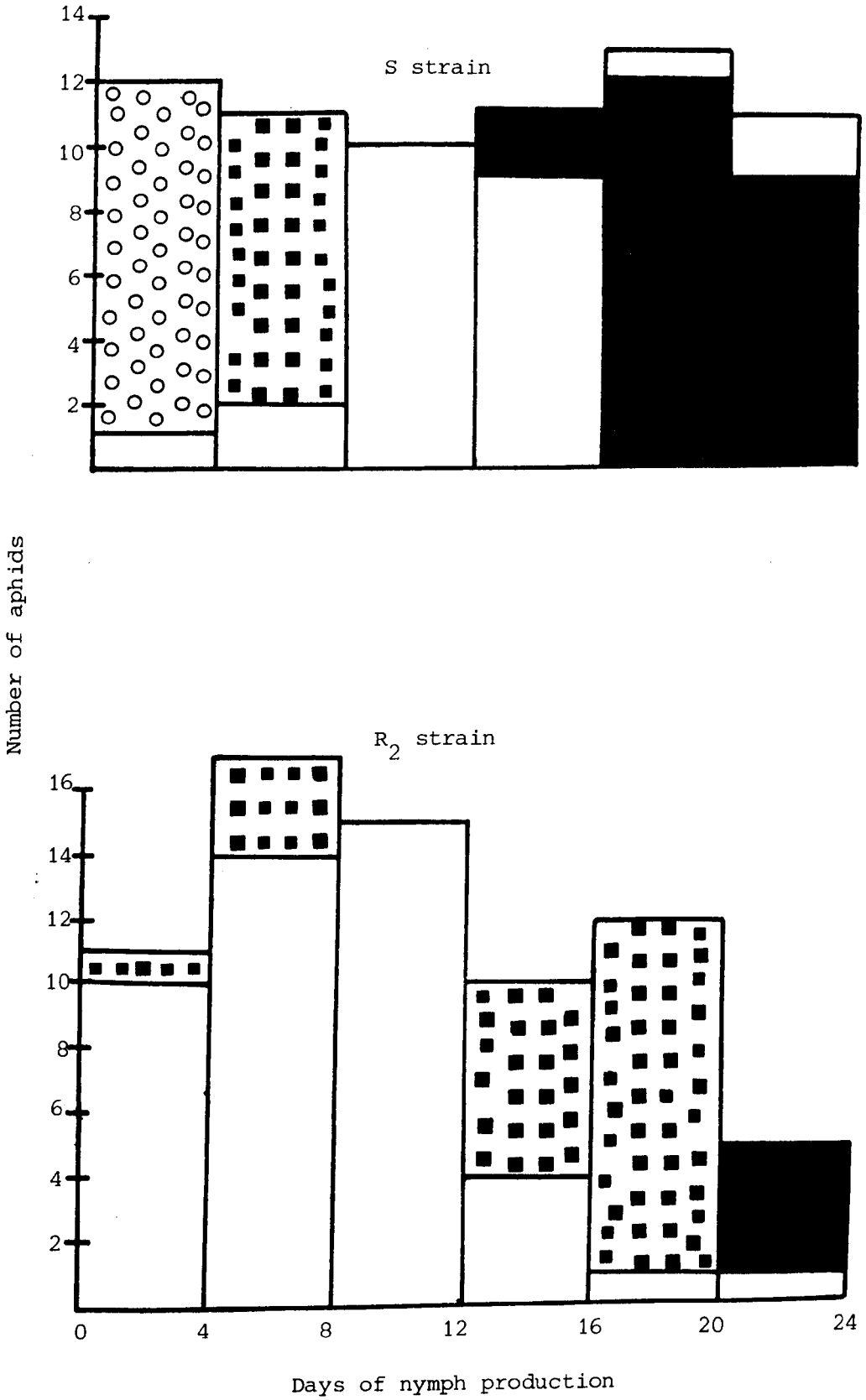
Method. This followed that of Blackman (1971) with minor modifications. Aphids were transferred from the culturing conditions at 20 $\pm$ 2°C and long photoperiod (18h/6h) to the experimental conditions of 18 $\pm$ 2°C and short photoperiod (10h/14h) in an 'aphid-bay' within a short photoperiod room. Aphids were fed on leaf discs of Chinese cabbage. After two generations, adult apterae (at least 3) from each strain were set up individually and a record of the progeny produced throughout the reproductive life of each individual made. Where sexual females (oviparae) and sexual males were ultimately produced the strain could be recorded as 'holocyclic'. Where reproduction produced only males and other morphs but no oviparae, the

strain was concluded as being 'androcyclic' (anholocyclic). Under control conditions of continuous culture with a long photoperiod, no sexual forms were ever recorded.

Results. Oviparae were easily recognised as they were dark pink with characteristic thickened hind tibiae covered in sensoria. Males were identified by the possession of a penis and claspers. Following this procedure the S strain was classified as holocyclic, while the R_1 and R_2 strains were apparently androcyclic, both producing a few males but no oviparae. An example of the progeny sequence for the holocyclic strain (S) and of an androcyclic strain (R_2) is given in Figure 2.1.

Figure 2.1 Examples of the progeny sequence of a third generation individual aptera from a holocyclic (S) and androcyclic (R_2) clone, maintained at 18 ± 2°C and 10h/14h photoperiod, on leaf discs of Chinese cabbage




S strain
R_2 strain
Number of aphids
Days of nymph production
0
4
8
12
16
20
24
2
4
6
8
10
12
14
16

INSECTICIDE RESISTANCE

The 'resistance stability' (Banks and Needham, 1970; Beranek, 1974b) of the three laboratory strains (S, R_1, and R_2) and subcultures used in experiments and the resistant-type of field collected populations of M. persicae was assessed and monitored at frequent intervals.

The elevated level of a certain carboxylesterase in resistant M. persicae was first demonstrated by Sudderuddin (1973) and this biochemical characterisation is used to distinguish between insecticide resistant and susceptible aphids (Beranek, 1974a; Baker, 1977b). This correlation is a causal relationship, the carboxylesterase from resistant aphids hydrolysing OP compounds (Beranek and Oppenoorth, 1977) and the mechanism of enzyme action further elucidated by Church et al (1982).

Two biochemical assays were used to assess insecticide resistance;

TILE TEST

This colorimetric test was developed by workers at Rothamsted Experimental Station to detect insecticide resistance by qualitatively assessing the total esterase levels in individual aphids (Sawicki et al, 1978). It is considered a measure of total carboxylesterase levels since variation between aphids is reflective of variation in the levels of the 'resistance associated carboxylesterase'. It is a simple and rapid test but is limited in that it only distinguishes very resistant aphids (R_2) from those of moderate (R_1) or no resistance (S). The method used was that detailed in Sawicki et al (1978) with a few modifications.

Stock solutions

A. Phosphate buffer (pH 7), 0.9g disodium hydrogen orthophosphate dodecahydrate and 0.34g potassium dihydrogen orthophosphate dissolved in 250ml distilled water. Stored at 5°C.

B. 1-naphthyl acetate in acetone (.6%, 0.6g/100ml)

C. 1-naphthol in acetone (.03%,0.03g/100ml)

stable at room temperature for several weeks if tightly stoppered

D. Sodium dodecyl (lauryl) sulphate in distilled water (5%, 3.5g/70ml).

Immediately prior to the test the following reagents were prepared from the above stock solutions;

E. 0.5ml B mixed well with 50ml A - enzyme substrate.

F. 0.15g Fast Blue B salt dissolved in 15ml distilled water and 35ml D added - coupling reagent.

Procedure

Approximately 0.2ml (1 drop from a Pasteur pipette = 0.05ml - sufficiently accurate when dispensing E and F) of substrate (E) was pipetted into the wells of a cavity staining tile. One adult aptera was added to each well (except those which were to form standards) crushed and thoroughly mixed with the substrate using a glass rod. Approximately 0.05ml of coupling reagent (F) was added to the wells, including the standards, after 2 minutes incubation of the aphids in the substrate. The homogenates were then mixed well and left for 10 minutes before assessment. 5µl of C (dispensed using a 'Microcap') was added to the standard wells and mixed.

The intensity of the colour which develops is related to the esterase (carboxylesterase) level in each aphid. Conversion of the 1-naphthyl acetate to 1-naphthol and acetic acid by the esterase results in the binding of the Fast Blue salt to the released 1-naphthol to give a coloured product (Gomori, 1953). The intensity of the colour which developed was then compared against that in the standard wells; those staining much more intense being scored as highly resistant aphids while those less or as intense were scored as susceptible or moderately resistant.

Results

Plate 5 shows a typical tile test for aphids from the S, R_1, R_2 and OH clones. Table 2.4 shows the resistant-type of the clones of M. persicae used during the research, based on tile test results. All except the S, R_1 and OSR clones gave carboxylesterase intensities greater than standards implying high insecticide resistance.

The clones were then further examined using gel electrophoresis to determine more precisely their resistance levels.

CONVENTIONAL POLYACRYLAMIDE GEL ELECTROPHORESIS (PAGE)

This technique was used to separate out the component esterases of individual aphids into their bands of activity, permitting direct comparison of the level of the carboxylesterase conferring resistance. This technique allows aphids to be classified more accurately as susceptible, moderately resistant or highly resistant (Beranek, 1974a; Devonshire, 1975; Baker, 1977b). In addition to this advantage over the tile test assay, it also eliminates possible confusion due to carboxylesterase contribution from other sources eg aphid parasites

Plate 5 Tile test of S, R_1, R_2 and OH clones of Myzus persicae; the intensity of the colour which develops, compared with the standard, indicates the insecticide resistant-type of the aphid.

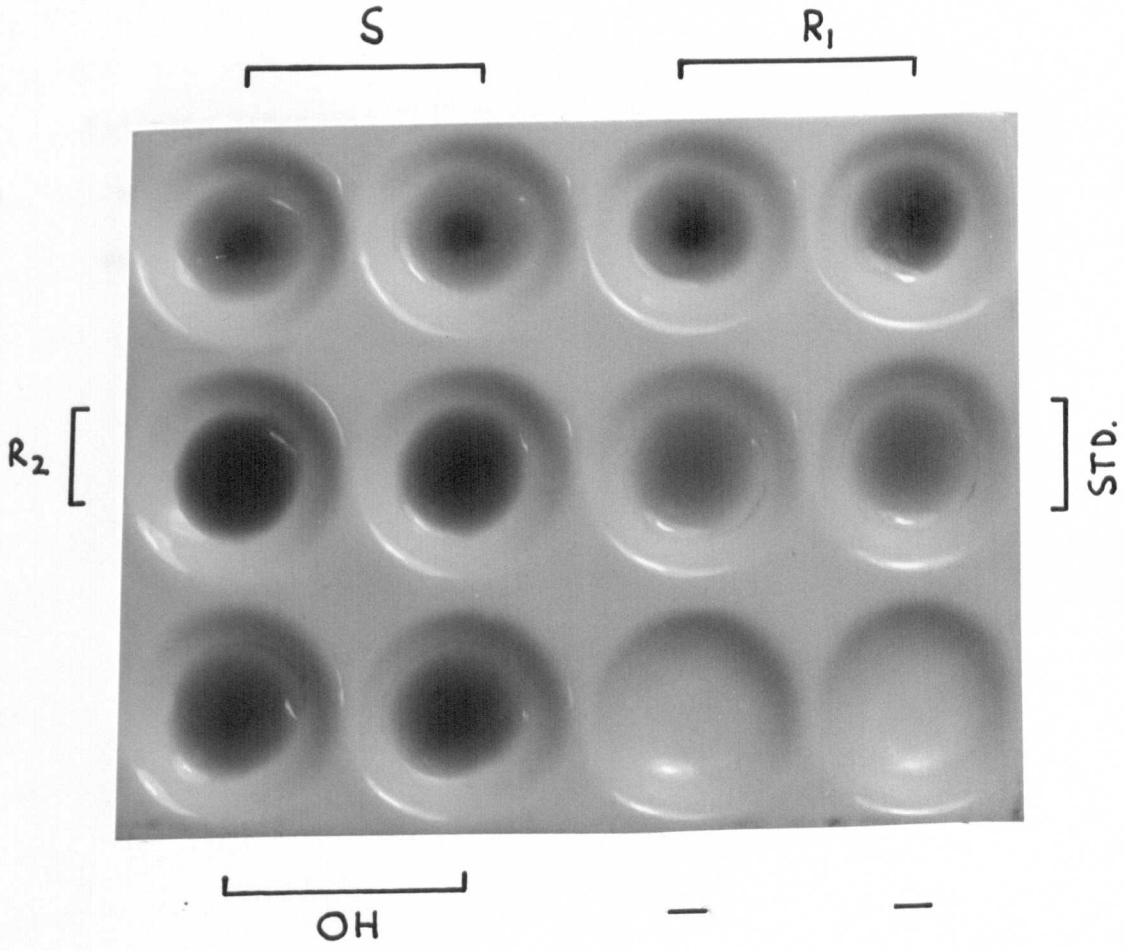


Table 2.4 Resistant-type of M. persicae clones as determined from the tile test assay.

Clone	Esterase intensity when compared against standard	Resistant-type
S	<	susceptible
R_1	=	moderately resistant
R_2	>	highly resistant
OSR	=	susceptible/moderately resistant
OH	>	highly resistant
Savoy	>	highly resistant
Kale	>	highly resistant
H	>	highly resistant

>: greater than standard

<: less than standard

=: equivalent to standard

(Castañera, Loxdale and Nowak, 1983). M. persicae possesses a distinct esterase pattern (Baker, 1978) so that electrophoresis serves to further confirm identification of field collected specimens. Following the demonstration of organophosphate hydrolysis by the 'resistance associated carboxylesterase' (Beranek and Oppenoorth, 1977), gel electrophoresis is now the most reliable and widely used measure of insecticide resistance in M. persicae, as well as in a number of other economically important aphid species (Stribley, Moores, Devonshire and Sawicki, 1983).

Method

The method of horizontal polyacrylamide slab gel electrophoresis followed a vertical method used previously by the author (Rose O'Doherty, 1981, unpublished B.Sc. Honours thesis, University of Glasgow; A Comparative Study of Two Clones of Myzus persicae (Sulz.) (Homoptera: Aphididae)) based partly on the methods of Devonshire (1975) and R.G. McKinlay (personal communication).

Solutions

1. Acrylamide solution.
   7.5g acrylamide, 0.25g bis-acrylamide (BIS) in 100ml TRIS citrate buffer (7.75% gel).
2. TRIS citrate buffer (gel buffer)
   4.59g TRIS (hydroxy methyl) aminomethane added to .525g citric acid in 100ml distilled water, made up to 1l.
3. Aphid homogenising fluid.
   100ml TRIS citrate buffer with 1g sucrose and 50µl Triton X-100.

4. Borate buffer (running buffer).

   7.27g boric acid, 15.75g sodium tetraborate (borax) (creamed in small amount of water first) in 1l distilled water.

5. Esterase stain.

   50mg 1-naphthyl acetate in 5ml acetone, 100ml phosphate buffer (see tile test section for recipe) and 50mg Fast Blue B salt. Made up immediately prior to staining.

6. Preserving solution.

   300ml ethanol, 100ml acetic acid and 100ml glycerol made up to 1l with distilled water.

Solutions 1 to 4 were stored at 5°C.

Preparation of gel

30ml acrylamide solution, 21.8µl tetramethylethylenediamine (TEMED) and 60µl Triton X-100 were mixed together. This solution was filtered through a sinterglass funnel (no. 3) under vacuum into a 250ml flask and then deaerated for 15 min. 1ml of a 12% ammonium persulphate (APS) solution (.12g in 1ml distilled water) was added and the solution then poured into a mould. The latter was assembled from a glass plate and slot former with dimensions, 230 x 115 x 2mm held together by a gasket. This gave a final gel thickness of 1mm. After polymerisation (1h), the slot former was separated from the glass plate and the gel (still on the glass plate) placed on the cooling plate of an LKB Multiphor (2117) electrophoretic unit. Filter paper wicks were used to make contact between the gel and the running (borate) buffer.

Preparation of aphid samples

Individual adult apterae were homogenised in 40µl of aphid homogenising fluid using a fine glass rod in a small glass vial. 10µl of each homogenate was added to each slot in the gel using a microsyringe. The two end slots were filled with the tracking dye, bromophenol blue (0.25% - 25mg in 10ml sample (TRIS citrate) buffer).

Running conditions

Gels were electrophoresed until the tracking dye had reached to within 1cm of the anodic (+ve) side, at a field strength of 7.5V/cm (current = 40mA). Throughout the run, the gel was maintained at 10°C by the circulation of mains tap water around the cooling plate.

Enzyme staining

Gels were stained overnight in 200ml esterase stain. They were then soaked in preserving solution overnight prior to permanent storage between plastic sheets after which they were photographed.

Results

Plates 6 and 7 show the esterases as separated in the various M. persicae strains following gel electrophoresis. 6 esterase bands were separated and from a study of the pattern in the S, R_1 and R_2 aphids, the third band from the origin (band 3) was identified as the 'resistance associated carboxylesterase'. This band corresponds to that referred to as E_4 by Devonshire (1975) and the resistance associated esterase (RAE) by Baker (1977b). Other bands were of similar activities with slightly different mobilities in some cases. Band 4 was of greater activity in most resistant M. persicae but was absent in the glasshouse derived OH clone. Devonshire (1975) recorded the absence of this band in other glasshouse resistant strains. The resistance status of the strains, from the comparison of

Plate 6 Bands of esterase activity stained with 1-naphthyl acetate following gel electrophoresis of individual adult apterae of the S, R_1, R_2 and OH clones of Myzus persicae (the reason for the 'cleared' area in band 3 of the R_2 aphids is unknown).

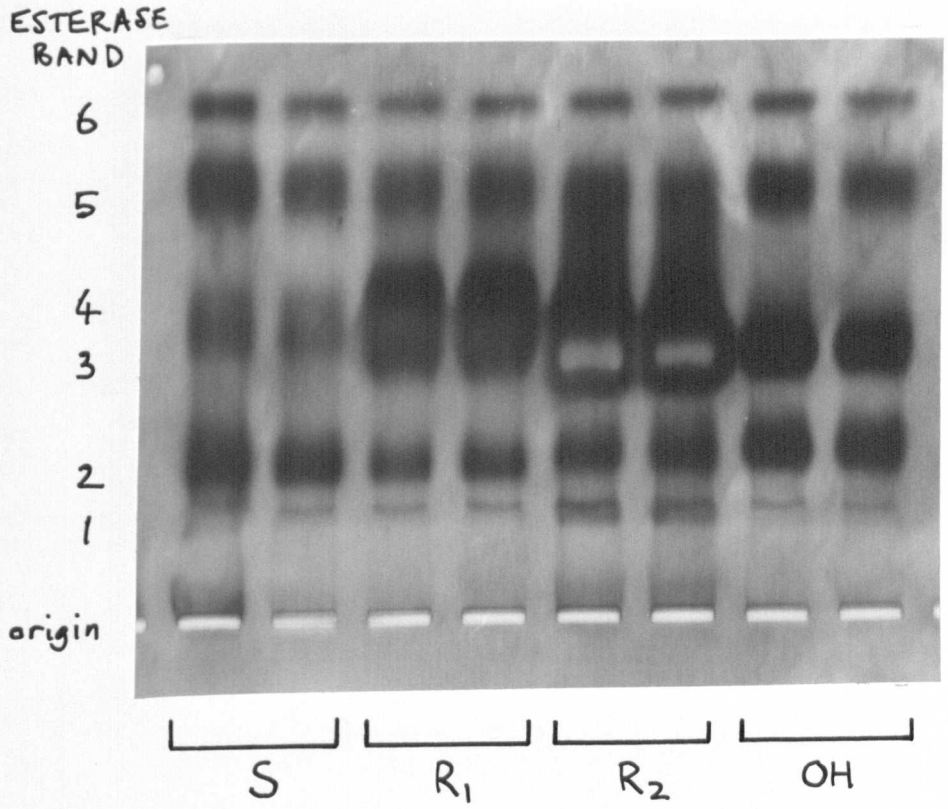
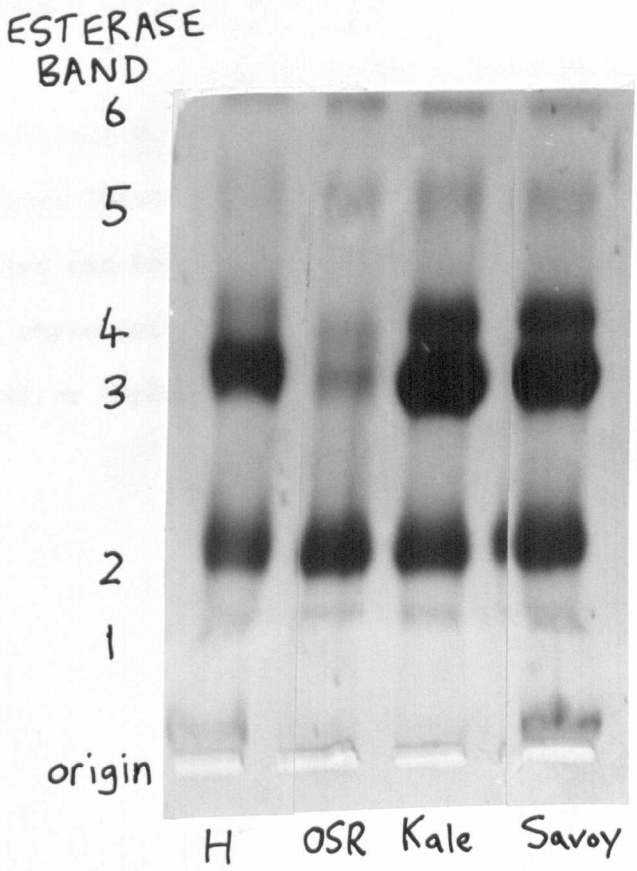

ESTERASE BAND
6
5
4
3
2
1
origin
S
R1
R2
OH

Plate 7 Bands of esterase activity stained with 1-naphthyl acetate following gel electrophoresis of individual adult apterae of the H, OSR, Kale and Savoy clones of *Myzus persicae*.


ESTERASE
BAND
6
5
4
3
2
1
origin
H
OSR
Kale
Savoy

band 3 is presented in Table 2.5. The highly resistant nature of all except the OSR clone was confirmed and the OSR clone classified as moderately resistant. Between strains classified as highly resistant and the R_2 strain itself there was a degree of quantitative variation in band 3 activity which corresponded with the tile test results.

Highly resistant M. persicae are of limited distribution in Britain at present (Devonshire, Foster and Sawicki, 1977; Woodford, Harrison, Aveyard and Gordon, 1983), with northern England being one of the regions where such aphids have previously been recorded, including Yorkshire (Sykes, 1977; Sawicki et al, 1978).

Table 2.5 Resistant-type of *M. persicae* clones as determined following gel electrophoresis

Clone	Band 3 activity (resistance associated esterase)	Band 4 activity	Resistant-type
S	+ (slightly increased mobility)	-	susceptible
R_1	++	X	moderately resistant
R_2	+++	X	highly resistant
OSR	++	-	moderately resistant
OH	+++	-	highly resistant
Savoy	+++	X	highly resistant
Kale	+++	X	highly resistant
H	+++ (slightly increased mobility)	-	highly resistant

\+ low activity
++ intermediate activity
+++ very high activity

X present
\- absent

CHAPTER THREE

DEVELOPMENT OF A SYSTEM TO DETERMINE APHID SUPERCOOLING POINTS

SUMMARY

In the two systems developed to determine aphid supercooling points, cooling was achieved by the use of a water-cooled, two-stage thermoelectric (Peltier) module, which could provide the cold stage with a lower minimum temperature of -55°C. The current was supplied by a bipolar controller, with manual control of cooling (1°C/min) in System 1. Aphids were attached to thermocouples (type T) using petroleum jelly and placed within a specimen holder mounted onto the surface of the cold stage. The thermocouples, connected via cold junction compensators to potentiometric chart recorders, sensed and recorded aphid body temperatures and supercooling points. The need for constant manual control in System 1 was overcome by the incorporation of a prototype automatic controller which provided a range of cooling rates and lower minimum temperatures, and a manual control option (System 2).

Recording of supercooling points was modified by using a 12-channel, cold junction compensated, thermocouple converter interfaced to a microcomputer, colour monitor and dot-matrix printer. A program was written in Basic (Applesoft) to incorporate individual polynomial descriptors for the thermocouples (mV vs °C), produce a digital and graphical display of cooling on the monitor, and to sense and record aphid supercooling points. On completion of experiments all supercooling point temperatures are output to the printer. The graphical display may also be printed or stored on disc.

System 2 constitutes a fully interfaced and compatible system for supercooling point determination and was used in all subsequent experiments.

INTRODUCTION

In freezing-susceptible species the supercooling point represents the instantaneous low-temperature death point (Sømme, 1982), since the formation of ice in the body tissues and fluids in such organisms is fatal. Freezing-susceptible and freezing-tolerant species are classified into their respective categories based on their ability to survive freezing at the supercooling point. Supercooling points have been widely used as an index of cold hardiness in freezing-susceptible arthropods (Sømme, 1982) since they provide rapid assessment of individual cold hardiness in the laboratory and when conducted in a standard manner, enable comparisons of cold hardiness to be made (Salt, 1961).

Equipment to determine supercooling points has two main features;

(a) a cooling system which provides a controllable and gradual rate of temperature decrease from the start temperature to below the lowest supercooling point of the sample

(b) a sensing and recording system to detect and display the rise in temperature which occurs at the supercooling point as a result of the release of the latent heat of fusion associated with ice formation in the organism. The supercooling point is the lowest temperature recorded prior to the rise in temperature.

Supercooling point equipment cannot be purchased as complete and integrated systems and consequently investigators have developed their own methods utilising various cooling units such as refrigerated alcohol baths (Block and Young, 1979; Ring and Tesar, 1980; Miller, 1982), freezers (Zachariassen, 1977) and cryostats (Powell, 1974;

Sømme and Conradi-Larsen, 1979) or thermoelectric systems (Luff, 1966, Bale, 1980). Potentiometric chart recorders are the most common method of recording the supercooling points.

This chapter describes the development of the method employed to determine the supercooling points of aphids, with modifications to incorporate automatic cooling and a computer-based recording system.

SYSTEM 1

COOLING

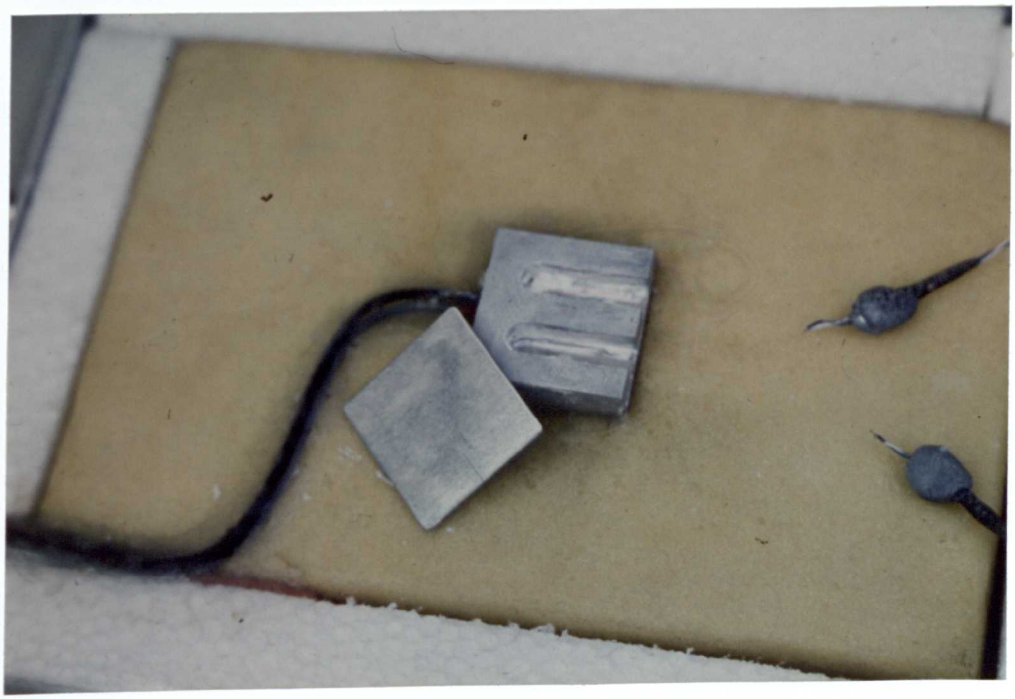
This was achieved thermoelectrically based on the Peltier effect; when a current is passed between two semiconductor elements, a temperature differential is created between the hot and cold surfaces of the module. Waste heat from the hot surface was removed by circulating mains tap water at a flow rate of 1l/min. A flow meter was incorporated into the system as a safety precaution; if the water supply fell below the set rate or if there was an interruption in the flow, the current supply was automatically terminated thus preventing overheating of the module.

A two-stage thermoelectric module (Cambion 806-1008-01) was used since it could provide a lower minimum temperature of -55°C. The cold surface of the module was 3.2cm^2 and an aluminium specimen holder with a removable lid was mounted onto the surface to accommodate the insects when attached to their thermocouples. The whole assembly was surrounded by expanded polystyrene within a purpose-built metal container to insulate the experimental chambers from the ambient temperature of the laboratory (Plates 8 and 9).

The current was supplied by a compatible bipolar controller (Cambion 809-3040-01), which used in conjunction with a range extender (Cambion 94-809-1099-01) achieved a temperature span of +25 to -50°C (Plate 10). The rate of cooling was controlled manually. The medium range on the extender was selected and the appropriate dial adjusted until the minimum temperature in this range was reached; the low range was then selected and the dial further adjusted until supercooling

Plate 8 The original two-chambered aluminium specimen holder, with removable lid, mounted onto the cold surface of the thermoelectric module. The thermocouples were held in position by using 'blu-tack' supports.

Plate 9 The purpose-built metal container, in which the module was surrounded by expanded polystyrene. The spring attachment at the front of the box held the thermocouples in position within the modified specimen holder. The inlet pipe for water circulation can be seen at the rear of the assembly.



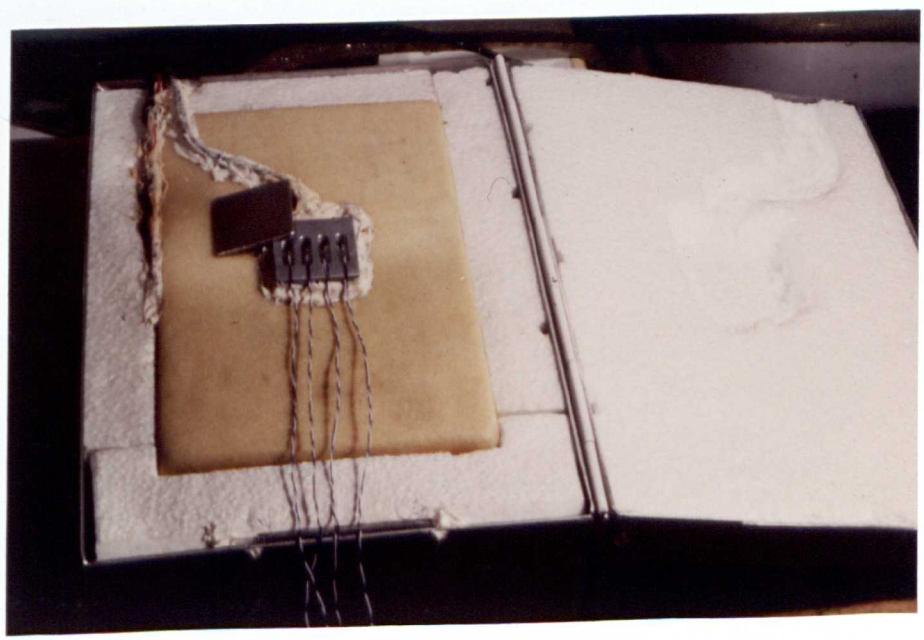


Plate 10 The bipolar controller and range extender used to cool the thermoelectric module.


COOL
HEAT
Bipolar Controller
POWER
CAMBION
MED
H

points were recorded. The medium range operated over a temperature range of +25 to 0°C and the low range over 0 to -50°C; after initial use as described the range extender was modified so that only the low range was required during cooling and the linearity improved, so allowing more dial divisions/°C and therefore easier manual control. Cooling was controlled at 1°C/min, in accordance with the proposal by Salt (1966b), by direct observation of the cooling curve. The purpose of such standardisation is to permit comparisons between studies (Sømme, 1982).

SENSING AND RECORDING OF APHID BODY TEMPERATURE AND SUPERCOOLING POINTS

Type T copper-constantan thermocouples were used as the temperature sensors. Thermocouples have been used to sense insect body temperature for some time (Nobili and Melloni, 1831; Bachmetjew, 1901). They are of particular value in insect supercooling point studies being small in size and thermal mass and therefore very sensitive to the relatively small rise in temperature at the supercooling point. In this study thermocouples were made from a pair of twisted PTFE-insulated, 0.2mm diameter wires which had a junction response time of less than 15msec. They were connected to potentiometric chart recorders (Pharmacia Rec-2, 2 channel and Servoscribe 1s, 1 channel) via cold junction compensators (Ancom, Omega MCJ-T); these provide a reference thermal emf, equivalent to 0°C from a resistor bridge and mercury battery with internal compensation for variation in the ambient temperature.

Thermocouples were individually calibrated prior to experimental use since their response is only approximately linear over their temperature range and there is variation between thermocouples. The response of each thermocouple, at intervals of 2°C over the temperature range of +20 to -50°C as measured by a platinum resistance thermometer (Ancom PTE 361) connected via an adaptor (Ancom RTC-1, resistance thermometer/dvm adaptor) to a digital multimeter (Thurlby 1503 high resolution), was marked on the appropriate chart. Supercooling point temperatures were read from these calibration charts and could be resolved to within 0.2 chart divisions, equivalent to $\pm$ 0.1°C (Fig. 3.1). Each thermocouple was 'permanently' coupled to the same compensator and chart recorder channel.

System 1, as described above (Plate 11), was used to assess the supercooling ability of (i) laboratory maintained Myzus persicae (inherent cold hardiness), (ii) most of the acclimated individuals (M. persicae) and (iii) in preliminary experiments with Aphis fabae, the results of which are included in Chapters Four and Seven.

The system proved reliable and accurate but had a number of limitations;

1. Constant manual control was necessary to maintain the cooling rate at 1°C/min.
2. The cold stage was initially provided with a specimen holder to accommodate two thermocouples, each of which held 1 adult or 2 small nymphal aphids, attached using a little petroleum jelly (Plate 8). This was later replaced with a 4-chamber holder, taking 4 experimental

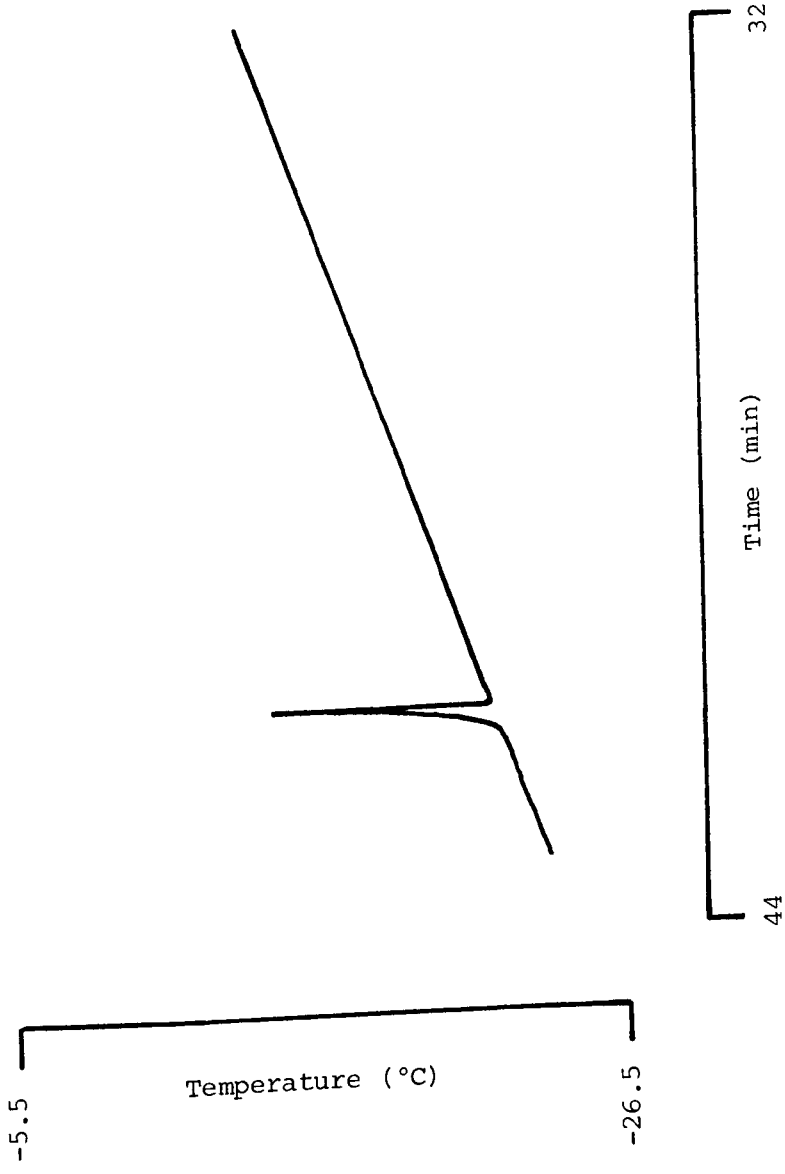


Figure 3.1 Part of the cooling profile for an adult M. persicae, as recorded on a potentiometric chart recorder, showing the rebound at the supercooling point (= -21.5°C)

Plate 11 System 1;

(a) cooling unit (thermoelectric module)

(b) bipolar controller

(c) range extender

(d) cold junction compensator

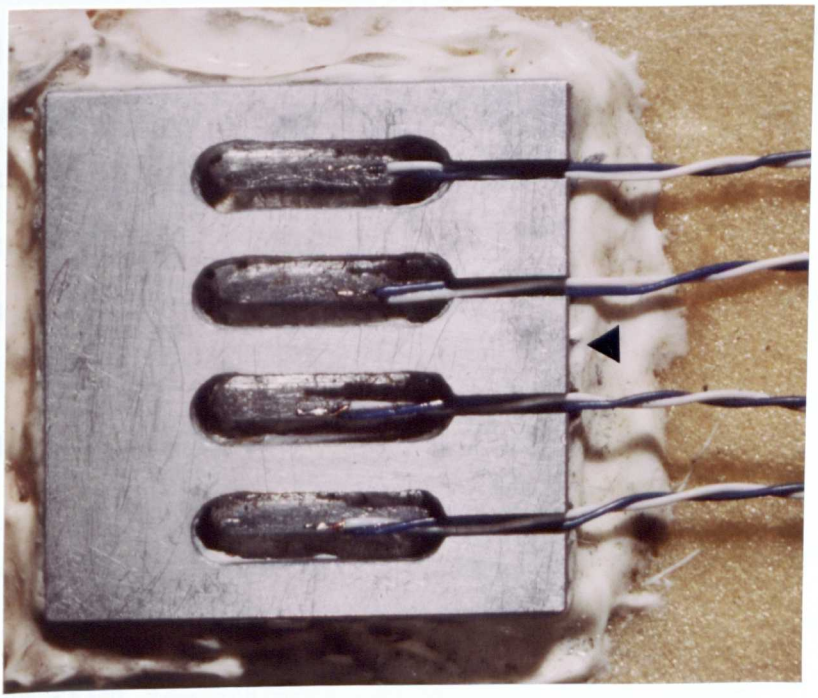
(e) potentiometric chart recorders


a
b
c
d
e

thermocouples and one fixed into the block (Plate 12). When both chart recorders were available, the maximum of three thermocouples could be used per experimental run. However the Pharmacia was of limited availability and therefore this potential was not always realised.

The incorporation of automatic cooling and multichannel, computer-based recording of supercooling points overcame these limitations, as described in the development of System 2 below.

Plate 12 Four-chamber specimen holder with experimental thermocouples (to hold aphids); the position of the block thermocouple (not shown) is indicated.



SYSTEM 2

COOLING

Automatic temperature controller.

Automatic control of thermoelectric cooling is only available commercially with special equipment, for example with cooled microscope stages in which the rate of cooling is often preset and therefore of limited use for other needs.

The requirements of an automatic controller to be used in this work were submitted as a preliminary design to Vickers Calibration (Southampton) who proceeded to build the prototype model (Plate 13). This replaced the range extender used in the first system. To use the controller in conjunction with the bipolar controller, the thermistor in the cold stage of the module was replaced by a linear sensor, a silicon diode (probe). Full electronic details of controller operation are given in Bale, O'Doherty, Atkinson and Stevenson (1984).

The controller provides cooling rates of 0.4 to 3°C/min, which is extended to 15°C/min under the manual control option. Under manual control different start temperatures can be used; under automatic control the start temperature was set at 15°C and was used in all supercooling point studies. A range of preset lower-limit temperatures, -5 to -45°C (later extended to -50°C), were included although in practice the selection of these prior to experiments was not necessary since the end of an experiment was a function of the time selected on the microcomputer (see below) and for this reason the lower-limit was always set at -50°C.

Plate 13 Cooling in System 2; bipolar controller and automatic temperature controller.


LOW LIMIT
DEVIATION
FREEZING RATE
MANUAL TUNE
MANUAL
AUTO
AUTOMATIC TEMPERATURE CONTROLLER
POWER
OFF
ON
START
STOP
RESET
PROBE
VICKERS CALIBRATION
WOOLSTON, SOUTHAMPTON
COOL
HEAT
TEMPERATURE SET
Bipolar Controller
POWER
CAMBION

Automatic cooling commenced when the start switch was depressed. Following each experiment the start temperature was re-established by depressing the reset switch; this took less than two minutes, saving excessive waiting time between successive experiments. Cooling could be terminated by using the stop switch, after which the reset temperature could be established. The centre-zero meter indicated when the start temperature had been achieved (reading = 0).

Water supply to the Peltier module.

A closed system to circulate water around the thermoelectric module later replaced the mains supply. A Grant circulator (FH 15) in conjunction with a Grant flow cooler (FC 15) circulated water at 10°C around the stage ensuring efficient removal of waste heat from the Peltier unit.

RECORDING

Computer-based visualisation and recording of supercooling points

The thermocouples were interfaced to an Apple II Europlus microcomputer via a compatible thermocouple converter (CIL Microsystems Ltd., PCI 1002) (Plates 14 and 15). The converter has 12 input channels (4 to 15) to accommodate type T thermocouples and each is provided with a platinum resistor cold junction compensation (CJC) signal from channel 3; CJC was therefore provided by one source, compared to the individual and therefore variable CJC sources associated with the Ancom cold junction compensators coupled to potentiometric recorders. The error associated with fluctuations in ambient temperature does not exceed 1°C for a 50°C shift during thermocouple converter use, compared with 1°C for a 15°C shift associated with Ancom cold junction compensators. Channels 1 and 2

Plate 14 Recording in System 2; microcomputer (Apple II Europlus) with disc drive operation, interfaced to the thermocouple converter.

Plate 15 The thermocouple converter with 12 input channels;

4 - experimental thermocouple 1

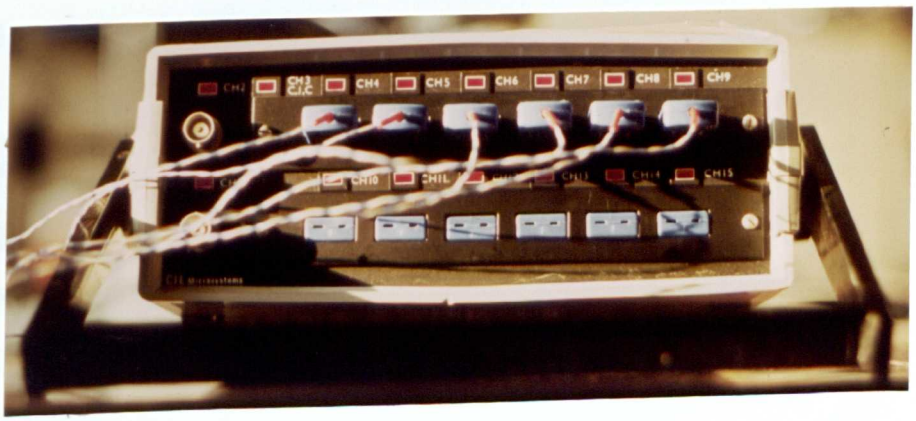
5 - reference (bath) thermocouple

6 - block thermocouple

7 - experimental thermocouple 2

8 - experimental thermocouple 3

9 - experimental thermocouple 4


CH2
CH3
CH4
CH5
CH6
CH7
CH8
CH9
CH10
CH11
CH15

are general-purpose millivolt inputs and channel 16 is connected internally to a 1V reference voltage.

Software written in Basic (Applesoft) was provided with the converter (program PCI 1002) and when used in conjunction with the programmable read-only memory (PROM) routine of the converter measured the millivoltage (mV) of each channel sequentially, providing 12 bit digital values which were then reproduced digitally on a monitor. This program used a quartic polynomial descriptor to convert the mV of each thermocouple to °C, and was subsequently modified to display digitally the mV of each thermocouple along with the temperature. The resulting program (COOL CAL) was used to individually calibrate each thermocouple, by determining the mV produced at a known temperature (measured using the platinum resistance thermometer as previously described) at 2°C intervals over the operational range of +20 to -50°C. This data was then analysed using an appropriate program (Nie et al, 1975) on a main-frame computer (Amdahl 470/V7) to produce fourth- or fifth-order polynomial regression descriptors (mV vs temperature) for each thermocouple. During calibration and in subsequent experiments each thermocouple was used in the same channel of the converter.

A program (COOLX3) was then written in Basic (Applesoft) to;

(1) produce a digital and graphical display of cooling on the colour monitor,

(2) sense and record aphid supercooling points.

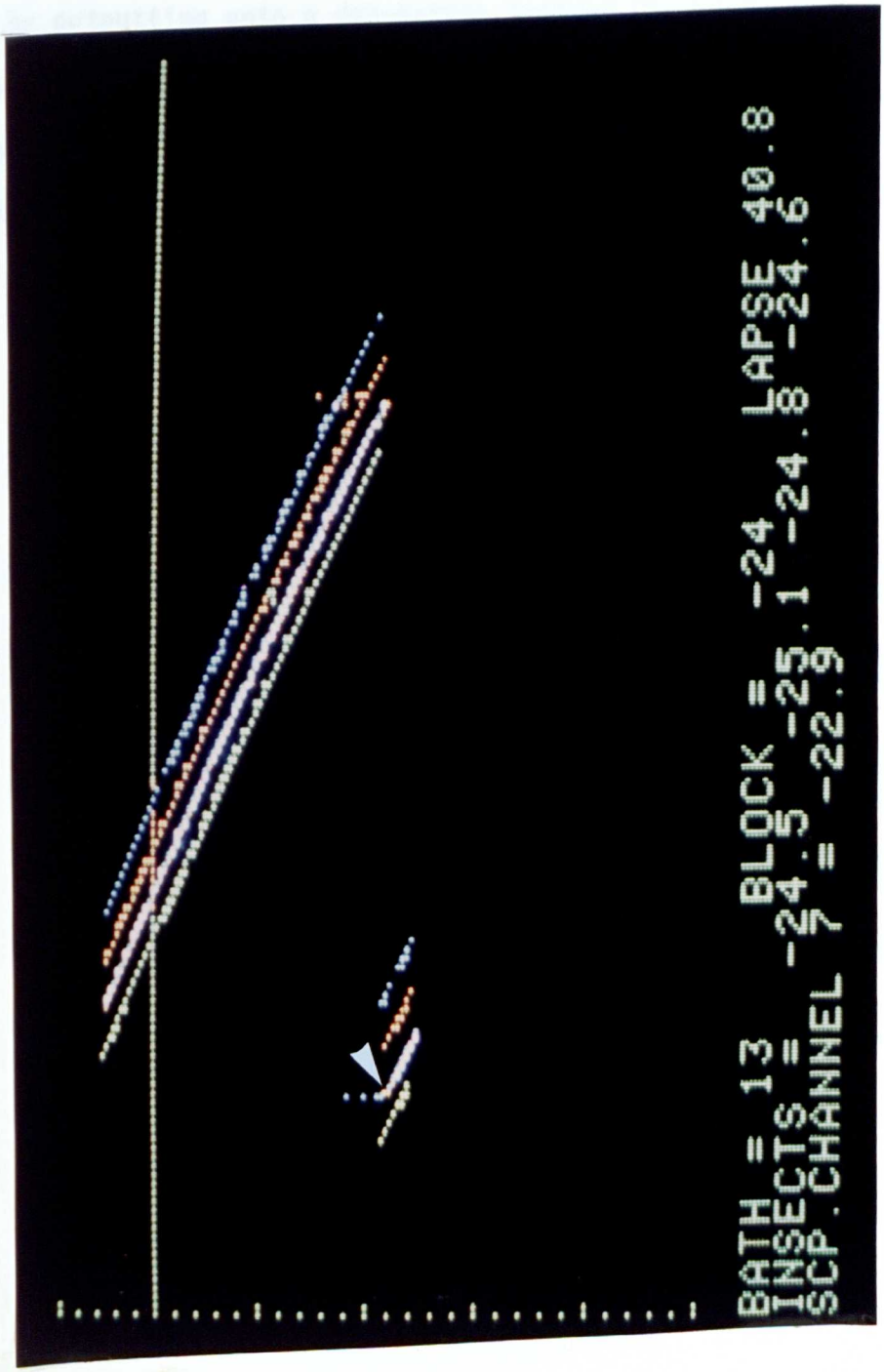
The program begins by requesting the experimental details, which are typed in together with the duration (minutes) of the experiment. Each thermocouple was sampled at 3s intervals and the digital values from

the PROM routine were converted to °C after reference to the incorporated polynomial descriptors. A continuous digital display of cooling for each thermocouple (4 experimental, 1 reference, 1 block) was then produced on the colour monitor.

When the block thermocouple reaches 5°C, the graphical display (high resolution graphics) of cooling is plotted on the monitor; this involves only the aphid bearing thermocouples, the temperatures of which are plotted in distinguishing colours against time. To accommodate the complete cooling curve as a single display, after 35 minutes the lines are reset to the left hand side of the screen. The duration of the run is also displayed together with the time elapsed (Plate 16).

Supercooling points were sensed as an increase in temperature in excess of a preset value over the previous reading for that thermocouple; this value was set at 0.2°C which was smaller than the heat release at all supercooling points but large enough to exclude all other 'background noise'. The supercooling point temperature (the lowest temperature recorded before the rise) is stored in the dimensional array of the program. When a supercooling point is recorded, this temperature is displayed digitally on the screen until replaced by the next supercooling point temperature. The recording of further supercooling points on the same channel is then suppressed for 40s so that the continued rise in temperature associated with a supercooling point is not regarded as further supercooling points. Cooling of a number of aphids (e.g. nymphs) on a single thermocouple is possible since the dimensional array can store up to 10 supercooling points per thermocouple. It is possible to extend

Plate 16 Graphical and digital display of cooling as observed on the colour monitor. Supercooling point ('rebound') on channel 7 is arrowed.


BATH = 13 BLOCK = -24 LAPSE 40.8
INSECTS = -24.5 -25.1 -24.8 -24.6
SCP.CHANNEL 7 = -22.9

experiments by intervals of 5 minutes, if necessary, to record the supercooling points of unfrozen individuals; otherwise the experiment is terminated by outputting onto a dot-matrix printer (Epson MX-80 F/T III) the recorded supercooling points for each thermocouple with accompanying block and reference thermocouple temperatures and the experimental details (Fig. 3.2). The graphical display may also be output to the printer (Fig. 3.3) or stored on disc for future reference.

MYZUS PERSICAE

INSECT 1 SUPERCOOLING PT = -24.5
BATH = 11.9
BLOCK = -25.4

INSECT 2 SUPERCOOLING PT = -25.1
BATH = 12.3
BLOCK = -25.8

INSECT 3 SUPERCOOLING PT = -24.2
BATH = 12
BLOCK = -25.4

INSECT 4 SUPERCOOLING PT = -21.4
BATH = 11.5
BLOCK = -22.9

Figure 3.2 The print-out of supercooling points recorded for four adult M. persicae (using System 2), along with the block and reference (bath) temperatures

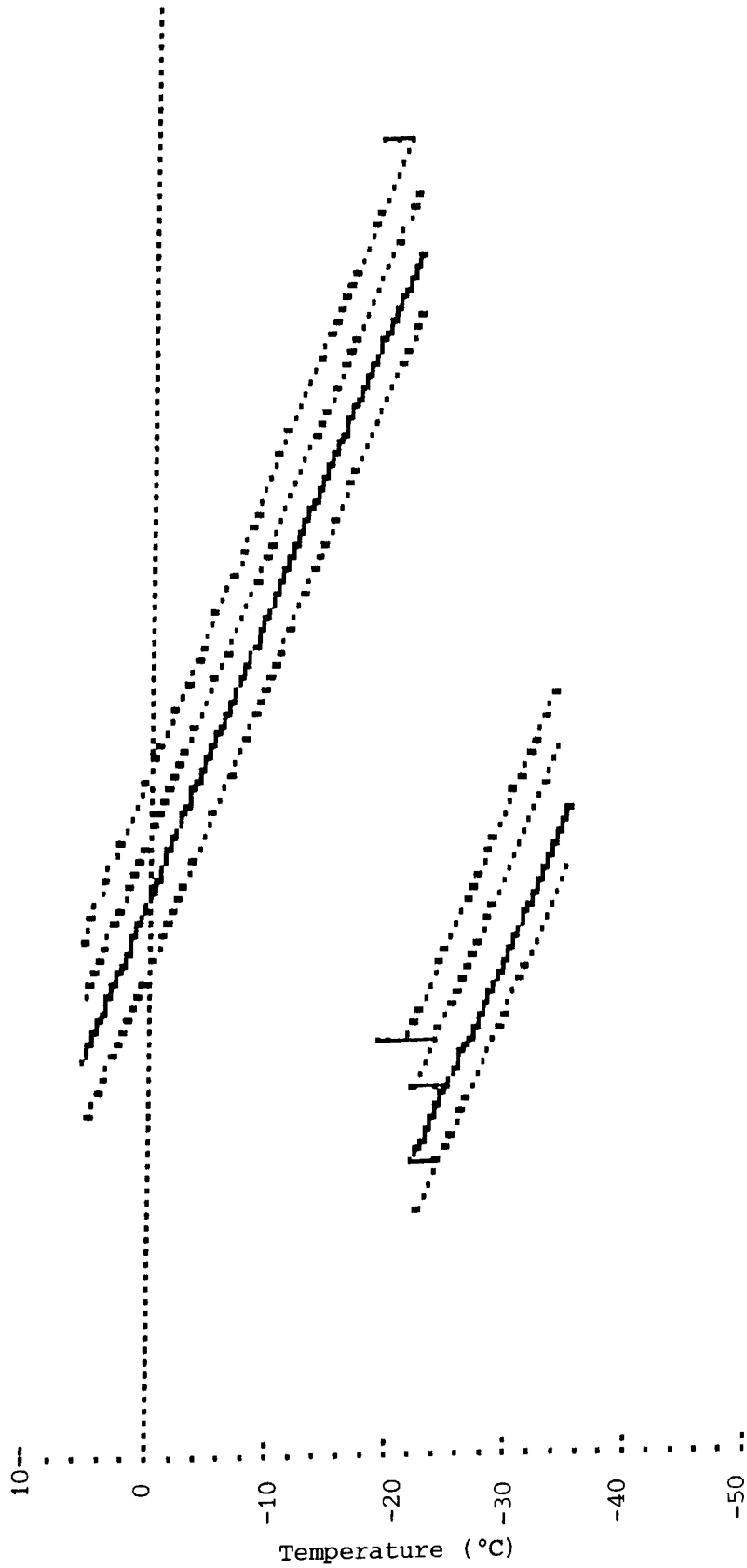


Figure 3.3 The print-out of the graphical display of cooling; the temporary rise in temperature ('rebound') at each of the four supercooling points shown (see Fig. 3.2) has been drawn in as a continuous line to indicate the channels from which they originate

CORRECTION FACTOR

Supercooling points determined using System 2 were consistently more cold hardy e.g. the mean supercooling point of S strain adult apterae (reproductive) was -25.6 ± 0.20°C (n=20) compared with -24.2 ± 0.29°C on System 1. This was attributed to the increased accuracy of the sensing and recording technique in the automatic computer-based system, especially,

(a) the use of a polynomial regression term for each thermocouple to match mV against °C,

(b) the method of CJC in System 2 which was inherently more accurate (using a platinum resistance thermometer) compared with the error associated with less accurate and multiple CJC in System 1.

It was necessary therefore to calculate a 'correction factor' to make the data from System 1 comparable with that of the second system, particularly since many of the results collected using the first system served as control data. To calculate correction factors the three thermocouples connected to the Pharmacia and Servoscribe chart recorders and the platinum resistance thermometer were taped together and placed in a low temperature bath (Haake F3) filled with an antifreeze solution (polyethylene glycol (PEG)).

Starting at -28.6°C and at approximately 1°C intervals until 5°C (as measured by the thermometer), the temperature recorded by each thermocouple, based on the chart recorder calibration charts, was noted. The correction factor for supercooling points falling into each interval was calculted as the difference between the mean temperature of the three thermocouples and the known temperature (Table 3.1).

Table 3.1 Correction factors for chart recorder supercooling point temperatures from System 1

Temperature (°C)					
Platinum resistance thermometer	Chart recorder			Mean recorded temperature	Correction factor
	Servoscribe	Pharmacia			
		1	2		
-28.6	-26.7	-27.8	-27.9	-27.5	1.1
-27.9	-26.3	-27.2	-27.4	-27.0	0.9
-26.7	-25.3	-25.9	-26.0	-25.7	1.0
-25.7	-24.4	-24.9	-24.5	-24.6	1.1
-24.7	-23.5	-23.8	-24.1	-23.8	0.9
-23.7	-22.5	-22.7	-23.2	-22.8	0.9
-22.7	-21.6	-21.9	-22.0	-21.8	0.9
-21.8	-26.7	-20.7	-21.0	-20.8	1.0
-20.8	-19.5	-19.9	-20.0	-19.8	1.0
-19.8	-18.7	-18.8	-19.0	-18.8	1.0
-18.9	-17.8	-17.9	-18.1	-17.9	1.0
-17.8	-16.8	-16.8	-17.2	-16.9	0.8
-16.7	-15.6	-15.8	-16.1	-15.8	0.9
-15.7	-14.8	-14.7	-15.2	-14.9	0.8
-14.7	-14.0	-13.7	-14.3	-14.0	0.7
-13.9	-13.0	-12.8	-13.4	-13.1	0.8
-12.0	-11.5	-10.5	-11.5	-11.2	0.8
-11.0	-10.6	-9.8	-10.3	-10.2	0.8
-9.9	-9.3	-8.7	-9.0	-9.0	0.9
-9.0	-8.3	-7.8	-8.3	-8.1	0.9
-8.0	-7.2	-7.0	-7.2	-7.1	0.9
-7.0	-6.8	-6.0	-6.3	-6.4	0.6
-6.0	-5.4	-5.2	-5.0	-5.2	0.8
-5.0	-4.5	-4.3	-4.0	-4.3	0.7

All supercooling point data collected using System 1 was therefore adjusted accordingly, with means and standard errors recalculated prior to statistical analysis.

All data in this thesis is corrected (except O'Doherty and Bale (1982) which was published before development of System 2).

DISCUSSION

System 2 (Plate 17) has made use of commercially available components, includes a specially constructed prototype automatic controller and involved the development of software for the microcomputer; as such it constitutes a fully interfaced and compatible system for aphid supercooling point determination. The original idea and the development of the equipment owes much to the expertise of several colleagues and not merely that of the author of this thesis. The system was developed entirely during the course of this research and thus initially its use was confined to studies on aphid cold hardiness. Subsequent work however has included supercooling point determination of nematodes and slugs as well as arthropods other than aphids.

The cooling system with an electronic basis, is easy to use and includes reliable automatic control. Compared with systems using low temperature baths, it does not involve the use of liquids e.g. polyethylene glycol or alcohols which may be corrosive and hazardous; an additional minor but practical point is that it is also 'cleaner' to use, since such liquids are not used.

The computer-based recording system is compatible with other cooling methods and provides up to 12 experimental thermocouples which could all be used with cooling systems such as refrigerated baths and freezers. Individual calibration of thermocouples and the use of a thermocouple converter has resulted in a system providing maximised accuracy. At a cooling rate of 1°C/min, supercooling points are accurate to ± 0.2°C.

Plate 17 System 2;

(a) cooling unit (thermoelectric module)

(b) bipolar controller

(c) automatic temperature controller

(d) Grant circulator

(e) Grant flow cooler

(f) thermocouple converter

(g) microcomputer and disc drives

(h) dot-matrix printer


a
b
c
d
e
f
g
h

The use of a microcomputer confers a degree of flexibility not obtainable with chart recorders. Software can be specially designed to the demands of the experiment or organism, particularly in terms of the information displayed on the monitor. The program described here (COOLX3) has already been modified to produce a horizontal graphical display which facilitates visual recognition of the supercooling points of aphid nymphs or other small arthropods (J.D. Knight, personal communication). Since programs are written in Basic (Applesoft), modifications are straightforward. The use of machine language however would have the advantage of increasing the frequency of mV measurements of thermocouples during cooling. Another advantage of microcomputer use is that it can be used for general purposes or interfaced to other apparatus when not being used with the supercooling equipment, a feature less likely to be applicable to chart recorders.

As described the system has been used to study the supercooling capacity of all stages of aphid development from first instar nymphs to adults and their eggs (fresh weights of 40,500 and 25µg respectively). The supercooling ability of M. persicae is discussed in the next chapter, that of aphids in relation to feeding in Chapter Five and that of A. fabae in Chapter Seven.

CHAPTER FOUR

STUDIES ON THE SUPERCOOLING ABILITY OF LABORATORY MAINTAINED AND NATURAL POPULATIONS OF *MYZUS PERSICAE*

SUMMARY

The inherent supercooling ability of three strains of Myzus persicae, susceptible (S), moderately resistant (R_1) and highly resistant (R_2) to insecticides was assessed and was found to be similar; all stages of the aphid were capable of supercooling extensively to below -20°C. There was a progressive loss of cold hardiness with increasing age, with first instars the most cold hardy stage.

When development occurred at 5°C, first instars of the S and R_2 strains extended their supercooling potential and maintained this level of cold hardiness through to the third instar. Differences between the two strains were revealed in the fourth instars and adults, with R_2 individuals less cold hardy. In addition, R_2 adults acclimated at 5°C lost cold hardiness while S individuals showed no change. The progeny of adults (S and R_2) acclimated at 5°C were more cold hardy than those produced by unacclimated individuals.

Contact with surface moisture caused inoculative freezing in some first instar nymphs and adults when supercooled, but the majority were unaffected. When wetted in a detergent solution this protection was lost and all individuals experienced inoculative nucleation when the water froze. Holocyclic morphs (oviparae and males) produced under laboratory conditions, possessed similar levels of supercooling ability to individuals in parthenogenetic cultures. Eggs were capable of supercooling to below -30°C.

Seasonal studies of supercooling ability showed that first instars possessed considerable cold hardiness all year. As winter

progressed adults lost cold hardiness such that supercooling point distributions formed distinct high (HG) and low (LG) groups. Adults with poor (LG) supercooling ability formed variable proportions of winter samples collected in both 1982-83 and 1983-84, from a range of host plant species. Clonal differences and adult age did not account for these differing levels of supercooling ability within samples. In spring, cold hardiness increased so that adults possessed extensive and consistent supercooling ability throughout summer comparable to that of laboratory maintained individuals.

INTRODUCTION

Terrestrial arthropods in common with most invertebrate poikilotherms are broadly classified into two main groups according to the physiological and biochemical adaptations they adopt to survive low temperatures encountered during overwintering (Danks, 1978; Ring, 1980; Block, 1982b). Freezing-tolerant species can survive ice formation in their extracellular fluids and are characterised by poor supercooling ability (Sømme, 1982). Most overwintering arthropods are freezing-susceptible (Block, 1982b) and avoid the likelihood of a freezing death by supercooling, often to a considerable extent below their true freezing point, thus maintaining the body tissues and fluids in the unfrozen state. A measure or index of the cold hardiness of freezing-susceptible species can therefore be obtained by assessing their supercooling potential. As described in the previous chapter, this can be determined experimentally by supercooling point measurement and when the experimental procedure is standardised, the method provides a basis for comparing the cold hardiness of freezing-susceptible species (Salt, 1961).

Sømme (1982) reviewed the extensive literature on the supercooling ability of freezing-susceptible terrestrial arthropods; the majority demonstrate a general pattern of cold hardiness in which supercooling ability is influenced by a number of inherent and environmental factors.

Many species demonstrate seasonal changes in cold hardiness, undergoing a natural acclimatisation process in preparation for winter, observed as an increase in supercooling ability. That such

changes occur under natural conditions was first reported by Salt (1957) in freezing-tolerant Bracon cephi larvae. Since then the same pattern of increased supercooling ability with the onset of winter has been shown to exist in many freezing-susceptible species including those in temperate habitats (Sømme, 1981, 1982; Block, 1982b). In spring, supercooling ability decreases to the pre-winter level so that in most species cold hardiness is at a maximum in winter. This natural increase in cold hardiness occurs primarily in response to decreasing temperatures in autumn (Lee and Baust, 1981), as borne out by experimental acclimation in which such shifts in supercooling ability can be induced by laboratory exposure to low temperature (e.g. Young and Block, 1980).

The evacuation of the gut contents in preparation for overwintering, which occurs in most arthropods, accounts for some of the increased supercooling potential observed during acclimatisation (Salt, 1961; Sømme, 1982). The influence of gut contents and feeding on supercooling ability is considered in Chapter Five. The most common mechanism, however, by which this cold hardening is achieved is by the accumulation of natural antifreeze or cryoprotective substances which as a result of depressing the true freezing point, increases supercooling potential (Duman, 1982). Chapter Six considers possible biochemical correlates of aphid cold hardiness.

Another factor found to influence supercooling ability is contact with external moisture which can lead to inoculative nucleation and thus internal freezing in overwintering arthropods. Freezing mortality may therefore occur at sub-zero temperatures well above the level of inherent supercooling capacity (Salt, 1963; Sømme, 1982). In

comparison with other factors influencing supercooling ability there has been little research on surface moisture effects. Some freezing-susceptible arthropods avoid this potential mortality factor and seek out dry, sheltered overwintering sites or overwinter within protective cocoons (Danks, 1978). However Myzus persicae which overwinters anholocyclically on the aerial, exposed parts of its hosts does not have such protection and may be more affected by contact moisture.

Previous supercooling point studies on aphids have shown all species including M. persicae to be freezing-susceptible (Powell, 1974, 1976; Parry, 1978; Sømme, 1982). Powell (1973, 1974, 1976) studied tree feeding aphid species, assessing supercooling potential both in the laboratory and under field conditions, while previous studies of M. persicae (Parry, 1978) have been limited to assessment under laboratory culture.

The aim of the research described in this chapter was to assess the cold hardiness of M. persicae by studying age, seasonal and clonal variations in supercooling ability and to compare the cold hardiness characteristics of this species with those of other arthropods.

MATERIALS AND METHODS

The experimental procedures associated with the supercooling point studies are given in the relevant sections below.

Supercooling points were determined as indicated in Chapter Three. Following each supercooling point experiment aphid samples were saved for GLC analysis (carbohydrates) in a -70°C freezer.

Statistical analysis

Following the calculation of mean ($\bar{x}$) supercooling points and their standard errors (S.E.), the data was analysed using either a statistical package (Superstats, Lombardy Scientific Ltd.) on an Apple II Europlus microcomputer, or using appropriate programs (SPSS (Nie et al., 1975); SPSSX (Nie, 1983)) on an Amdahl 470/V7 main-frame computer. The statistical tests used were t-tests and analysis of variance.

Laboratory studies

A. Inherent supercooling ability

The inherent capacity for supercooling can be determined by maintaining cultures in standard conditions and excluding extrinsic influences. The results obtained can then be used as the 'control' when assessing the influence of a range of factors on the inherent potential to supercool.

Three strains of M. persicae susceptible (S), moderately resistant (R_1) and highly resistant (R_2) to insecticides were maintained on leaf discs of Chinese cabbage at 20°C; full details of laboratory culture are given in Chapter Two. After three generations

at these conditions, the supercooling ability of all the developmental instars (first, second, third and fourth) and adult virginoparae (both apterous and alate individuals) from the three strains was assessed. In addition, the supercooling ability of post-reproductive apterous virginoparae and first instars produced by them towards the end of their reproductive period, was also assessed (S strain individuals only).

B. Acclimation ability

To investigate whether M. persicae possessed a cold hardening mechanism, a series of experiments were conducted in which aphid supercooling ability was assessed following exposure to low temperature in the laboratory.

Aphids were acclimated at 5°C in incubators under a long photoperiod as described in Chapter Two. This temperature was chosen since it represents approximately the lower limit for physiological activity in aphids (Barlow, 1962; Smith, 1981; Pozarowska, 1983) and would therefore probably act as an effective thermal trigger in terms of an acclimation response; sub-zero temperatures would probably be too 'severe' producing cold injury/shock (Salt, 1961).

The S and R_2 strains were studied in these experiments as described below.

(i) First instar nymphs (>12 h old, to ensure they had fed) were collected from the cultures at 20°C, placed at 5°C on leaf discs of Chinese cabbage and maintained as described previously (see Plate 4). During subsequent development, nymphs were removed after 2 and 7 days.

In other experiments the nymphs developed to second, third and fourth instars, newly moulted apterae and alate virginoparae (R_2 strain only) and were then removed.

(ii) Fourth instars of both strains were taken from the 20°C cultures and their supercooling ability assessed following acclimation at 5°C on leaf discs for 2 days, and after moulting to pre-reproductive and reproductive adults.

(iii)Newly moulted adult apterae from the cultures at 20°C were acclimated at 5°C on leaf discs for 2, 7, 14, 21 and 28 days after which they were removed and their supercooling ability assessed.

In addition, the supercooling ability of first instar nymphs (1-2 days old) produced by acclimated adults of both strains was assessed, to investigate the possibility of a 'transovarial' transfer of cold tolerance from mother to progeny.

C. Contact moisture

To investigate the influence of contact with surface moisture on the inherent supercooling ability of M. persicae, S strain first instar nymphs and adult apterae (n=20) from the 20°C cultures were thoroughly wetted with: a) rainwater only

b) a rainwater and detergent solution (10%)

using a fine paintbrush, prior to supercooling point determination.

D. Holocyclic individuals

In an attempt to locate natural holocyclic populations of

M. persicae, a number of peach trees (the primary host) were examined in Leeds, parts of Yorkshire and in Kent (East Malling Research Station), in the autumn and winter of 1982; however, no holocyclic morphs or eggs were found. It had been hoped that such a study would have added further information to the comparison with Aphis fabae. However, holocyclic overwintering populations are not common and therefore the likelihood of obtaining sufficient numbers of such aphids for experimental work, occurring naturally in Britain is low (R.L. Blackman, personal communication).

Consequently, studies on holocyclic individuals were conducted in the laboratory with the H clone (see Chapter Two and Appendix 2).

After exposure of the clone to a 10 h photoperiod at 18 ± 2°C, apterous virginoparae which had developed under the short photoperiod, and the sexual females (oviparae) and males which were subsequently produced, were collected and their supercooling capacity assessed. Eggs (unfertilised) laid by oviparae were also examined.

Field studies

M. persicae has a world-wide distribution (van Emden, Eastop, Hughes and Way, 1969) and exists in a number of strains and races with inherent variation (Eastop, 1973), and therefore it is unwise to make generalisations about its cold hardiness based entirely on laboratory studies. Field studies were therefore conducted to investigate the cold hardiness of natural populations of M. persicae and to further examine potential influences on inherent supercooling ability. They commenced early in the winter of 1982-83 until the summer of 1984 and involved a number of independent studies as described below.

WINTER 1982-83

In October 1982 a number of brassica crops (oilseed rape, kale, savoy cabbage, brussel sprouts) in Yorkshire were inspected for overwintering M. persicae. The number of aphids found was low and was unlikely to provide sufficient samples for repeated experiments throughout the winter. As an alternative, protected crops in glasshouses were inspected. These plants allow continued parthenogenesis in most winters and are therefore often the main source of overwintering M. persicae.

In November 1982, a large infestation of M. persicae was located in an unheated and naturally lit glasshouse at the University Gardens (Oxley Hall) in Leeds. The aphids were found on four-month old Cineraria (multiflora nana maxima) plants and a sample of 10 plants was removed to a similar glasshouse close to the laboratory (Plate 18).

These plants were maintained in this glasshouse until May 1983 when the study was terminated. During this period the supercooling ability of samples of first instar nymphs and adult apterae was assessed at monthly intervals (n=20 except March 1983 when only 10 adults were collected; no first instars were collected in March or April 1983 because of the low numbers of reproductive adults).

(a) Clonal differences

The population of M. persicae sampled from the glasshouse Cineraria was unlikely to represent a single clone. Five clones (designated OH 2, OH 3, OH 4 (see Appendix 2), OH 5A and OH A) were established in the laboratory from single aphids taken from the

Plate 18 Unheated and naturally lit glasshouse, in which experiments were conducted in the winters of 1982-83 and 1983-84.

Plate 19 Field cage used in winter 1983-84, sited on the department rooftop.

glasshouse population in January 1983 and maintained at 20°C in the normal procedure for laboratory cultures (see Chapter Two). After at least three generations in culture, the supercooling ability of samples of first instar nymphs and adult apterae from each clone was assessed.

(b) Field sample

In December 1982 a population of M. persicae from savoy cabbage, located at High Mowthorpe EHS, North Yorkshire was obtained from ADAS, Leeds. Samples of first instars and adult apterae were used to determine the supercooling ability of this population.

In addition, a clone (Savoy) was established from the population and maintained in the laboratory at 20°C. After three generations, the inherent supercooling potential of first instar nymphs and adults was determined.

SUMMER/AUTUMN 1983

The glasshouse experiment was terminated in May 1983 and to continue the seasonal assessment of supercooling ability, aphid samples were collected from the original glasshouse at Oxley Hall during the summer and autumn of 1983. First instars were collected in the first month only, while adult apterae were collected monthly. The aphids were collected from a range of host plants.

Also in July 1983, a M. persicae population was located on a kale crop at the University experimental garden (Ridgeway), and samples of first instar nymphs and adult apterae were collected and their supercooling ability assessed.

WINTER 1983-84

A number of studies were conducted in this winter, the main aim of which was to investigate the influence of certain individual factors on supercooling levels during winter.

(a) Field cage experiment.

Protected sites such as glasshouses are an important source of overwintering anholocyclic M. persicae but cannot be regarded as a true field situation since solar heating occurs on sunny days and the aphids are not subjected to the continuous severe conditions of winter (Fig. 4.1 and Appendix 4). A large cage was therefore constructed to represent more closely the field situation. The cage consisted of a timber frame (2.43 x 1.91 x 1.23m) covered with a very fine mesh nylon bolting (nybolt 50 GG-355; John Staniar and Co., Manchester) to prevent aphid emigration or immigration during the experiment.

Aphids were derived from a single clone (OH 4) and placed onto the host plants in a single introduction at the same age so that the subsequent age and reproductive status of sampled aphids would be as close as possible. The insecticide resistance status of this clone had been previously assessed as highly resistant.

Oilseed rape plants (var. Jet Neuf; about three months old, grown from seed in John Innes (J.I.) no. 2 compost) were used as the host plants since they are regularly colonised by natural overwintering populations. A single caged oilseed rape plant was colonised with OH 4 individuals in early October 1983 to provide the first sample of adults later that month.

On 27 October 1983, 100 late fourth instar nymphs/newly moulted adults were placed on each of the 12 host plants. The cage was originally sited in close proximity to the glasshouse but following vandalism a few weeks later it was moved to a more isolated site on the roof (Plate 19).

The main experimental plan had been to remove monthly samples of clonal adults of known age, reproductive status and insecticide resistant-type to assess their supercooling potential. However, by the 13 November just over 30 of the original adults were recovered. Samples later in November and in December were therefore made up from progeny of the original adults. A sample of first instars was collected in December with samples of second and third instars also examined for supercooling ability levels, to investigate developmental acclimatisation.

Heavy rains in late November/early December reduced the population such that the experiment was terminated in January 1984.

(b) Field Sample

In December 1983 a sample of adults of M. persicae was obtained from oilseed rape at the University Farm (Tadcaster, North Yorkshire) and their supercooling ability assessed.

(c) Glasshouse experiments

A number of studies were carried out in the glasshouse, previously used in winter 1982-83, to investigate factors which may have influenced the supercooling point results obtained in that winter.

(i) 10 Cineraria plants (grown from seed in J.1. no. 2 compost) were artificially infested with aphids from the OH 4 clone, to continue the

1982-83 experiment. The colony did not establish well however and only samples of adult apterae were collected and their supercooling ability determined at monthly intervals until February 1984.

(ii) To further investigate clonal variation of cold hardiness individuals from the Savoy, OSR (collected from oilseed rape in December 1982 - see Appendix 2) and the Kale (collected from kale in July 1983 - see Summer/Autumn 1983) clones, which had been maintained in the laboratory at 20°C, were established on oilseed rape plants in individual cages and then placed in the glasshouse. In December 1983, adults from each clonal culture were collected and their supercooling ability assessed.

(iii)Cineraria is not a cold hardy host and this factor may have influenced supercooling levels in winter experiments involving this host plant. To investigate this, late fourth instar nymphs or newly moulted adults of the OH 4 clone, were established on caged oilseed rape plants (a cold hardy host) in October 1983. Samples of adults were removed in November and December to assess supercooling ability.

SUMMER 1984

A sample of adult apterae of M. persicae was collected from a brussel sprout crop at the University experimental garden (Ridgeway) in July and their supercooling ability assessed.

Temperature records

The maximum and minimum temperatures recorded inside the glasshouse and in the field during the winter study of 1982-83 are shown in Figure 4.1. The data collected for inside the glasshouse and field cage, and in the field during the winter 1983-84 studies is shown in Figure 4.2. All the temperature data, recorded using minimum-maximum thermometers and the dates of the measurements are given in Appendix 4 (Lewis and Siddorn, 1972).

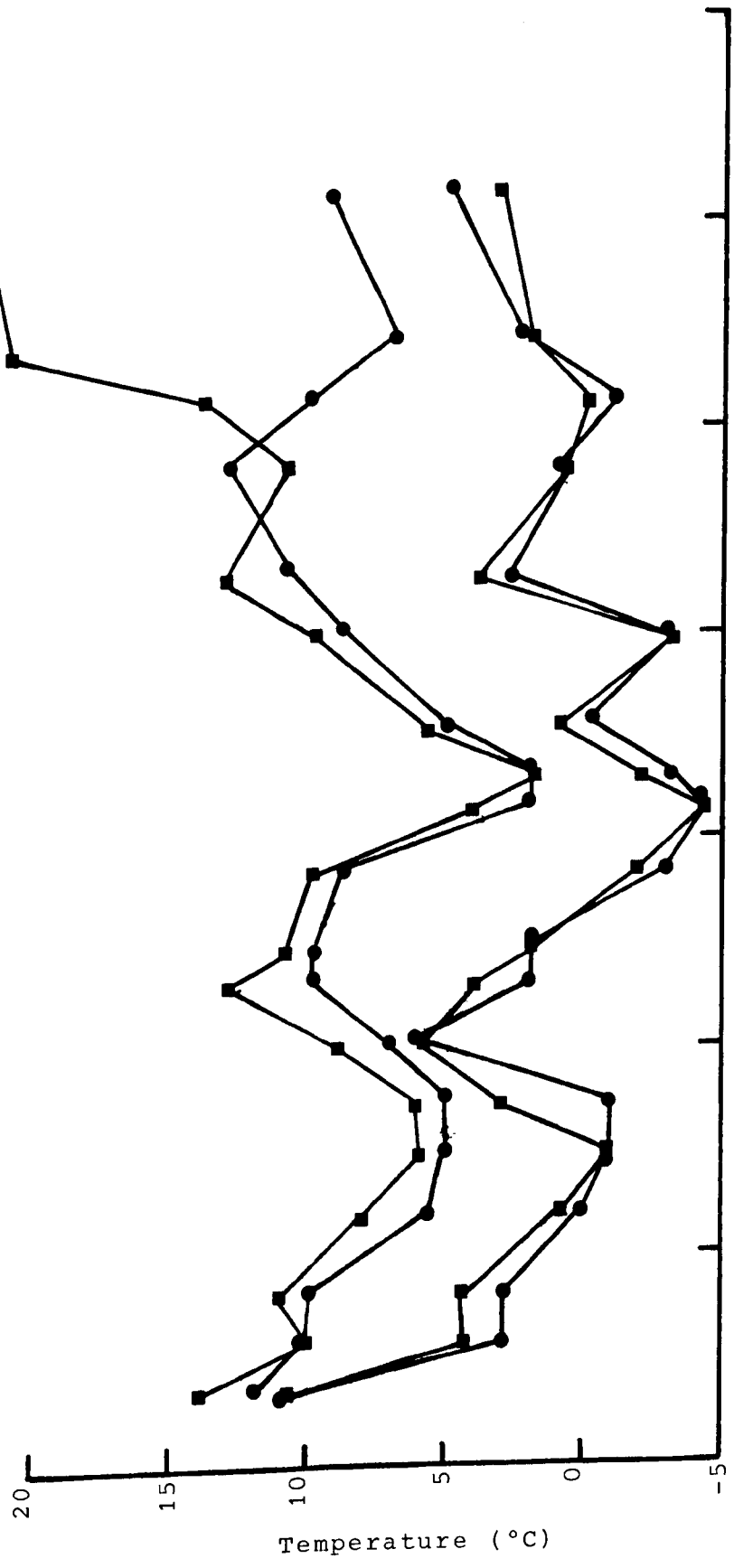


Figure 4.1 Maximum and minimum temperatures recorded in the unheated glasshouse (■) and in the field (●) in 1982-83

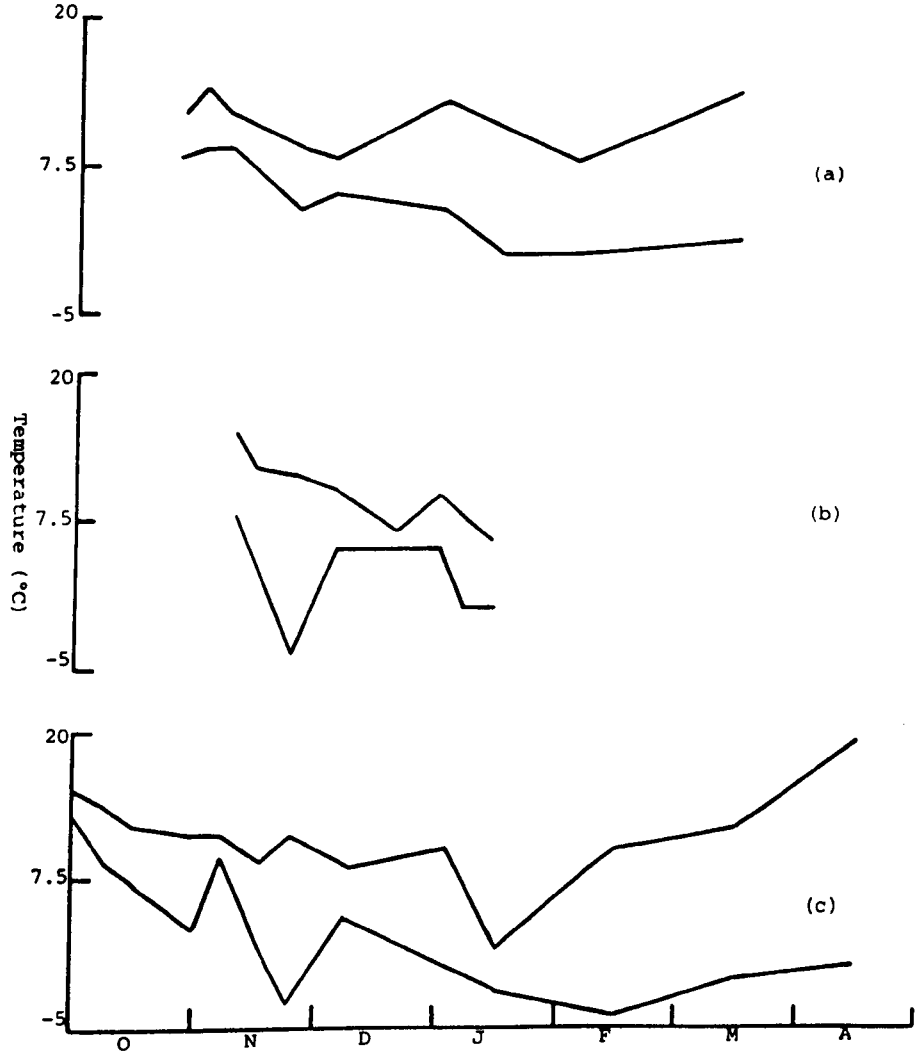


Figure 4.2 Maximum and minimum temperatures recorded in the unheated glasshouse (a), field cage (b) and in the field (c), in 1983-84

RESULTS

Inherent cold hardiness

No aphids were found to survive after supercooling point experiments, confirming that M. persicae was a freezing-susceptible species.

The inherent supercooling ability of the various life cycle stages of the S, R_1 and R_2 strains is presented in Table 4.1. All the aphid samples in these experiments had a mean supercooling point below -20°C, indicating that all stages were capable of extensive supercooling; within each sample there was little variation in supercooling potential, as indicated by the small standard errors. There was no difference in supercooling ability between the three strains.

Within each strain there was a progressive loss of cold hardiness with increasing age, a trend which was significant ($P<0.05$). In each strain therefore first instar nymphs were the most cold hardy although only significantly so in the S and R_2 strains ($P<0.05$).

Influences on inherent levels of cold hardiness

Acclimation ability

In all supercooling point determinations, irrespective of previous treatment, aphids experience a brief exposure to ambient temperature and then 15°C at the start of cooling. To confirm that this short exposure to a temperature higher than that at which aphids were acclimated did not reduce any cold hardening which may have occurred, first instar nymphs (S) maintained at 5°C for 14 days, were cooled from 5°C (using the manual option of the supercooling point equipment). The mean supercooling point of -25.2 $\pm$ 0.15°C did not differ from that of the corresponding acclimated sample, cooled

Table 4.1 Inherent supercooling ability (°C) of S, R_1 and R_2 strains of *M. persicae* maintained continuously at 20°C

Strain	Mean ± S.E. (n=20) Range								
	First instar	Second instar	Third instar	Fourth instar	Newly moulted adult	Reproductive adult	Alate adult	Post-reproductive adult	First instars produced by 'late' reproductive adults
S	-26.0 ± 0.25	-25.1 ± 0.25	-23.9 ± 0.36	-24.3 ± 0.30	-24.3 ± 0.40	-24.2 ± 0.29	-23.8 ± 0.27	-23.9 ± 0.21	-26.8 ± 0.39
	-23.1/-27.4	-23.6/-27.6	-21.2/-27.0	-22.0/-27.1	-18.8/-27.1	-21.3/-26.1	-21.1/-25.6	-21.7/-26.0	-22.4/-28.4
R_1	-25.4 ± 0.47	-25.0 ± 0.27	-24.1 ± 0.29	-23.3 ± 0.40	-24.7 ± 0.21	-23.2 ± 0.49	-23.9 ± 0.25	-	-
	-21.1/-27.9	-22.9/-26.9	-22.0/-27.1	-19.1/-25.4	-23.3/-26.2	-18.2/-26.3	-21.8/-25.8		
R_2	-25.9 ± 0.25	-24.4 ± 0.34	-23.5 ± 0.30	-24.3 ± 0.29	-23.5 ± 0.42	-23.8 ± 0.20	-23.9 ± 0.38	-	-
	-23.7/-28.1	-21.3/-26.3	-20.2/-26.0	-21.5/-27.1	-17.7/-25.5	-23.3/-26.1	-20.5/-26.2		

using the normal start temperature of 15°C, indicating that there was no change in supercooling ability associated with short exposures to higher temperatures.

At 5°C there was a significant trend towards poorer supercooling ability with increasing aphid age ($P<0.001$), with first instar nymphs in both the S and R_2 strains being the most cold hardy stage (Table 4.2).

In both strains first instars were more cold hardy after 7 days at 5°C than those maintained continuously at 20°C ($P<0.05$; see Table 4.1 for control data). This increase in supercooling ability was maintained into the second and third instar stages in both strains so that they were also more cold hardy than the corresponding aphids at 20°C ($P<0.001$). After moulting to the fourth instar while S strain individuals remained more cold hardy than those at 20°C ($P<0.05$), R_2 fourth instars which had developed at 5°C, showed poorer supercooling ability than when maintained throughout development at 20°C ($P<0.001$). This small, but significant difference in cold hardiness between the two strains was again observed in the adult stage when the supercooling potential of S individuals did not differ from those at 20°C, but R_2 adults (apterae and alatae) were less cold hardy than if allowed to develop at 20°C ($P<0.01$).

Fourth instar nymphs of both strains acclimated at 5°C showed no increase or loss of supercooling potential compared to the corresponding individuals at 20°C (Table 4.3).

Further differences between the S and R_2 strains following low temperature exposure were found when R_2 adults acclimated at 5°C lost supercooling ability after 14 days (Table 4.4). This loss

Table 4.2 Supercooling ability (°C) of M. persicae acclimated at 5°C

Strain	Mean ± S.E. (n=20) Range						
	First instar		Second instar	Third instar	Fourth instar	Adult (newly moulted)	Adult (alate)
	Days acclimated						
	2	7					
S	-26.1 ± 0.19	-26.9 ± 0.23	-26.6 ± 0.22	-26.3 ± 0.15	-25.3 ± 0.33	-23.8 ± 0.25	-
	-26.7/-27.9	-24.9/-28.5	-23.7/-28.0	-24.1/-27.2	-21.3/-27.0	-22.2/-25.5	
R_2	-26.6 ± 0.17	-26.5 ± 0.20	-25.8 ± 0.26	-25.4 ± 0.42	-21.6 ± 0.18	-21.9 ± 0.31	-21.5 ± 0.44
	-25.2/-27.7	-25.0/-28.3	-23.6/-27.3	-20.1/-27.2	-20.4/-23.4	-19.6/-24.8	-17.6/-25.4

Table 4.3 Supercooling ability (°C) of fourth instar nymphs of *M. persicae* acclimated at 5°C

Strain	Mean ± S.E. (n=20) Range Days acclimated		
	2	7	14
S	-23.5 ± 0.42	-25.1 ± 0.26	-24.5 ± 0.32
	-19.6/-26.4	-22.8/-27.1	-20.6/-26.5
R_2	-23.9 ± 0.22	-23.5 ± 0.33	-24.4 ± 0.40
	-21.4/-25.9	-19.0/-25.4	-21.0/-27.5

Table 4.4 Supercooling ability (°C) of adult *M. persicae* acclimated at 5°C

Strain	Mean ± S.E. (n=20) Range Days acclimated				
	2	7	14	21	28
S	-24.6 ± 0.27	-23.8 ± 0.29	-23.7 ± 0.30	-24.9 ± 0.32	-24.5 ± 0.29
	-22.2/-26.8	-20.6/-25.7	-20.4/-26.3	-21.8/-26.5	-22.5/-26.1
R_2	-22.8 ± 0.37	-22.7 ± 0.21	-21.6 ± 0.33	-23.4 ± 0.36	-22.4 ± 0.49
	-19.4/-25.2	-20.4/-24.3	-17.1/-24.5	-19.5/-26.0	-14.2/-25.9

however, was apparently regained with longer exposures of 21 and 28 days. Adults of the S strain showed no changes in supercooling potential under any of the exposure times.

First instar nymphs produced by acclimated S adults possessed greater supercooling ability than either those produced at 20°C or maintained at 5°C for 2 days ($P<0.05$; Table 4.5). Those produced by R_2 adults were more cold hardy than 20°C nymphs ($P<0.05$) but did not differ from those acclimated at 5°C for 2 days. Nymphs produced by acclimated adults in both strains are therefore more cold hardy than those produced by unacclimated adults.

Contact moisture

After wetting with rainwater only a small proportion of first instar nymphs and adults showed reduced supercooling ability compared to dry samples (Fig. 4.3). The graphical display of cooling showed a single temperature 'rebound' (which occurs at freezing) in those aphids with reduced supercooling ability, but two rebounds in the majority of wet aphids (Plate 20). When a water droplet was supercooled on a thermocouple, only one rebound was observed, corresponding to the single rebound seen in only a few of the wet aphids, and the first of the rebounds observed in the majority of wet aphids. This suggests that a single rebound occurs when freezing of the contact moisture subsequently inoculates the aphid and two rebounds are observed when there is no inoculative freezing and the aphid has a normal supercooling point. Aphids removed from the thermocouples after the first rebound but before the second were still alive, further demonstrating their resistance to inoculation by frozen contact moisture.

Table 4.5 Supercooling ability (°C) of first instar nymphs of *M. persicae*, acclimated at 5°C or produced by adults at 5°C and at 20°C

Strain	Mean ± S.E. (n=20) Range		
	Acclimated for 2 days at 5°C	Produced by adults acclimated at 5°C	Produced by adults at 20°C
S	-26.1 ± 0.19	-27.5 ± 0.21	-26.0 ± 0.25
	-24.7/-27.9	-25.2/-29.1	-23.1/-27.4
R_2	-26.6 ± 0.17	-27.0 ± 0.25	-25.9 ± 0.25
	-25.2/-27.9	-25.1/-28.2	-23.7/-28.1

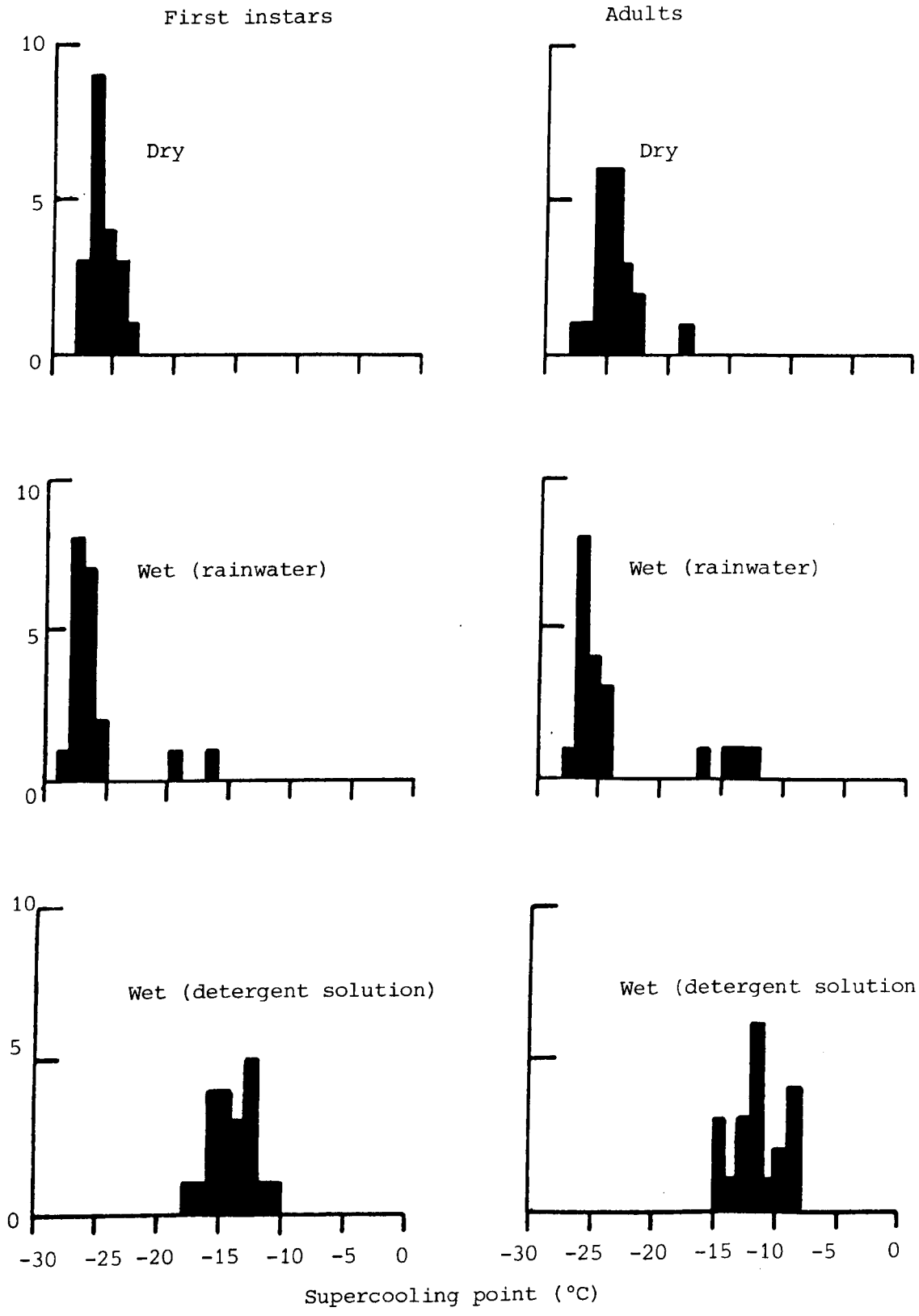
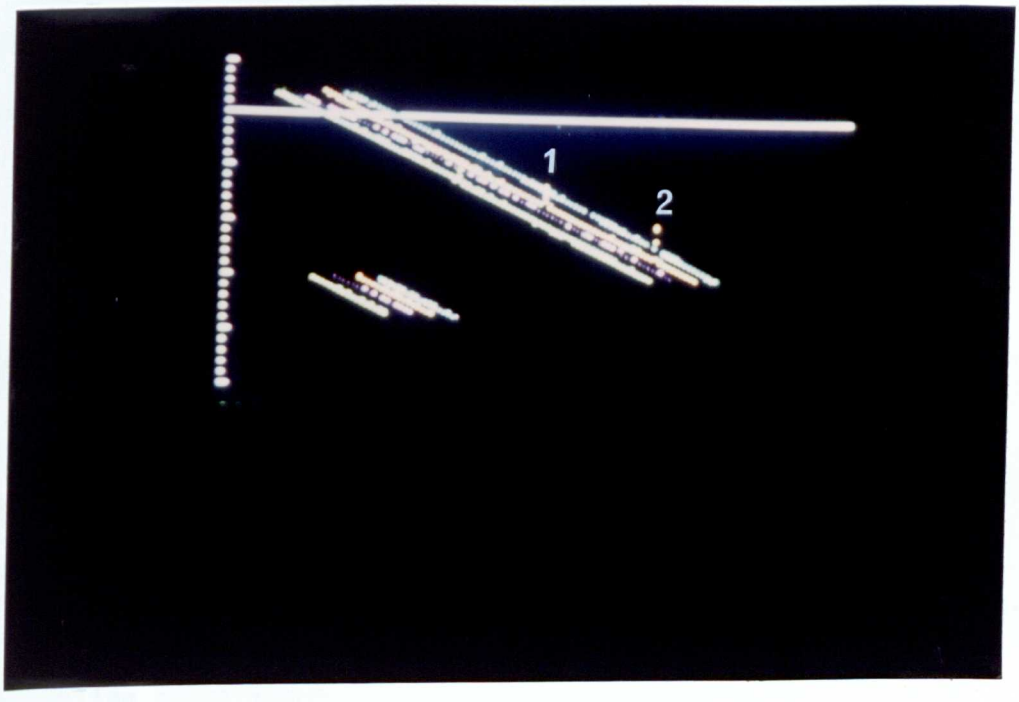


Figure 4.3 Supercooling point distribution histograms of first instar nymphs and adults of M. persicae when dry (control) and after wetting with rainwater or a detergent solution

Plate 20 Graphical display of cooling showing two 'rebounds' (thermocouple 3; orange curve) typical of wet aphids which were not inoculated when supercooled. Rebound 1 represents freezing of water while rebound 2 occurs when the aphid freezes.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2

When wet with the detergent solution all aphids had single rebounds above -20°C, suggesting that they all experienced inoculative freezing when the contact moisture froze.

Cold hardiness of holocyclic aphids

The inherent supercooling capacity of males, oviparae and virginoparae, produced and maintained under the short photoperiod conditions, did not differ from that of aphids maintained by continued parthenogenesis under long photoperiod culture (Table 4.6).

Eggs possessed greater supercooling potential, to below -30°C.

Seasonal cold hardiness

WINTER 1982-83

The supercooling ability of first instar nymphs varied little with season (Table 4.7), although the December sample was less cold hardy than those collected in February and May ($P<0.05$). Among all the samples collected only one individual was recorded with a supercooling point above -20°C. They therefore maintained consistent and extensive supercooling ability throughout the sampling period.

The overall seasonal trend of supercooling ability in adult M. persicae during the same period, was of a slight loss in cold hardiness in the whole population as winter progressed, with a substantial decrease in supercooling potential in a variable proportion in mid to late winter. In spring there was a distinct increase in supercooling ability so that May collected individuals were capable of extensive supercooling.

Table 4.6 Supercooling ability (°C) of holocyclic *M. persicae* maintained under short photoperiod conditions

	Mean ± S.E. Range		
Virginoparae (n=20)	Oviparae (n=20)	Males (n=10)	Eggs (n=10)
-24.3 ± 0.42	-24.9 ± 0.35	-24.3 ± 0.43	-31.8 ± 0.36
-21.0/-26.3	-21.2/-26.8	-21.5/-25.4	-30.1/-33.2

Table 4.7 Supercooling ability (°C) of first instar nymphs of *M. persicae* collected in 1982-83

Collection date/source	Mean ± S.E. (n=20)	Range
3 November 1982	-26.2 ± 0.43	-21.7/-27.8
7 December 1982	-25.6 ± 0.50	-20.0/-27.7
13 January 1983	-26.6 ± 0.46	-19.5/-28.4
9 February 1983	-27.5 ± 0.27	-25.1/-28.9
23 May 1983	-27.1 ± 0.15	-25.9/-28.3

The supercooling points of adult samples collected over winter and into spring 1983 were often not normally distributed (Fig. 4.4). The distribution of supercooling points in adults collected in November showed a slight skew towards reduced cold hardiness and as winter progressed this trend continued, so that in December most adults showed some loss of cold hardiness, with a distribution around a mean of -20.7°C. In January, the distribution was bimodal with a distinct high (HG) and low (LG) group, with adults in the latter category showing a marked reduction in supercooling ability. The HG was composed of individuals with little variation in their supercooling points of around -20°C. A very similar pattern was found in February and March. In April, although the LG was still represented, supercooling ability had increased so that the HG showed an overall shift towards improved cold hardiness. By May, only the HG was present and all adults exhibited extensive supercooling ability, with a minimum supercooling point of -24.2°C.

In addition to the marked decrease in cold hardiness in a proportion of the population in winter there was also a less obvious loss in all individuals over the same period; the mean supercooling point of the HG in November and that of the May sample was lower (more cold hardy) than that of the HG in the other winter months ($P<0.05$).

(a) Clonal variation in cold hardiness

All of the aphids from the five clones established from the glasshouse population demonstrated extensive supercooling potential when maintained at 20°C, with first instars the most cold hardy (Table 4.8). There was no difference in cold hardiness between any of the clones when tested under these conditions.

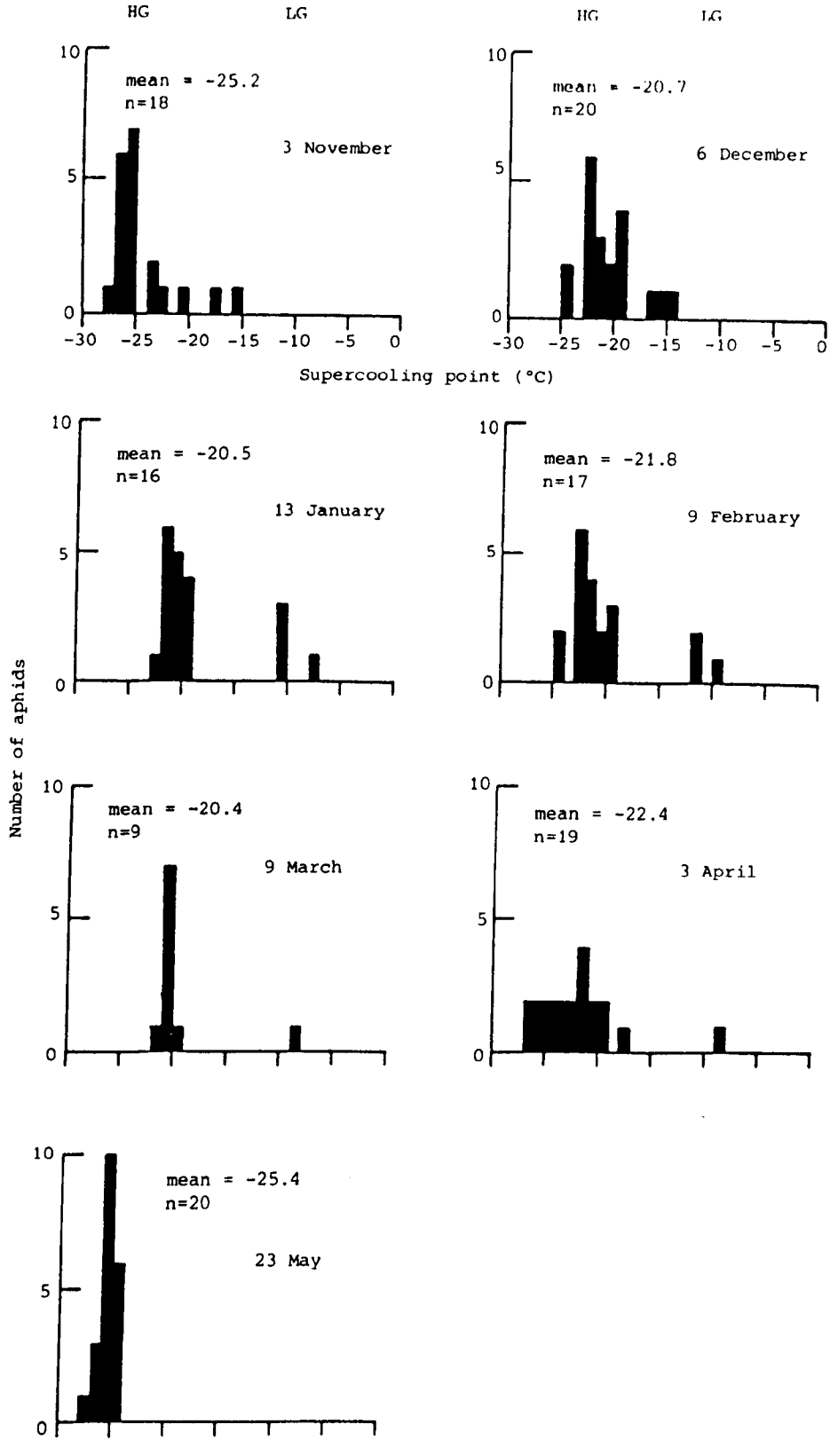


Figure 4.4 Supercooling point distribution histograms of adult apterae of M. persicae collected from Cineraria within the unheated glasshouse in 1982-83

Table 4.8 Supercooling ability (°C) of M. persicae clones established from the glasshouse population and maintained at 20°C

Clone	Mean ± S.E. (n=20) Range	
	First instar	Adult apterae
OH 2	-27.1 ± 0.19	-24.9 ± 0.30
	-25.4/-29.2	-22.1/-26.6
OH 3	-27.1 ± 0.21	-25.6 ± 0.19
	-25.5/-28.8	-22.3/-26.9
OH 4	-27.4 ± 0.15	-25.8 ± 0.27
	-26.0/-28.6	-23.3/-27.2
OH 5A	-27.5 ± 0.14	-25.8 ± 0.10
	-26.0/-28.5	-24.5/-26.6
OH A	-27.3 ± 0.12	-25.6 ± 0.19
	-26.4/-28.3	-23.6/-27.2

(b) Cold hardiness of field collected aphids

The mean supercooling potential of first instar nymphs collected from savoy cabbage in December 1982 was -26.4 ± 0.60, (range -20.7/-29.2), and therefore did not differ from that of first instars sampled from the glasshouse maintained Cineraria in the same month (see Table 4.7) nor individuals (clone Savoy) maintained in the laboratory which had a mean supercooling point of -27.3 ± 0.15 and a range of -25.5/-28.7. The range of supercooling points of the field collected adults was also similar to that of glasshouse collected individuals in December, with a number of the adults displaying poorer supercooling potential; when maintained in the laboratory at 20°C all possessed extensive and similar levels of supercooling (Fig. 4.5).

SUMMER/AUTUMN 1983

All the aphid samples collected from July to October were capable of extensive supercooling with first instars possessing the greatest supercooling potential (Table 4.9). While all the adults had supercooling points below -20°C, distributed in one group around the mean of the sample, some of the adults collected from kale in July had slightly poorer supercooling potential.

WINTER 1983-84

(a) Cold hardiness of field cage maintained M. persicae

All adults sampled from October to December possessed similar levels of good supercooling ability (Fig. 4.6). The final adult sample at the end of December included a number of individuals with somewhat poorer supercooling ability, comparable to that of HG adults in mid winter the previous year.

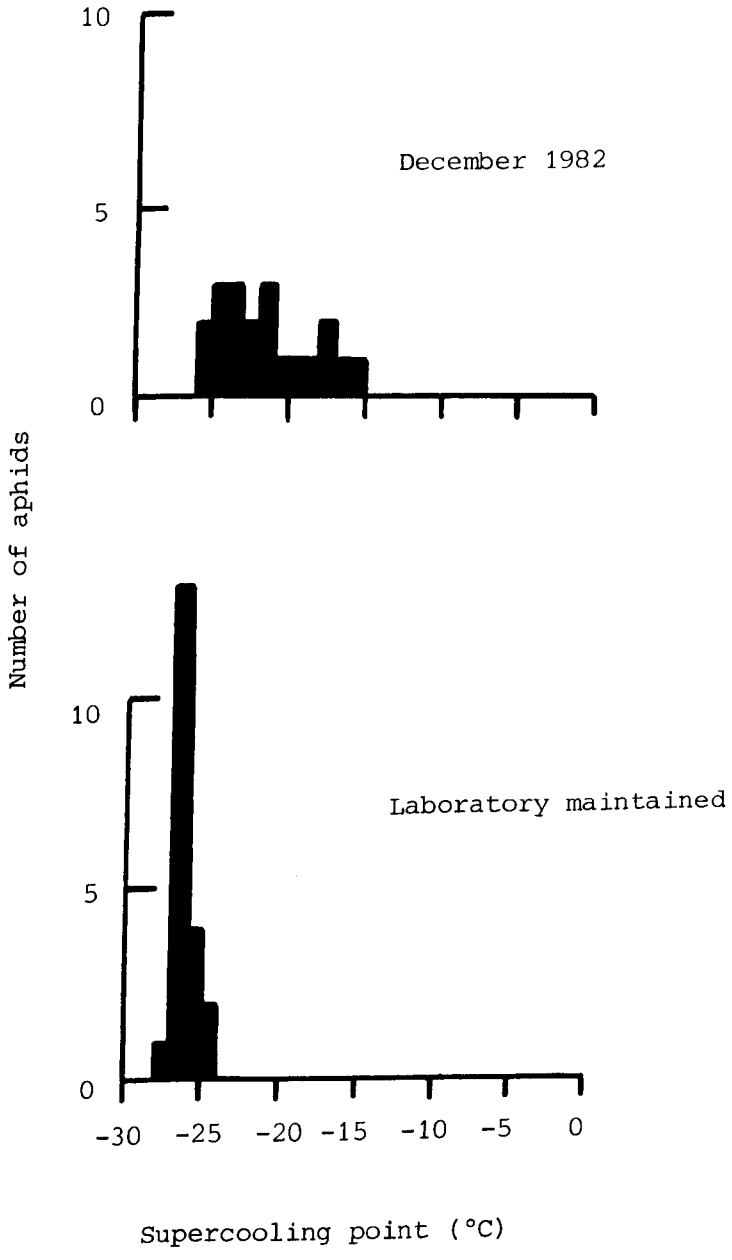


Figure 4.5 Supercooling point distribution histograms of adult M. persicae collected from savoy cabbage in December 1982 or maintained in the laboratory (Savoy clone)

Table 4.9 Supercooling ability (°C) of M. persicae collected in summer and autumn 1983

Collection date	Source	Mean ± S.E. Range		
		Adult apterae		First instar nymphs
5 July	Impatiens	-25.6 ± 0.25	(n=20)	-26.5 ± 0.20
		-23.3/-27.4		-24.7/-28.4
30 July	Kale	-23.6 ± 0.51	(n=10)	-26.5 ± 0.29
		-20.7/-26.0		-25.0/-27.4
3 August	Primula	-25.7 ± 0.17	(n=20)	-
		-23.7/-26.9		
21 September	Cineraria	-25.6 ± 0.14	(n=20)	-
		-24.2/-26.5		
19 October	Cineraria	-24.9 ± 0.32	(n=20)	-
		-22.3/-27.5		

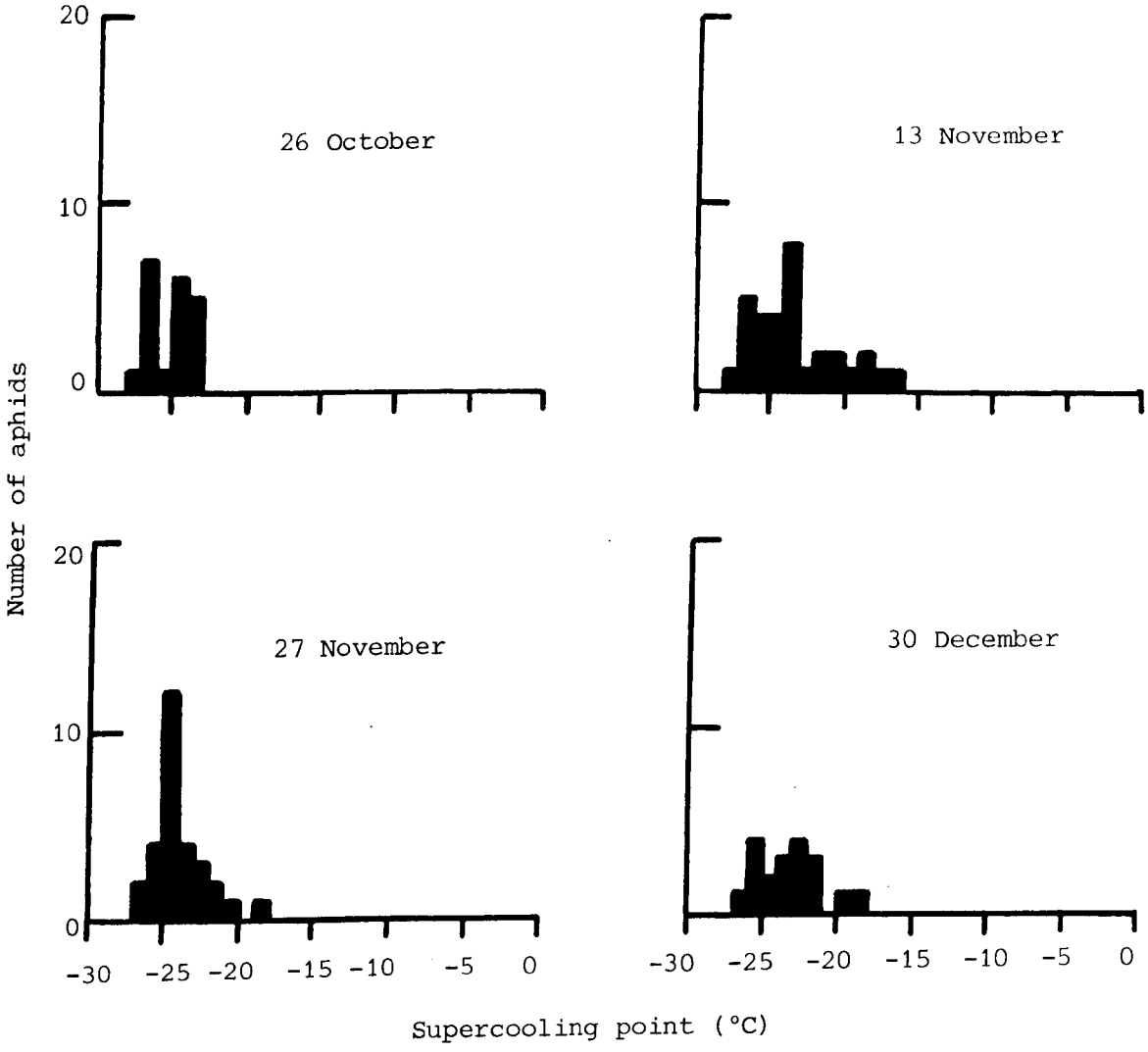


Figure 4.6 Supercooling point distribution histograms of adult *M. persicae* collected from the field cage experiment in 1983-84

First instar supercooling ability was typically extensive and this level of cold hardiness was maintained following development under field conditions to second and third instars (Table 4.10); these instars were more cold hardy than corresponding nymphs which had developed under favourable laboratory conditions ($P<0.05$).

(b) Cold hardiness of field collected aphids

The distribution of supercooling points in this sample of adults collected from oilseed rape was not normal with 20% of the individuals in a distinct LG comparable with those recorded in earlier winter samples (e.g. January, February, March 1983; Fig. 4.7). The remainder of the sample possessed supercooling ability of around -20°C, with the maximum supercooling point of -23.1°C.

(c) Cold hardiness of glasshouse maintained M. persicae

(i) On Cineraria. All adults sampled from December to February possessed good supercooling ability (Table 4.11), although as winter progressed individuals with poorer supercooling potential were recorded.

(ii) Clonal variation. All adults from the three clones (Savoy, OSR and Kale) possessed good supercooling ability with no difference between the clones (Table 4.12).

(iii)On oilseed rape. All adults collected in November were capable of extensive supercooling (Fig. 4.8). In December the sample included a number of adults with poor (LG) supercooling ability, while the remainder were still capable of considerable supercooling potential.

Table 4.10 Supercooling ability (°C) of first, second and third instars of M. persicae collected from the field cage in winter 1983-84

Sampling date	Mean ± S.E. (n=10) Range		
	Instar		
	First	Second	Third
18 December 1983	-27.1 ± 0.25 -26.1/-28.2		
15 January 1984		-25.9 ± 0.29 -24.1/-27.1	-26.6 ± 0.21 -25.7/-27.6

Figure 4.7 Supercooling point distribution histogram of adult *M. persicae* collected from oilseed rape at the University Farm, North Yorkshire in 1983

Figure 4.8 Supercooling point distribution histograms of adult *M. persicae* (clone OSR) collected from oilseed rape maintained in the unheated glasshouse in 1983-84

Figure 4.9 Supercooling point distribution histogram of adult *M. persicae* collected from brussel sprout at the University experimental garden (Ridgeway) in 1984

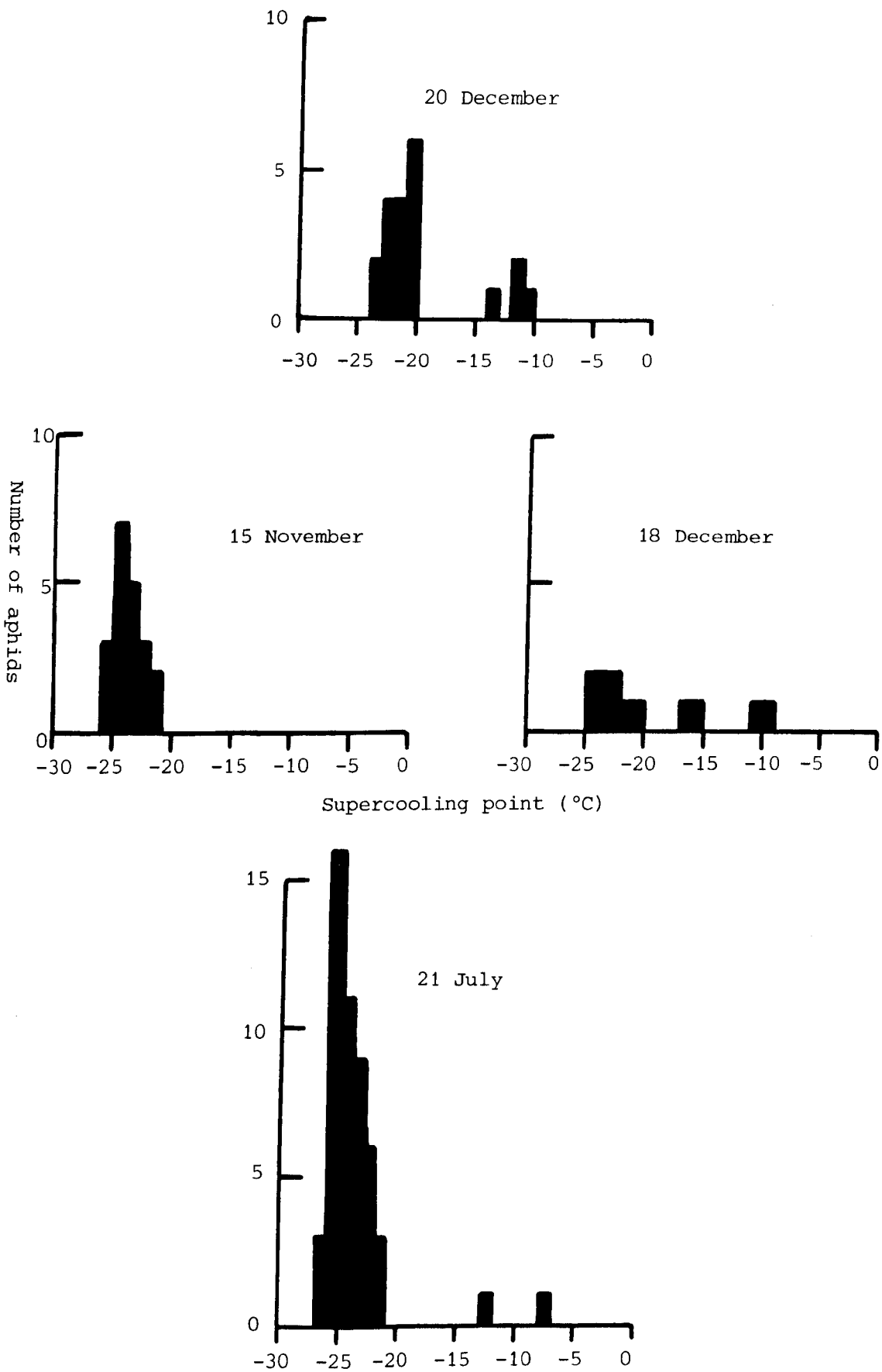

10
5
0
20 December
-30 -25 -20 -15 -10 -5 0
10
5
0
Number of aphids
15 November
-30 -25 -20 -15 -10 -5 0
18 December
-30 -25 -20 -15 -10 -5 0
Supercooling point (°C)
15
10
5
0
21 July
-30 -25 -20 -15 -10 -5 0

Table 4.11 Supercooling ability (°C) of *M. persicae* adults on glasshouse maintained *Cineraria* in winter 1983-84

Sampling date	Mean ± S.E. (n=10)	Range
15 November 1983	-25.0 ± 0.61	-20.3/-26.8
15 December 1983	-23.6 ± 0.68	-20.8/-26.4
16 January 1984	-24.3 ± 0.70	-19.8/-26.5
28 February 1984	-23.1 ± 0.90	-16.9/-26.7

Table 4.12 Supercooling ability (°C) of adults from Savoy, OSR and Kale clones of *M. persicae* maintained on oilseed rape in December 1983

Clone	Mean ± S.E. (n=20)	Range
Savoy	-25.0 ± 0.65	-19.8/-26.8
OSR	-24.5 ± 0.24	-23.1/-25.3
Kale	-25.7 ± 0.26	-24.4/-27.0

SUMMER 1984

While 48 adults in this sample from brussel sprout possessed extensive supercooling potential comparable with previous summer collected samples, 2 individuals were recorded with poor supercooling points, typical of LG aphids and previously only recorded in winter sample individuals (Fig. 4.9).

DISCUSSION

The results from the work described in this chapter have shown that when maintained under favourable laboratory conditions with a food supply, the inherent supercooling capacity of all strains, clones and age groups of M. persicae was consistent and extensive, comparable with that found in arthropod species inhabiting alpine and polar areas (Sømme, 1981). Powell (1973, 1974) found that all the developmental stages and adults of Elatobium abietinum (green spruce aphid) possessed inherent supercooling ability to below -15°C; field collected specimens showed equivalent levels of cold hardiness and although a seasonal pattern of acclimatisation, with increased supercooling ability, was observed in one winter, no such pattern was found the following year. Further studies led Powell (1976) to conclude that the supercooling ability of E. abietinum, Drepanosiphum platanoides (sycamore aphid) and Eucallipterus tiliae (lime aphid) was related to food (sap) quality. Parry (1978) assessed the supercooling ability of laboratory reared M. persicae and found that feeding position as well as age influenced supercooling capacity in this species; young nymphs possessed a greater supercooling potential which was at its maximum, below -20°C, when they were unfed. Because of the relationship between supercooling ability and feeding suggested in these aphids, further discussion and comparison of aphid cold hardiness in general is considered in Chapter Five and the General Discussion.

First instar nymphs were the most cold hardy stage with supercooling ability decreasing as aphid development proceeded. This trend suggests that the potential for supercooling may be related to

aphid age or size. Increasing age within the adult stage did not however result in decreased supercooling potential; post-reproductive adults possessed comparable supercooling ability to newly moulted adults. Zettel (1982) found a similar trend in four species of Collembola and concluded that decreasing supercooling ability was related to physiological changes associated with development, or increasing body size. The small size and thus volume of first instar nymphs may reduce nucleator concentration thereby enhancing supercooling potential; Powell (1976) found that while such a relationship between size and supercooling ability existed within first instar E. abietinum, it did not extend to the older instars and attributed this to the intake of nucleators in phloem sap. Cannon (1983) found very little difference in supercooling potential of the instars of the psyllid Strophingia ericae feeding on heather phloem. Whatever the basis of the relationship, the same trend of decreased supercooling ability during aphid development was also observed in the acclimation experiments, where first instars were also the most cold hardy stage.

While first instars of the S and R_2 strains did show an acclimation response, which was maintained through to the third instar stages, it never exceeded 2.4°C. Therefore, although biologically interesting, this acclimation ability is unlikely to influence winter survival since inherent cold hardiness is itself extensive. In addition, when reproduction continues overwinter it would seem that nymphs produced by adults which do not themselves show any acclimation response, and in some cases lose cold hardiness, are more cold hardy than unacclimated nymphs. Low temperature acting on the mother must

indirectly influence nymphal cold hardiness, perhaps involving a type of 'transovarial' transfer, as is the case for example with aphid symbiotes (Auclair, 1963).

The results of adult acclimation, in which R_2 strain adults lost cold hardiness during both development and continuous acclimation at 5°C, with no changes in supercooling ability observed in S strain individuals are of particular interest. This loss of supercooling potential during exposure to low temperature represented the only difference in cold hardiness between these insecticide susceptible and resistant strains of M. persicae. However, while statistically significant the decrease was small and overall of little biological importance since with acclimation periods of longer than 14 days the losses were regained. A possible explanation is that during adult acclimation after feeding for 14 days, aphids were transferred onto new leaf discs. It is possible that access to a fresh food source may have resulted in improved supercooling potential. The influence of food quality on supercooling ability is considered in Chapter Five.

M. persicae is unlikely to experience high mortality from instantaneous inoculative nucleation when in contact with surface moisture; this may be an important aid to winter survival in a species which overwinters in diverse and often unsheltered sites. Most of the aphids were not inoculated when surrounded by frozen moisture, and if removed from the thermocouples before reaching their inherent supercooling temperature, appeared unharmed. Young (1979) found that the freezing-susceptible mite Alaskozetes antarcticus could survive when encased within ice.

Inoculative nucleation is thought to result from the passage of ice through cuticular pores and orifices, thus leading to internal freezing (Salt, 1963). When wet with a detergent solution, all aphids died from inoculative nucleation when the water froze; this treatment would reduce the surface tension and allow penetration of water/ice through the hydrophobic cuticular layers. Salt (1963) found that pre-treatment of the cuticle of sawfly larvae (Cephus cintus), by boiling or soaking in detergent hastened inoculative nucleation. Bevan and Carter (1980) attributed the increased survival of wet green spruce aphid E. abietinum when exposed to low temperature, to its waxy bloom not possessed by Megoura viciae (vetch aphid). However A. fabae and Brevicoryne brassicae (cabbage aphid) both with waxy cuticles, showed reduced supercooling ability compared to M. persicae and Sitobion avenae when in contact with external moisture (E.J. Major, personal communication). Griffiths and Wratten (1979) found that the LT_{50} of the cereal aphids S. avenae and Rhopalosiphum padi at sub-zero temperatures was unaffected by wetting, but reduced that of Metopolophium dirhodum.

Sømme (1981, 1982) has suggested that differences in cuticular structure may account for differences in the response to contact moisture, but it would appear that aphid cuticles may be generally resistant to instantaneous inoculative nucleation compared with most other freezing-susceptible species (Sømme, 1982). However under field conditions aphids will experience repeated frosts and longer exposures to low temperature when wet and unless there is continued resistance to external moisture, considerable accumulated mortality may occur. The physical effect of precipitation including rainfall in dislodging

and drowning aphids may be a more influential mortality factor in wet winters (Agyen-Sampong, 1972; Smith, 1981; Harrington and Xia-Nian, 1984), regulating overwintering survival of M. persicae in some parts of Britain (Taylor, 1977).

All the M. persicae eggs tested, although unfertilised, were capable of supercooling to below -30°C. Oviparae and males possessed consistent and extensive supercooling ability, comparable with virginoparae maintained at 20°C. These aphids however were produced at 18 ± 2°C on a herbaceous host plant (Chinese cabbage) and as no holocyclic field populations of M. persicae were located during the research, it is possible that the levels of supercooling exhibited by these laboratory maintained holocyclic morphs may not be comparable with those of natural populations. Egg values however were similar to those recorded for the overwintering eggs of a number of aphid species (Sømme, 1964, 1969; Parry, 1979b; James and Luff, 1982; Parry, 1985 and see Chapter Seven); extensive supercooling potential is a typical feature of freezing-susceptible arthropod eggs (Somme, 1982). Recent studies (Baust, 1982; Horwarth and Duman, 1982) have suggested that photoperiod may act as an environmental stimulus for various cold hardening mechanisms. The role of photoperiod in inducing the sexual phase in holocyclic aphids in autumn does not apparently extend to act as a trigger for a cold hardening response in these aphids as virginoparae maintained under short photoperiod conditions were no more cold hardy than those reared in longer day lengths. In addition the acclimation response of M. persicae individuals described earlier in this chapter took place with only a low temperature stimulus and without the influence of short photoperiod.

There was a seasonal pattern of cold hardiness observed in M. persicae in the two successive winters and summers; this pattern

however is quite the reverse of that found in most freezing-susceptible arthropods. Aphids were most cold hardy in summer, with levels of supercooling ability at this time comparable with the inherent capacity of laboratory reared individuals. While younger instars maintained inherent levels of cold hardiness throughout the year, with some degree of acclimatisation in winter, there was a gradual loss of cold hardiness in all adults as winter progressed, with a substantial loss of supercooling potential in a variable proportion of the adult population. These seasonal changes in supercooling potential confirmed the results that had already been obtained in laboratory acclimation experiments.

Summer collected adults always possessed extensive supercooling potential. In only one sample (July 1984 from kale) did two individuals (4%) possess poor levels of supercooling ability equivalent to LG individuals, which in natural winter populations formed up to 25% of the population; such individuals (LG) were never found in other summer collected or laboratory maintained samples of adults. This suggests that poor supercooling ability in adults is related to low temperture in winter or its influence on adult aphids. It further suggests that the loss cannot be attributed to an inherent feature of the aphid itself, such as ageing, since it would then be expected that LG individuals would be observed at times other than during winter.

Some Cineraria plants in the first glasshouse experiment froze during exposure to sub-zero temperatures (January 1983). Adults (n=10) removed from the plants and allowed to feed for a week on healthy Cineraria at 10 ± 1°C, showed supercooling points between -19.6°C to -21.9°C, that is comparable with the levels of supercooling

observed earlier in the December sample (see Fig. 4.4), suggesting that the overall (HG) loss in cold hardiness had not been induced merely as a result feeding on a non-hardy and frozen host (Cineraria). In addition an adult sample collected from Oxley Hall in the same month, where Cineraria plants were continuously maintained at 10°C, had a mean supercooling point of -24.5 ± 0.34°C (n=20), thus showing neither the overall loss in cold hardiness or substantial reduction in supercooling ability associated with LG individuals. This is further evidence that low temperature acting on the glasshouse M. persicae induced, by whatever mechanism, losses of cold hardiness. While solar heating reduced the severity of the temperature inside the glasshouse on sunny days, minimum temperatures were comparable with the outside environment (Fig. 4.1 and Appendix 4). One possibility is that the ability to continue feeding under the Oxley Hall conditions maintained cold hardiness levels.

Overall the experiments conducted in the winter of 1983-84 confirmed previous results; younger instars maintain inherent levels of cold hardiness throughout the year, while adults show a gradual loss of supercooling ability as winter progresses and temperatures fall. Nevertheless conditions prevailing in this winter resulted in the experiments being less productive than had been envisaged. There was poor establishment of artificial M. persicae populations which subsequently reduced the availability of experimental adults. Aphid development was faster under the mild winter conditions (Fig. 4.2 and Appendix 4) resulting in some overburdening of plants together with decreased aphid longevity. The winter was also very wet with large numbers of aphids being 'washed off' the plants. These events merely

serve to illustrate the inherent difficulties associated with field-based experiments, particularly in winter where it is impossible to anticipate or control climatic variables.

These experiments however did suggest that the LG/HG distribution of adult supercooling points were unlikely to be the result of clonal differences within samples. When a single clone of M. persicae was used to establish the glasshouse experiment in 1983-84 on oil seed rape, a similar bimodal distribution of supercooling points was observed. Levels of supercooling ability observed in several clones of M. persicae during winter (e.g. Savoy, Kale, OSR) were similar, again suggesting that there is little clonal variation in cold hardiness.

This loss of cold hardiness in overwintering adult M. persicae thus appears to be independent of a number of factors. LG individuals were associated with several host plant species and under favourable conditions, again irrespective of host plants species adult supercooling levels were comparable. Aphid cold hardiness, particularly in adults, was most extensive and least variable when feeding under summer field or favourable laboratory conditions, a complete contrast to the influence of feeding and gut contents in other freezing-susceptible arthropods (Sømme, 1982). One theory which developed from these results was that if extensive supercooling potential occurred under favourable feeding regimes, then the losses observed during winter might be related to the inability to obtain food, particularly when chill-coma temperatures were reached and the aphids become immobile. In other freezing-susceptible arthrpods,

chill-coma results only in a loss of activity, since feeding ceases before winter (Sømme, 1982). Thus, the influence of feeding on aphid cold hardiness was investigated and this is the subject of the next chapter.

CHAPTER FIVE

THE INFLUENCE OF FEEDING ON THE SUPERCOOLING ABILITY OF APHIDS

SUMMARY

Adult Myzus persicae starved at 20°C, 5°C and 0°C survived for 2, 14, and 20 days respectively. First instar nymphs survived for 2 days at 20°C and 7 days at 5°C and 0°C when starved; factors other than starvation are thought to have induced mortality in the latter two groups. Adults and first instars starved at 20°C for 1 and 2 days showed no changes in supercooling ability compared with controls. When starved at 5°C for 1, 2 and 7 days, first instars maintained extensive supercooling ability; adults showed little change in supercooling potential after 1 and 2 days but after 7 days starvation, showed reduced cold hardiness with a proportion losing up to 10°C of supercooling potential, compared with adults maintained at 5°C with food which demonstrated no losses. In subsequent starvation experiments at 5°C and 0°C adults did not show such losses; reproductive phase, feeding status and imbibing of water prior to starvation treatment did not account for the unreproducibility, suggesting that the deprivation of food alone at low temperature may not be the only factor contributing to the feeding influence.

M. persicae fed on a range of growth stages of Chinese cabbage were all capable of considerable supercooling ability although S strain adults feeding on six-week old whole plants were to some extent less capable of supercooling.

Aphis fabae feeding on the primary host, spindle, were poorer supercoolers than those associated with bean plants. In a series of transfer experiments, aphids transferred to bean from spindle acquired good levels of supercooling and then lost over 10°C of supercooling potential when transferred back to spindle. There was evidence to

indicate that the shift from poor (LG/spindle) to good (HG/bean) supercooling ability was more difficult to achieve, suggesting the presence of a nucleating agent in spindle sap.

The influence of feeding status on the cold hardiness of overwintering aphids was discussed with respect to the levels of supercooling observed in these experiments.

INTRODUCTION

The presence of food in the gut of a number of freezing-susceptible arthropods has been shown to detract from the inherent supercooling ability of such individuals (e.g. Salt, 1953; Krunic, 1971; Sømme and Conradi-Larsen, 1977; Block and Zettel, 1980; Sømme, 1982). It is the presence of agents within the food or the food particles themselves, which act as nucleators, and are thought to induce freezing at temperatures above the 'normal' supercooling point (Zachariassen, 1982). The food of some species such as leaf chewing insects for example, may be contaminated with 'foreign' particles which act as efficient nucleating agents (Salt, 1953; 1961). Thus, animals with empty guts often possess greater supercooling potential; experimental starvation of Collembola resulted in an increase in their supercooling capacity (Sømme, 1976).

The acclimatisation observed in many overwintering freezing-susceptible species, represented by an increase in supercooling potential, results partly from gut evacuation in preparation for winter (Barson, 1974; Danks, 1978). If feeding occurs during winter, decreases in supercooling potential are observed as a result of the presence of nucleating agents (Block, 1982a). The supercooling ability of the adult weevil, Rhynchaenus fagi decreases when feeding restarts in spring (Bale, 1980). Many freezing-susceptible species therefore show maximum cold hardiness in the winter, only to lose such levels when feeding resumes (Sømme, 1982).

The supercooling point studies on Myzus persicae, (Chapter Four) revealed a markedly different pattern to that observed for most

freezing-susceptible species. Indeed all stages of the aphid exhibited maximum supercooling while feeding, under favourable laboratory culture conditions or in the field during summer (O'Doherty and Bale, in press).

Aphids which overwinter in the active stage continue to feed throughout this period, a feature again in contrast with many other overwintering insect species. This thesis has already discussed the overall loss of supercooling ability in overwintering adult M. persicae, and the substantial loss observed in a proportion of the population as winter progressed. It appeared that a possible explanation for these losses in cold hardiness might be associated with 'feeding status' overwinter.

The aim of the work described in this chapter therefore, was to investigate the influence of feeding on the supercooling ability of aphids. The quantity of food intake, and the quality of such food, was examined in experiments using M. persicae and the black bean aphid Aphis fabae.

MATERIALS AND METHODS

The influence of feeding on the cold hardiness of adults was of particular interest because of their seasonal pattern of cold hardiness. During winter however, continued reproduction would result in first instars forming part of the population and they were therefore also studied in most experiments.

The influence of starvation (restricted feeding) on supercooling ability

(1) Preliminary experiments were carried out to determine the longevity of M. persicae when subjected to starvation at various temperatures. Such information was necessary to plan the starvation experiments, following which supercooling ability would be determined.

Adult apterae (newly moulted) and first instar nymphs were removed from leaf discs of Chinese cabbage under 20°C culture conditions, and maintained as detailed in Chapter Two (see Plate 4) but without food. Aphids were starved at 20°C, 5°C and 0°C in cooled, illuminated incubators. In each sample (n=20), the period just before the first mortality occurred was recorded; this was to ensure that in planning the subsequent experiments, supercooling ability would be assessed before the aphids began to die as a direct result of starvation.

(2) Using the information obtained in (1), experiments were set up in which adults and first instar nymphs were starved at 20° and 5°C as described below.

a) 20°C - 1 and 2 days

5°C - 1, 2 and 7 days

The supercooling ability of the starved aphids was then assessed.

Subsequent experiments were carried out in which adults only were starved:

b) 5°C - 7, 14 and 17 days

0°C - 1, 2, 7, 14 and 24 days

(3) The results from (2b) contrasted with those of the first experiment (2a) and therefore a set of experiments were carried out to investigate factors which might have accounted for this unreproducibility. The following treatments were used:

(a) - newly moulted adults which had not fed since moulting

(b) - newly moulted adults which had fed but were still in the pre-reproductive phase

(c) - newly moulted adults, fed and reproductive

These three groups were starved at 5°C for 17 days.

(d) - newly moulted adults, fed and reproductive, kept at 20°C in dishes for 2h before being starved at 5°C for 17 days. This period at 20°C was to allow time for imbibing of water (from moist tissue) to occur.

(e) - as (d) but then starved at 0°C for 17 days.

(f) - post-reproductive adults, taken from the 20°C culture and starved at 5°C for 17 days.

Following each treatment, supercooling ability was assessed.

(4) To investigate the supercooling ability of aphids with no food in the gut, newly born (<45 min old) first instar nymphs were collected following production by adults at 20°C.

Aphids for all the above experiments were taken from the S strain culture.

The influence of food 'quality' on supercooling ability

(1) The supercooling capacity of M. persicae on a range of host plant species under natural conditions has already been discussed in Chapter Four. It would appear that under favourable conditions, supercooling potential is more or less comparable whatever the host species. In addition, losses of supercooling potential in M. persicae occurred on several host plant species.

Experiments to investigate the influence of food quality on supercooling ability involved rearing aphids on different growth stages of Chinese cabbage at 20°C. The stages used were 1-week old seedlings, 3-week old seedlings, 6-week old plants and leaf discs as normally used in experiments. In addition, adults which had developed on senescent leaf discs at 20°C, and others allowed to feed on senescent discs for 1 week at 5°C were tested. All aphids were from the S strain except for the experiment with 6-week old plants where S and R_2 first instar nymphs and adults were compared.

(2) Observations were made on the feeding positions of first instar nymphs and adults of M. persicae on whole plants and leaf discs. It was observed that while both settled on the veins, adults were more likely to be on or close to large veins, while first instar nymphs

were nearer the smaller veins. To investigate whether feeding site might account for the observed difference in inherent supercooling ability, adults were placed on sections of leaf tissue (Chinese cabbage) incorporating main veins only and the following day, first instar nymphs (12h old) were removed and their supercooling ability assessed.

(3) During studies on the cold hardiness of the black bean aphid Aphis fabae, which are discussed in Chapter Seven, it was found that aphids feeding on the woody host, spindle (Euonymus europaeus) in autumn and winter exhibited much poorer supercooling ability compared to summer individuals, feeding on herbaceous hosts such as sugar beet and bean plants. This suggested a food influence related to sap quality. Thus, experiments were conducted in which aphids were transferred between woody and herbaceous host plants, followed by assessment of supercooling ability.

Samples of fundatrigeniae (progeny of fundatrices which had hatched from overwintering eggs) were collected from spindle bushes between 13-17 May 1984. Both samples of alate and apterous individuals were supercooled and another sample of apterous individuals were maintained on branches of spindle standing in water, in an 'aphid bay' at 20°C with a long photoperiod. Bean plants were the same as those used for maintaining laboratory cultures (see Chapter Seven).

The programme of transfer experiments, summarised in Figure 5.1, was then conducted under the above conditions, followed by supercooling ability assessment of each sample; the number of aphids tested is indicated under the experimental group from which they were taken. In addition, on 9 July 1984 a residual natural population of

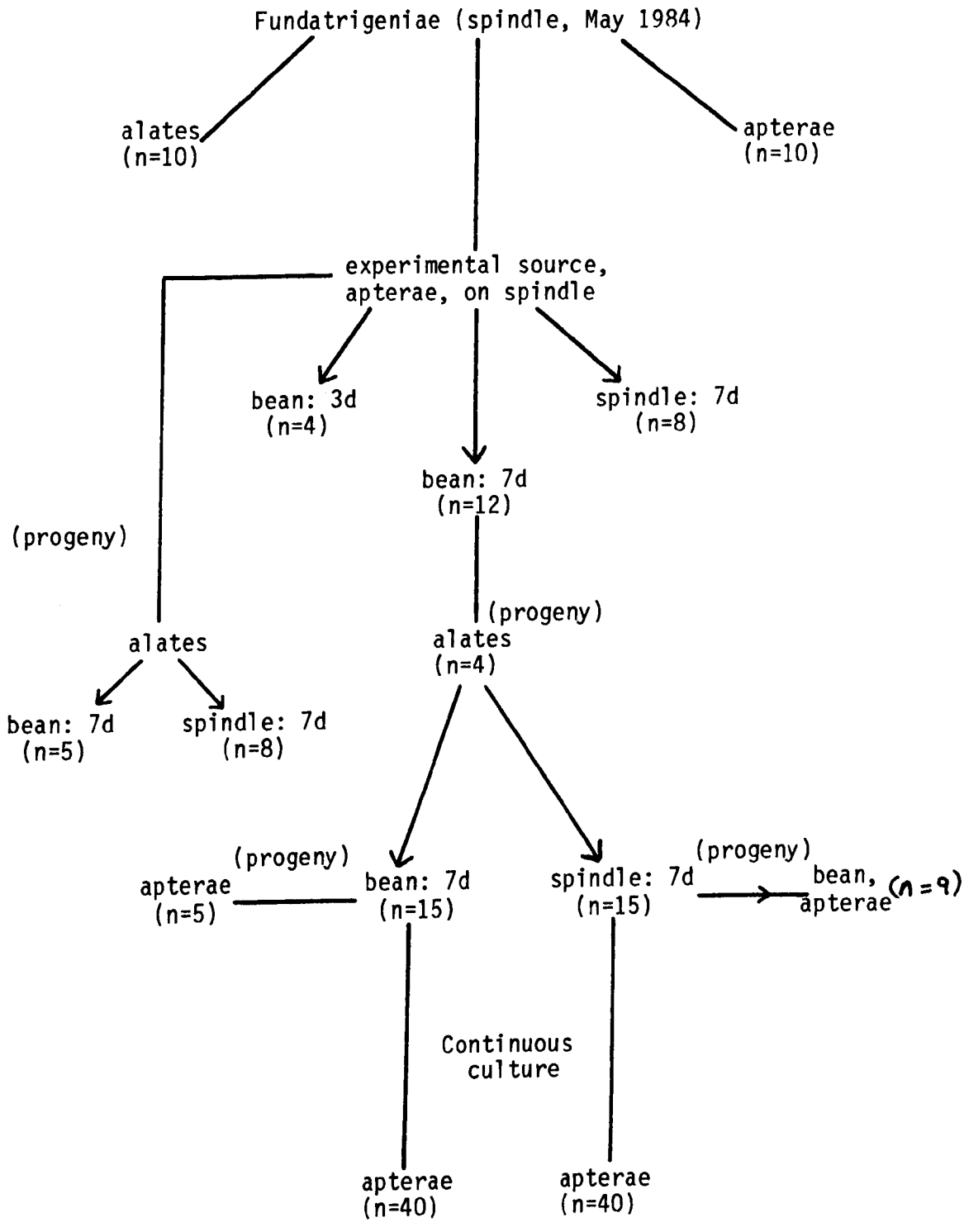


Figure 5.1 *Aphis fabae* host plant transfer experiments

A. fabae on spindle, provided a sample of apterae for supercooling point determination.

Statistical Analysis

The procedures outlined in Chapter Four were used to analyse the results from the above experiments.

RESULTS

The influence of food availability on survival and supercooling ability of M. persicae

Survival of starved aphids

Table 5.1 shows the periods of maximum (i.e. 100%) survival of samples of adult apterae and first instar nymphs following starvation at the three test temperatures. Both stages survived only a few days at 20°C without food. As the temperature decreased adults survived longer, for up to 20 days at 0°C. First instars survived for up to 7 days at 5°C, but subsequent mortality may have been induced prematurely. Without food, all the aphids were restless and subsequent wandering meant that it became difficult to contain the small nymphs. Therefore, Petri dish bottoms without a cover of mesh netting replaced the modified bottoms normally used (see Chapter Two). Consequently, humidity levels increased, leading to extensive condensation and first instars may have 'drowned' rather than died from the effect of starvation. For this reason the survival periods for first instars at 5°C and 0°C should be regarded with some caution.

Supercooling potential following starvation

Table 5.2 shows the supercooling abilities of aphids following starvation at 20°C. Neither adults nor first instar nymphs starved for 1 or 2 days showed any difference in their ability to supercool, compared with aphids allowed continued access to food (see Chapter Four).

There was also no difference in supercooling potential between first instars starved at 5°C or those allowed to feed under the same conditions (Table 5.3).

Table 5.1 Survival periods of *M. persicae* when starved at 20°C, 5°C and 0°C

Temperature	Survival (days)	
	Adults	First instar nymphs
20°C	2	2
5°C	14	7
0°C	20	7

Table 5.2 Supercooling ability (°C) of adults and first instar nymphs of M. persicae starved at 20°C

	Mean ± S.E. (n=20) Range	
	Days starved	
Aphid stage	1	2
Adult	-24.1 ± 0.20	-25.0 ± 0.21
	-21.0/-26.3	-23.1/-26.7
First instar nymphs	-26.5 ± 0.12	-26.0 ± 0.23
	-25.7/-27.6	-24.2/-27.4

Table 5.3 Supercooling ability (°C) of first instar nymphs of *M. persicae* starved at 5°C and control (7 days at 5°C with food, Chinese cabbage)

Mean ± S.E. (n=20)
Range

Starvation period (days)			
1	2	7	Control
-26.3 ± 0.16	-26.6 ± 0.18	-26.7 ± 0.15	-26.9 ± 0.23
-24.6/-27.5	-24.2/-27.7	-25.8/-28.6	-24.9/-28.5

Adult M. persicae starved for 1 and 2 days at 5°C also showed little change in supercooling ability (Fig. 5.2). However, after 7 days starvation, the distribution of supercooling points was skewed with an overall shift towards reduced supercooling potential, with a very obvious loss in a proportion of the sample. By comparison, adults maintained at 5°C with access to food showed no such losses of supercooling ability.

The extension of these starvation experiments with adults only at 5°C and 0°C firstly failed to continue the trend which was evident in the earlier experiments, and subsequently failed to reproduce the losses of cold hardiness which were observed after 7 days starvation at 5°C, with all individuals showing good supercooling potential (Table 5.4).

Unreproducibility of starvation experiments

Reproductive phase, feeding status following moult to adult or imbibing water prior to starvation, did not seem to account for the unreproducibility of the starvation data (Table 5.5); experiments 3(a) to 3(f), designed to investigate these possible influencing factors, showed that after 17 days all adults possessed good supercooling potential with no LG individuals.

Supercooling ability of aphids with no food content in the gut

The mean supercooling ability of first instars collected before commencing to feed was -28.1 ± 0.13 (range, -26.7/-29.5; n=20), which was more cold hardy than individuals which had fed (-26.0 ± 0.25) ($P<0.001$). The presence of food in the gut does therefore reduce supercooling potential to some extent.

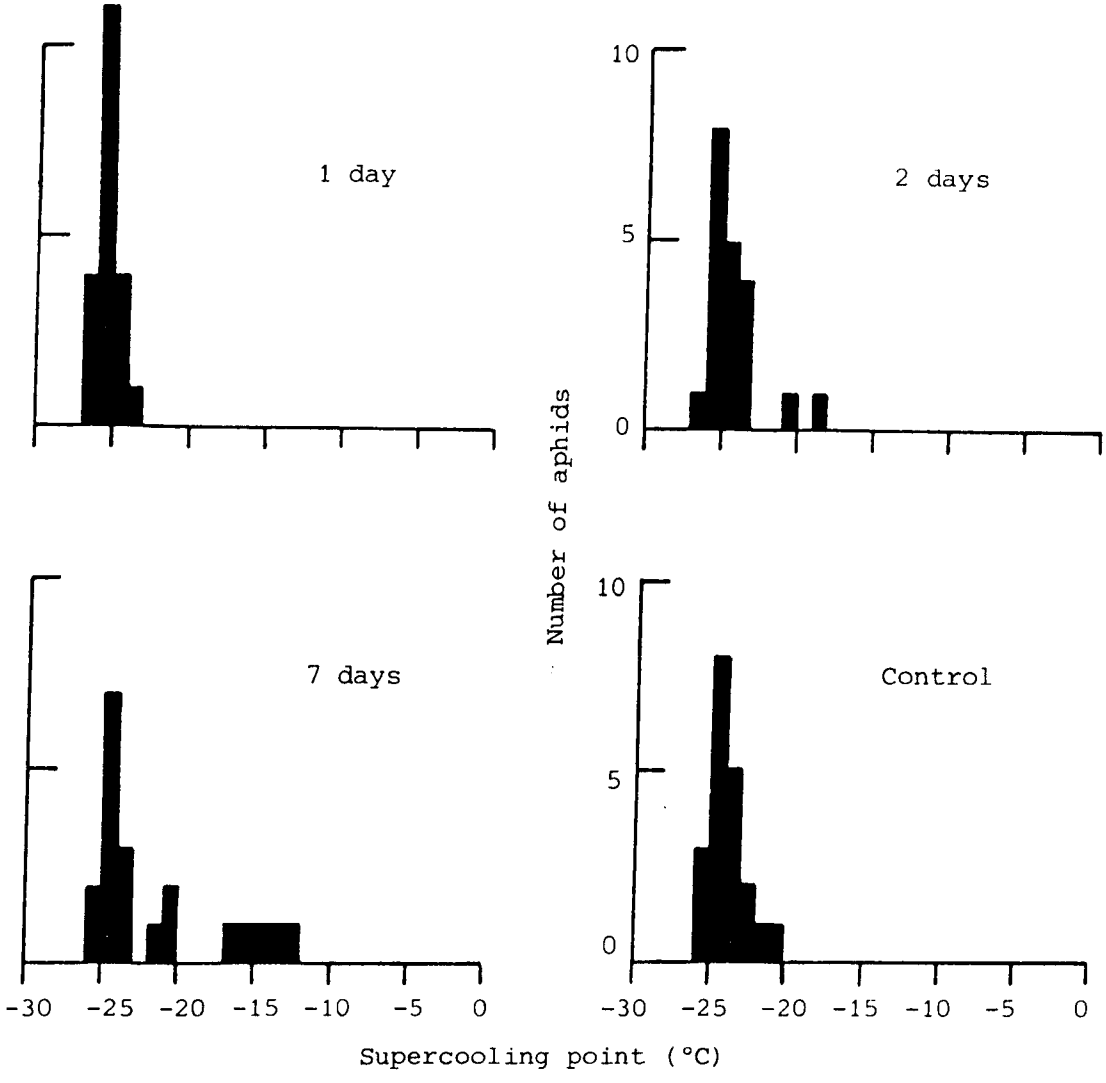


Figure 5.2 Supercooling point distribution histograms of adults of M. persicae starved at 5°C for varying periods of time and control (maintained at 5°C for 7 days with food)

Table 5.4 Supercooling ability (°C) of adult M. persicae starved for various periods of time at 5°C and 0°C

Time (days)	Mean ± S.E. (n=20) Range	
	5°C	0°C
1	-	-24.4 ± 0.44 -19.6/-26.6
2	-	-25.1 ± 0.35 -21.2/-26.7
7	-24.3 ± 0.42 -20.2/-26.7	-24.0 ± 0.35 -21.1/-26.3
14	-23.7 ± 0.34 -21.0/-25.9	-23.9 ± 0.31 -20.1/-25.7
17	-23.3 ± 0.62 -13.3/-25.8	-
24	-	-23.6 ± 0.39 -19.4/-25.6

Table 5.5 Supercooling ability (°C) of adult M. persicae starved following various treatments

Treatment	Mean ± S.E. (n=20)	Range
3(a) newly moulted adults, unfed, starved 17 days at 5°C	-24.7 ± 0.29	-21.8/-26.8
3(b) newly moulted adults, fed, pre-reproductive, starved 17 days at 5°C	-25.2 ± 0.19	-23.4/-26.5
3(c) newly moulted adults, fed, reproductive, starved 17 days at 5°C	-23.0 ± 0.27	-20.4/-25.4
3(d) newly moulted adults, fed, reproductive, on moist tissue at 20°C, prior to being starved for 17 days at 5°C	-22.5 ± 0.42	-17.7/-25.3
3(e) newly moulted adults, fed, reproductive, on moist tissue at 20°C, prior to being starved for 17 days at 0°C	-23.3 ± 0.39	-19.3/-25.7
3(f) post-reproductive adults, starved for 17 days at 5°C	-24.4 ± 0.40	-21.3/-27.2

The influence of food quality on supercooling ability

The supercooling ability of M. persicae on different growth stages of Chinese cabbage

The only significant difference between the different food sources was between 6-week old plants, and the seedlings and leaf discs; S adults had poorer supercooling potential when feeding on plants ($P<0.001$) (Table 5.6). Nymphs of the same strain however did not differ from those maintained on leaf discs. R_2 adults therefore were comparatively better supercoolers when feeding on these older plants than S adults ($P<0.001$) while aphids feeding on all the other food sources were capable of similar supercooling ability.

Feeding position and supercooling ability

When only allowed access to larger veins of Chinese cabbage, first instar nymphs of both the S and R_2 strains were capable of more extensive supercooling (S = -27.8 ± 0.31, range -26.0/-27.3: R_2= -27.3 ± 0.37, range -25.2/-28.5). This level of supercooling ability does not differ from that recorded for unfed first instars (see above), and therefore implies that feeding may not have occurred. However, even if it is assumed the aphids had fed, this result would suggest that feeding on large veins would not itself account for the poorer inherent supercooling ability of adults.

Influence of the host plant on the supercooling ability of A. fabae

Both apterae and alatae collected from the spindle in May showed poor supercooling ability (Table 5.7), typical of A. fabae

Table 5.6 Supercooling ability (°C) of M. persicae on different growth stages of Chinese cabbage

Mean ± S.E. (n=20)
Range

Growth stage	Strain and stage of aphid			
	S, adult	S, first instar	R_2, adult	R_2, first instar
1-week old seedlings	-24.4 ± 0.60 -14.5/-26.1			
3-week old seedings	-23.1 ± 0.26 -23.2/-26.2			
6-week old plants	-22.2 ± 1.83 -18.0/-24.9	-26.0 ± 0.39 -22.5/-28.4	-24.8 ± 0.24 -23.9/-26.9	-26.8 ± 0.46 -20.1/-28.9
Leaf discs	-24.2 ± 0.29 -21.3/-26.1	-26.0 ± 0.25 -23.1/-27.4	-23.8 ± 0.20 -22.3/-26.1	-25.9 ± 0.25 -23.7/-28.1
Senescent leaf discs	-23.1 ± 0.29 -23.3/-26.3			
Senescent leaf discs at 5°C	-24.8 ± 0.53 -17.7/-27.1			

associated with this host (see Chapter Seven). When transferred to bean plants, apterae from this source (2) were still poor supercoolers after 3 days but by 7 days most individuals were capable of considerable supercooling ability, almost comparable with laboratory maintained or summer collected A. fabae collected from herbaceous hosts (see Chapter Seven), with only three aphids possessing supercooling points above -20°C. By comparison, aphids maintained on spindle for 7 days were poor supercoolers.

Alate progeny (3) of the apterae used in the above transfers, were, after being transferred to bean for 7 days, still poor supercoolers and therefore no different from those transferred to fresh spindle for 7 days.

Alate progeny (A), originating on bean plants and transferred onto the same host for 7 days maintained their extensive supercooling ability as did their progeny. However, when a sample of these alates was transferred to spindle for 7 days the aphids showed a shift back to reduced supercooling ability, comparable with aphids maintained continuously on spindle. First instar nymphs produced by this latter transfer group, were transferred to, and allowed to develop to adult on bean plants, following which they showed extensive supercooling potential with only three individuals showing intermediate levels of supercooling ability.

When continuously cultured on bean and spindle for two months, the aphids within these groups remained good and poor supercoolers respectively (5). The natural population sampled from spindle at the same time were poor supercoolers.

Table 5.7 Supercooling ability (°C) of *A. fabae* following host transfer experiments (see Figure 5.1)

Mean ± S.E.
Range

SOURCE	TRANSFER/TREATMENT					
	Apterae	Alatae	Spindle:7d	Bean:3d	Bean:7d	Progeny
(1) Spindle, 13-17 May 1984	-9.8 ± 0.08 -9.5/-10.4	-10.2 ± 0.14 -9.7/-10.9				
(2) Colony established on spindle with apterae from (1)			-10.2 ± 0.13 -9.8/-10.6	-10.2 ± 0.12 -10.0/-10.5	-21.2 ± 1.07 -14.6/-24.9	-22.2 ± 0.89 -20.3/-24.3 (A)
(3) Progeny (alate) of (2)			-10.5 ± 0.45 -9.7/-13.6		-10.3 ± 0.23 -9.6/-10.9	
(4) Alates (A)			-9.9 ± 0.10 -9.0/-10.4		-23.7 ± 0.46 -18.8/-25.6	-22.7 ± 1.35 -19.1/-25.7
(5) Continuous culture of (4)			-11.5 ± 0.30 -9.5/-14.9		-23.8 ± 0.23 -19.6/-25.8	-21.8 ± 1.44 -14.8/-26.1
(6) Natural population, spindle, 9 July 1984	-13.0 ± 0.39 -10.7/-14.8					

DISCUSSION

All stages of the two aphid species studied during this research have possessed maximum supercooling potential when either maintained in the laboratory under favourable culture conditions, or when sampled from natural populations in summer, while feeding on a range of secondary, herbaceous hosts. Most freezing-susceptible arthropod species are by comparison capable of only limited supercooling when feeding, or when there is food in the gut (Sømme, 1982).

This contrast may be related to the specialised diet of aphids; as plant feeders, they use their modified mouthparts to extract phloem sap from host plants (Auclair, 1963; Forbes, 1977). This liquid diet is likely to be free of the efficient nucleators which often contaminate the diet of other plant feeders. In this respect, aphids may act as efficient vessels in which supercooling is favoured (Sømme, 1982). Sømme and Zachariassen (1981) concluded that the ability to obtain such a diet contributed to the extensive supercooling capacity demonstrated in the aphid Brachycaudus helichrysi sampled from Mount Kenya.

The continued feeding activity of overwintering aphids contrasts with most other freezing-susceptible species which actually cease feeding during this period. Mortality observed during the preliminary survival experiments would appear to be be the direct result of starvation, since during the acclimation experiments described in Chapter Four, longevity at low temperature in the presence of food was much longer. Starvation experiments were therefore designed to terminate before this 'senescent' phase began since this might itself be associated with a change in supercooling potential.

When starved experimentally in the first set of experiments at 5°C, the supercooling point distributions had a distinct HG and LG, which was almost identical to the field winter distributions. These results suggested the possibility of a starvation influence overwinter, particularly since a number of other potential influences had been eliminated. However, the failure of subsequent starvation experiments to confirm and/or extend this trend, together with the results from the experiments to identify an aphid or experimental factor which may have accounted for this subsequent unreproducibility, suggests that the solution is not so straightforward. Thus, the actual deprivation of food may not itself induce cold hardiness losses and the situation is apparently complicated by as yet unidentified factors.

Even when feeding is possible during winter, aphids are likely to have access to phloem sap from a variety of sources of varying quality. Sap flow is reduced overwinter and may even cease (Kennedy and Stroyan, 1959), and in addition reduced photosynthesis and growth during winter will result in a reduced carbohydrate and amino acid load in the phloem, which will therefore be of poorer quality for feeding aphids (Dadd and Mittler, 1965). Overall, aphids will be confined to older, poorer quality leaves and when collected from field hosts during this research, aphids were nearly always located on the senescent leaves. Harrington (1984) and Harrington and Xia-Nian (1984) suggested that as M. persicae preferentially colonises these older leaves, the aphids are likely to fall from the plant with the leaves during leaf abscission and subsequent cold periods may prevent these individuals from regaining a feeding position. Under natural overwintering conditions therefore a whole complex of interrelated

factors may act to increase the likelihood of aphid starvation, or the availability of poor quality sap and possibly induce the cold hardiness losses as observed in the field studies in 1982-83 and 1983-84.

When fed on Chinese cabbage of differing physiological 'quality', no LG individuals were observed even on senescent leaf discs at 5°C, although aphids were in the latter example, associated with these food sources for only one week. R_2 adults 'regained' supercooling potential when transferred after 14 days from senescent to fresh leaf discs during acclimation experiments (Chapter Four). It has been suggested that leaf excision results in the accumulation of synthates and the mobilisation of nutrients in leaf discs (van Emden, Eastop, Hughes and Way, 1969). This transfer to fresh plant material may therefore account for the regained supercooling potential in the R_2 adults. However, S strain adults did possess slightly poorer supercooling ability when feeding on whole plants compared to other food sources, including leaf discs, while R_2 individuals possessed similar levels of supercooling when feeding on whole plants and leaf discs.

A dramatic influence on aphid supercooling potential was observed during the host plant transfer experiments with A. fabae; the HG to LG shift was reversible and during transfers intermediate levels of supercooling were revealed. The LG to HG shift occurred between 3 and 7 days of feeding on beans; the length of time required if attributable to sap intake may have been because of low feeding rates, the initial transfer being of 'spindle adapted' morphs onto a herbaceous host. Even after feeding for 7 days on bean however,

alates from the original colony on spindle were still poor supercoolers. This may also be accounted for by the often more restless nature (Dixon, 1978) and therefore possibly lower feeding rates of alate migrants. However, alates transferred to spindle from bean became LG individuals within 7 days, suggesting that the shift from HG to LG is easier to produce than the reverse. That this poor supercooling ability was not the result of maintaining the aphids on excised branches of spindle was supported by the same poor supercooling ability observed in aphids from the natural population on the primary host.

Changes in the nucleator concentration of phloem sap ingested by the aphids Elatobium abietinum, Drepanosiphum platanoides and Eucallipterus tiliae influenced the supercooling point distributions of these aphids (Powell, 1976), and particularly that of first instar E. abietinum which were capable of extensive supercooling until they fed. The dramatic shift in supercooling potential achieved by transferring A. fabae to spindle indicates that a nucleator may also be involved here; such an explanation would also account for the greater difficulty experienced in obtaining the LG to HG response, if the nucleator was for example, retained in the aphid gut even after bean sap had been excreted. Further evidence for a nucleator in the sap of spindle was indicated by experiments in which the HG to LG shift was achieved in A. fabae samples within 2 days of transfer to spindle; however the LG to HG (bean) shift could not be demonstrated (A.F. Gash, personal communication). Since autumn collected spindle was used in the latter experiments whereas that in the present research was spring collected, this may suggest a greater nucleator concentration in the spindle sap in autumn. However oviparae are

better supercoolers than fundatrices/fundatrigeniae (see Chapter Seven), and therefore a more likely explanation may be greater retention of the nucleator within the aphid gut in autumn.

While nucleator activity seems a very plausible explanation for shifts in A. fabae supercooling ability, it is doubtful whether in retrospect a nucleator might account for such losses in M. persicae. If the losses observed overwinter in adults were the result of nucleator uptake in plant sap, a greater proportion of the sample might be expected to have shown a loss of supercooling potential assuming more aphids would be in the fed state. Also, in the first set of starvation experiments, where all aphids were treated identically, the HG/LG distribution was similar to that observed in winter. This latter result might suggest that if a nucleator is involved it may be aphid derived and present in LG individuals only. Salt (1953) suggested that food may reduce cold hardiness by direct inoculation or by an insect tissue reaction to the 'foreign matter'. Parry (1978) suggested that losses of supercooling ability in starved M. persicae might result from a gut associated reaction. In practice it is difficult to starve aphids experimentally to the extent that gut evacuation occurs, since unlike many other insects the gut contents tend to be retained during starvation rather than excreted (Auclair, 1963). During starvation experiments or while overwintering, adult aphids would probably possess some gut content; losses of supercooling potential may therefore be due to a reaction within the aphid gut.

Starvation did not affect first instar M. persicae cold hardiness; this instar is clearly less susceptible than adults to this factor together with others, as a modifying effect on inherent supercooling levels. Furthermore, this agrees with the seasonal

pattern observed, in which first instars maintained extensive supercooling potential all year. Once sap was imbibed, first instars lost only about 2°C of supercooling potential; this contrasts with the initial feed of E. abietinum first instars in which supercooling potential was reduced by up to 10°C (Powell, 1976). Parry (1978) also recorded the difference in supercooling ability between fed and unfed first instar nymphs of M. persicae to be nearer that observed in this work. This perhaps further suggests that a sap nucleator is not responsible for changes in the supercooling ability of M. persicae.

Powell and Parry (1976) attributed some of the winter mortality experienced by E. abietinum to the effect of starvation following extended chill-coma, which aphids will experience at temperatures well above those which would induce freezing death. Smith (1981) and Powell (1973) observed that in the cereal aphid Sitobion avenae and E. abietinum the temperatures inducing chill-coma were -4°C and approximately 0°C respectively. Whilst in chill-coma the aphids would become incapable of movement and possibly also would be prevented from feeding. With reference to the data in this chapter, it is tempting to suggest that an alternative explanation to Powell and Parry's theory is the likelihood of reduced supercooling potential in starved overwintering adults, and thus a freezing related death.

Despite much research effort concerned with various aspects of aphid feeding (Auclair, 1963, 1964), very little of it has been concerned with the feeding behaviour and nutritional requirements of aphids at low temperature or overwinter (T.E. Mittler, personal communication). Without such basic information it becomes difficult to interpret further the inter-relationships between feeding

behaviour, starvation and poor quality hosts as an influence on supercooling potential during winter. However these results pose a number of interesting questions. Firstly, if the inherent supercooling potential of feeding aphids is comparable with that of freezing-susceptible arthropods which often have accumulated natural antifreezes/ cryoprotectants (Sømme, 1982), does the phloem sap provide a basis for 'cryoprotection' in aphids, in addition to its nucleator-free nature? Extending this analogy, in starved M. persicae or those feeding on poor quality hosts, might the observed loss in supercooling potential be associated with the deprivation of some substance(s) within the sap, or with the subsequent inability of the aphid to 'metabolise' a substance from the plant sap contents?

This suggested that a study of the carbohydrates in both M. persicae and A. fabae might provide further information to support the 'feeding theory' on which the above ideas are based. The next chapter is concerned with these studies.

CHAPTER SIX

CARBOHYDRATE CORRELATES OF APHID COLD HARDINESS

SUMMARY

The carbohydrates of Myzus persicae and Aphis fabae, extracted from whole body homogenates were analysed by gas-liquid chromatography using a trimethylsilylation derivatisation technique. Separations were initially achieved on a packed column but the gas chromatograph was then converted for use with a capillary column, with an inlet splitter injection system, which resulted in better resolution and enabled the use of a temperature programme. Carbohydrates were identified on the basis of relative retention times and quantified by integration of peak areas; arabitol was used as the internal standard.

Glucose and glycerol were present in all M. persicae, together with fructose, mannitol, sucrose and trehalose in some samples. A number of unidentified carbohydrates were also present in trace amounts in many samples. The same carbohydrate spectrum was found in A. fabae, but no unidentified compounds were detected. Dulcitol was present only in A. fabae which had fed on spindle.

There was no obvious correlation between the presence or absence of individual substances and the extent of aphid supercooling ability. Total percentage body carbohydrate levels were however high, particularly in first instar M. persicae at over 12%,with half of this amount attributable to glycerol alone. In adult M. persicae and A. fabae total carbohydrate composed almost 7% and 5% of the fresh body weight respectively. It is suggested that such levels of body carbohydrate may provide a basis for maximised supercooling potential in laboratory maintained and feeding individuals.

INTRODUCTION

The accumulation of cryoprotective substances in both freezing-tolerant and freezing-susceptible terrestrial arthropods is well documented and has recently been discussed in several comprehensive reviews (Danks, 1978; Zachariassen, 1980; Baust, 1981; Duman, 1982). In freezing-tolerant species these natural antifreezes minimise cellular damage while in the frozen state (Baust, 1981). In freezing-susceptible species they act to enhance supercooling ability by depression of the true freezing point; it is likely that they have other 'cryoprotective functions' and probably play some part in metabolism (Duman, Horwarth, Tomchaney and Patterson, 1982). The production of these cryoprotectants accounts for the increased supercooling ability (acclimatisation/cold hardening) observed in overwintering freezing-susceptible species (Sømme, 1982), and this synthesis can be triggered by acclimation at low temperatures (Storey, 1984). This ability to increase freezing avoidance makes their production an essential part of the cold hardiness strategy of many of these species (Duman, 1982).

A number of low molecular weight compounds and particularly polyhydric alcohols (polyols) and sugars have been correlated with the increases in cold hardiness observed in terrestrial arthropods during winter (Duman, 1982). Free amino acids have also been suggested as functioning as antifreezes (Sømme, 1967). The most commonly reported low molecular weight carbohydrate is the polyol glycerol (Danks, 1978; Duman, 1982; Sømme, 1982), the first of such substances to be associated with arthropod cold hardiness (Salt, 1957, 1959). The

predominance of reports of glycerol as a cryoprotectant has been suggested to result partly from the fact that it is often the most commonly and sometimes the only such substance tested for (Danks, 1978); however the importance of a substance as a cryoprotectant depends not only on its presence, but also its concentration, and in this respect such a relationship has been substantiated not only for glycerol but also for many of the other cold hardiness correlated substances (Sømme, 1982). Recently a group of high molecular weight substances, known as thermal hysteresis proteins (THPs), have been shown to be important as antifreeze agents particularly in freezing-susceptible terrestrial arthropods where they act in a non-colligative manner to reduce the freezing point and consequently supercooling point of these species (Duman, 1982). These compounds are similar to the protein and glycoprotein antifreezes of polar, marine fish (DeVries, 1982; Tomchaney, Morris, Kang and Duman, 1982).

As this research developed it seemed unlikely that Myzus persicae synthesised cryoprotectants in the traditional sense as described above, because although younger instars demonstrated an acclimatisation response, it was relatively small, and adults actually lost cold hardiness as winter progressed. However, the considerable inherent supercooling potential of laboratory maintained and summer collected M. persicae suggested that the phloem sap on which the aphids fed, in addition to being nucleator-free, may provide an external 'cryoprotectant' source since starved aphids lost supercooling potential; it has been suggested that the same losses observed in natural populations during winter may be due to the inability to obtain food during winter.

The poor supercooling ability associated with individuals of Aphis fabae feeding on the primary host, spindle, and the dramatic losses observed when aphids were transferred to spindle from bean plants on which they were capable of extensive supercooling potential, has been attributed to the possibility of a nucleator in the spindle sap. Powell (1976) has suggested that changes in the nucleator concentration of the phloem sap of the aphids Elatobium abietinum, Drepanosiphum platanoides and Eucallipterus tiliae influenced the supercooling point distribution of these aphids. Krog, Zachariassen, Larsen and Smidrød (1979) have reported the presence of a high molecular weight carbohydrate with nucleator activity from Afro-alpine plants (Lobelia telekii); most biological nucleators identified to date are of a proteinaceous nature (Duman, 1982; Zachariassen, 1982).

The work described in this chapter therefore investigates the carbohydrates present in samples of M. persicae and A. fabae by gas-liquid chromatography (GLC) of trimethylsilyl (TMS) derivatives (Sweeley, Bentley, Makita and Wells, 1963; Blau and King, 1977; Block and Sømme, 1982).

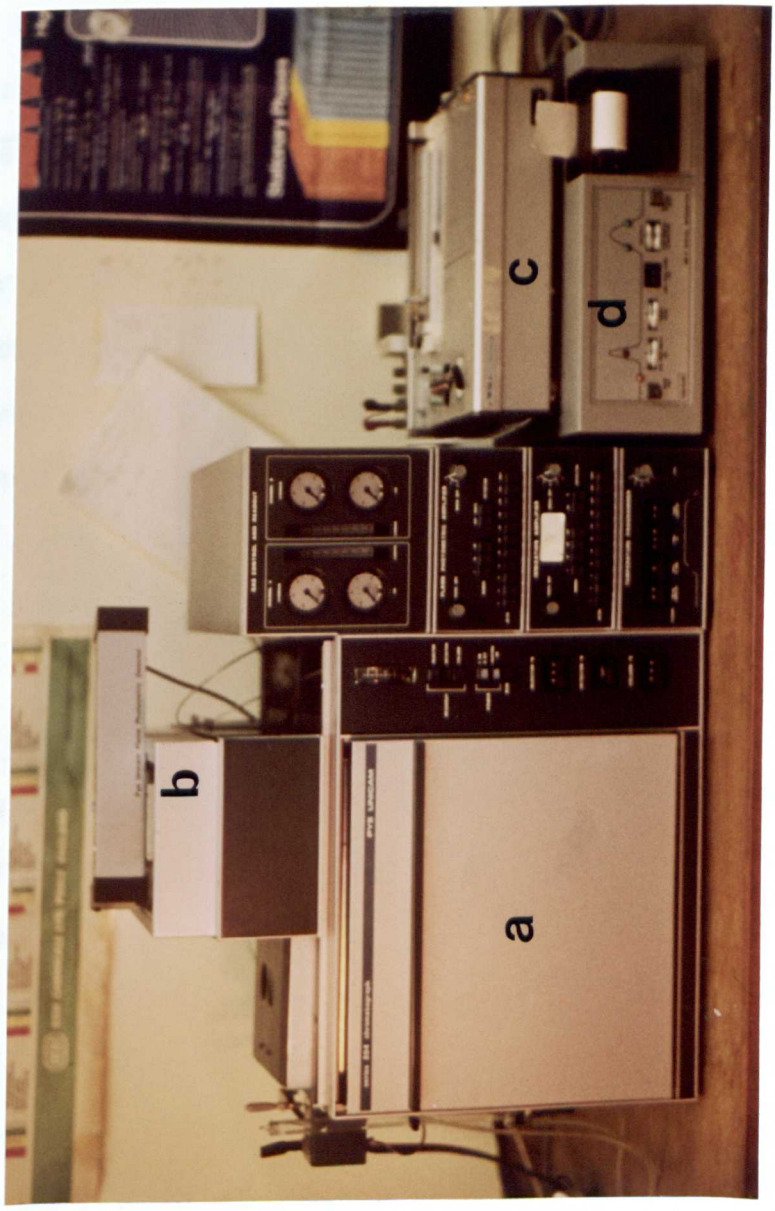
MATERIALS AND METHODS

Gas chromatography

A Pye-Unicam Series 204 gas chromatograph was used (Plate 21), and in preliminary work employed the use of a glass, packed column, a 3% OV-1 (100/120, Chromosorb). Separations were carried out at isothermal oven temperatures of 190°C and 250°C; injector and detector temperatures were 250°C. Nitrogen, at 30ml/min, was used as the carrier gas. Using a hydrogen flame ionisation detector (FID; hydrogen and air at 30ml/min), a range of carbohydrate standards were run to determine retention times, and a few aphid samples were derivatised (see below) and analysed to obtain preliminary information on the carbohydrate content. A number of carbohydrates were separated and identified but the use of isothermal temperatures resulted in excessively long running times. An attempt to overcome this problem by using a temperature programme was unsuccessful because of the high levels of stationary phase bleed from the packed column.

A decision was made to use a capillary column which not only enabled temperature programming to be used, so reducing running times, but also gave much improved resolution of the separated substances, due to the increased length of the capillary column (Adam and Jennings, 1975; Jellum et al, 1976). The gas chromatograph was therefore 'converted' for use with capillary columns by fitting a capillary 'inlet splitter' injection system. This system 'splits' the sample in a set ratio so that only a small portion of the injected volume actually goes onto the column with the rest split off. This is necessary because of the lower capacity of capillary columns compared to packed columns. Full details of conversion are given in Anon (1981).

Plate 21 Gas chromatograph (a), with flame ionisation detector (b), as used in conjunction with single channel chart recorder (c) and digital integrator (d).


a
b
c
d

A fused silica (quartz) 25m SE-30 WCOT capillary column was selected to achieve carbohydrate separations (Pelletier and Cadieux, 1982). Helium was used as the carrier gas at a flow rate of 1ml/min; this rate was set and checked daily prior to use, by adjusting the retention time of injected methane to 100s using the carrier gas pressure regulator. (This corresponds to an average linear carrier gas velocity, $\bar{u}$ of about 25cm/s for helium through a 25m column). The split was set at 20:1. The injector temperature was 210°C while that of the detector was 250°C. Hydrogen and air flow rates were 30 and 300ml/min respectively. The oven temperature operated under the following programme: 110°C for 5min, increasing at 2°C/min, until 240°C, and then held for 20min, giving a total running time of 90min. This programme was selected following good separations of standard carbohydrates.

Derivatisation of carbohydrates

Carbohydrate standards (obtained in the purest form available from a range of chemical companies) were dried over P_2O_5 (phosphorus pentoxide) before use. A known amount of standard (approximately 1mg) was weighed out on a Beckman microbalance and placed in a 1ml reactivial (Pierce Chemical Co.) and internal standard (arabitol) then added. The carbohydrates were then dissolved in distilled water (Rumpf, 1969) and the mixture evaporated to dryness under nitrogen on a heating block (Silli-Vap and Silli-Therm, Pierce Chemical Co.) at 40°C (Worland and Block, personal communication).

The derivatising reagent was Sigma-Sil-A (Sigma Chemical Co.), a mixture of pyridine, hexamethyldisilazane (HMDS) and

trimethylchlorosilane (TMCS) in a 9:3:1 ratio (Sweeley, Wells and Bently, 1966; Laine, Esselman and Sweeley, 1972). This was added to the dried standards (100µl per mg), the mixture agitated on a whirlimix and then reacted overnight on the heating block at 40°C (Narumi, Arita, Kitagawa, Kumazawa and Tsumita, 1969). Injections were made from the mixture directly into the gas chromatograph (Womersley, 1981).

Calibration procedure

The retention times (RT) and relative retention times (RRT) of each carbohydrate standard was determined following three experimental runs. Peaks were displayed on a chart recorder (Servoscribe 1s) and retention data collected on a digital integrator (Venture Mk II). The peak areas of standard and sample sugars were computed by the integrator.

Quantitative analysis of the sugars and polyols in aphid samples was achieved by firstly calculating the relative response factor (RRF) between each carbohydrate standard and the internal standard during calibration runs using the following calculation, based on a mean of three runs per standard;

$$\frac{\text{integrator units (carbohydrate standard)}}{\text{integrator units (internal standard)}}$$

Thus since a known amount of internal standard was included in each aphid sample, the concentration of each identified carbohydrate could be calculated by using the following equation (Chapman, 1981);

$$\frac{\text{integrator units (sugar or polyol)}}{\text{integrator units (internal standard)}} \times \frac{\text{concentration of internal standard}}{\text{RRF (for sugar or polyol)}}$$

Preparation of aphid samples

All aphids (M. persicae and A. fabae) to be analysed had been stored in a -70°C freezer following supercooling point determination or were taken directly from the laboratory cultures. All samples therefore contained 20 aphids except laboratory maintained M. periscae samples and A. fabae apterae maintained continuously on bean and spindle, where larger sample sizes were available to quantify the identified sugars and polyols (see Tables 6.5 and 6.6).

Aphid samples were homogenised (by hand) in 60% ethanol together with a known amount of internal standard until the aphids were well disintegrated. The mixture was centrifuged until the supernatant was clear (approximately 5min at 6000rpm), and then pipetted off into a 1ml reactivial. The precipitate was resuspended in ethanol, respun, the supernatant pooled with the first and the procedure repeated once more. The pooled supernatants were dried under nitrogen on the heating block and then left over P_2O_5 overnight. Sigma-Sil-A was then added at a volume of 200µl per 20 adult aphids or proportions thereof, depending on the sample weight. The mixture was allowed to derivatise overnight at 40°C. The samples were then analysed by gas-liquid chromatography on the basis of at least 3 injections per sample.

RESULTS

Retention times of carbohydrate standards

The actual and relative retention data for the TMS derivatives of carbohydrate standards are shown in Table 6.1. Complete separation of all standards was achieved even where retention times were close, as demonstrated in Figure 6.1 for dulcitol and mannitol. The majority of substances were resolved as single peaks although glucose gave two peaks representing the α and β forms. Fructose, arabinose, ribose and dihydroxyacetone each gave a number of peaks (see Table 6.1). Arabitol which was resolved as a single peak, eluted at 36.42min (approximately mid run), had not been detected in any aphid samples and was therefore selected as the internal standard (IS).

Carbohydrate content of aphids

Sugars and polyols identified from aphid samples

Table 6.2 shows the carbohydrates identified from the M. persicae samples. All aphids contained glycerol and glucose (α and β), and all laboratory reared and acclimated individuals contained mannitol. Sucrose was found in all laboratory cultures except alate adults but in the acclimated samples was found in third instar nymphs only. Trehalose was present in all laboratory cultured aphids with very high levels in alates. Adults of both the S and R_2 strains which had developed at 5°C contained trehalose but while S strain adults acclimated at 5°C for 14 days also contained trehalose, the comparable R_2 sample did not. Third instars of both strains at 5°C contained trehalose but second instars did not. Fructose occurred in all

Table 6.1 Actual and relative retention times (minutes) of TMS derivatives of carbohydrate standards

Carbohydrate		Actual retention time (RT)	mean	Relative retention time (RRT)
D (+) Arabitol (internal standard)		36.72; 36.73; 36.42	36.62	1.0
Sucrose		82.25; 82.24; 81.98	82.16	2.25
D-Glucose	α	44.65; 44.60; 44.64	44.63	1.23
	β	49.51; 49.46; 49.57	49.51	1.36
β-D (-) Fructose	1.	40.70; 40.62; 40.50	40.61	1.12
	2.	41.05; 40.97; 40.88	40.97	1.13
	3.	41.30; 41.22; 41.12	41.21	1.13
D-Mannitol		47.77; 47.53; 47.51	47.60	1.30
Sorbitol		48.05; 47.44; 48.69	48.06	1.31
D (+) Trehalose dihydrate		89.98; 89.46; 89.72	89.72	2.44
Dulcitol		47.99; 48.07; 47.88	47.98	1.31
Ribitol (adonitol)		36.75; 36.83; 36.65	36.74	1.01
L (+) Arabinose	1.	29.77; 29.03; 29.86	29.55	.81
	2.	29.97; 29.27; 30.07	29.77	.82
	3.	31.42; 30.90; 31.51	31.28	.86
	4.	32.96; 32.55; 33.03	32.85	.90
myo-Inositol		54.53; 54.52; 54.48	54.51	1.50
D (-) Ribose	1.	30.96; 30.72; 30.83	30.84	.85
	2.	31.70; 31.52; 31.57	31.60	.87
	3.	32.03; 31.87; 31.90	31.93	.88
	4.	32.64; 32.48; 32.51	32.54	.89
Glycerol		11.49; 11.50; 11.61	11.53	.32
i-Erythritol		24.48; 23.59; 24.50	24.19	.67
Threitol		23.85; 23.48; 23.76	23.69	.65
Dihydroxyacetone		34.14; 33.92; 33.18	33.75	.91
		35.80; 35.57; 34.78	35.38	.95
		36.01; 35.76; 35.00	35.59	.96
		37.99; 36.89; 37.78	37.55	1.01

Figure 6.1 Chromatogram of TMS derivatives of carbohydrate standards as separated on a SE-30 fused silica capillary column (see Materials and Methods). The peaks correspond to the internal standard, arabitol (1), α glucose (2), mannitol (3), dulcitol (4) and β glucose (5)

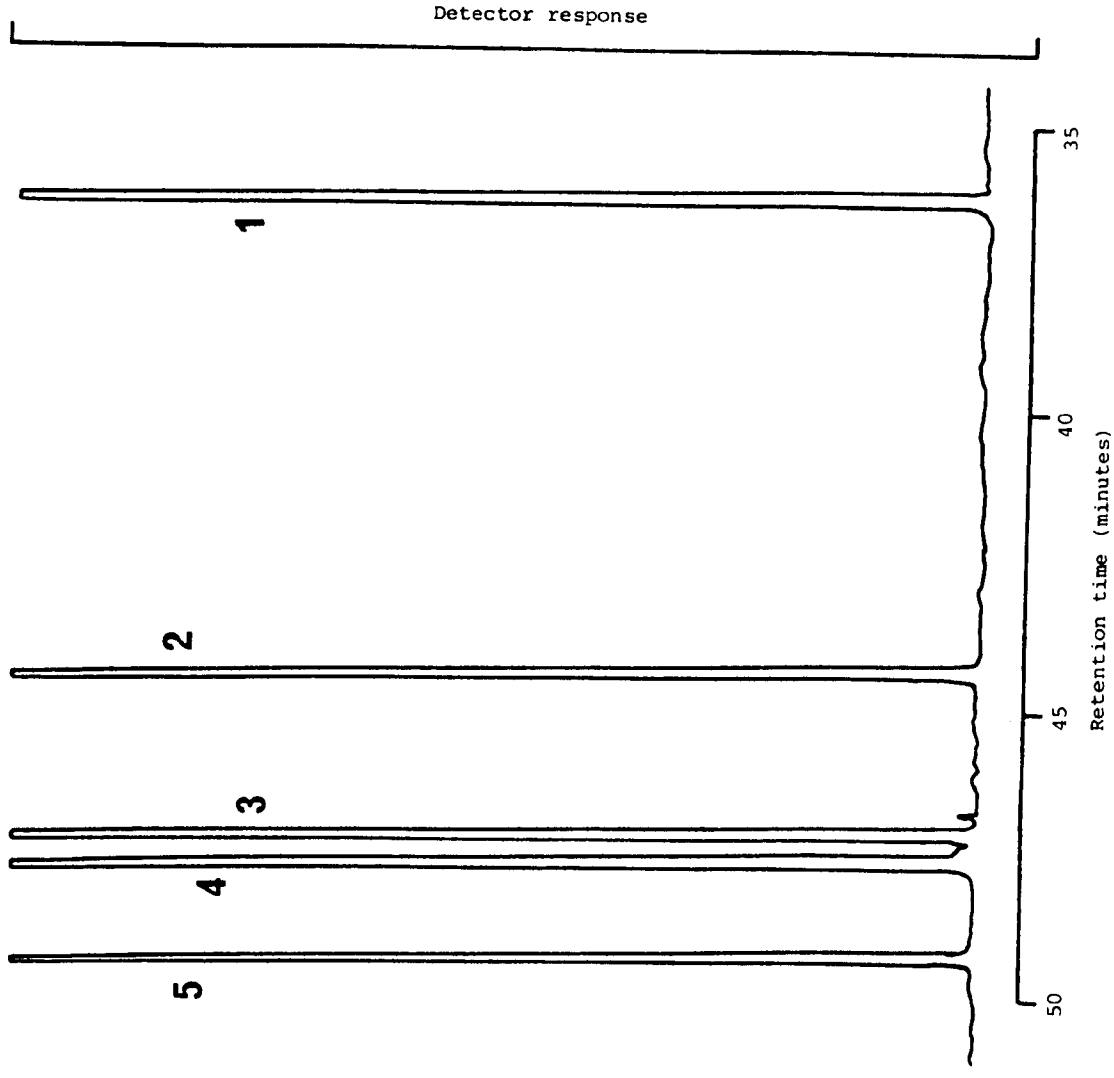

Detector response
1
2
3
4
5
35
40
45
50
Retention time (minutes)

Table 6.2 Carbohydrate content of *M. persicae* samples, as identified using gas liquid chromatography

	Glycerol	Fructose	Glucose (α and β)	Mannitol	Sucrose	Trehalose	Unidentified (RRT)
20°C CULTURE (R_2 STRAIN)							
First instar nymphs	X	X	X	X	X	X	
Second instar nymphs	X	X	X	X	X	X	0.55, 0.66, 1.33 1.59, 1.74
Third instar nymphs	X	X	X	X	X	X	1.18
Fourth instar nymphs	X	X	X	X	X	X	0.34, 0.62, 1.27 1.60
Adult apterae	X	X	X	X	X	X	0.33, 0.79
Adult alate	X	-	X	X	-	X	
ACCLIMATED APHIDS							
S, adults, development at 5°C	X	X	X	X	-	X	0.29
R_2, adults, development at 5°C	X	X	X	X	-	X	0.29, 1.60
S, adults, 14d at 5°C	X	X	X	X	-	X	0.28
R_2, adults, 14d at 5°C	X	X	X	X	-	-	0.28
S, second instar, development at 5°C	X	X	X	X	-	-	0.28

X = present
- = absent

Table 6.2 (Continued)

	Glycerol	Fructose	Glucose (α and β)	Mannitol	Sucrose	Trehalose	Unidentified (RRT)
R_2, second instar, development at 5°C	X	X	X	X	-	-	0.29, 1.57
S, third instar, development at 5°C	X	X	X	X	X	X	0.28
R_2, third instar, development at 5°C	X	X	X	X	X	X	1.18, 1.49, 1.82
FIELD COLLECTED APHIDS							
OH population, November 1982, adults	X	-	X	-	-	-	
OH population, December 1982, adults	X	-	X	X	-	-	0.23, 1.58, 1.71
OH population, January 1983, adults	X	-	X	X	-	-	
OH population, May 1983, adults	X	-	X	X	-	-	
Field population, December 1983, oilseed rape, adults, LG individuals only	X	X	X	X	X	X	

X = present
- = absent

acclimated and laboratory reared samples except alates. In the field samples, mannitol occurred in all but the November collected adults. Fructose, sucrose and trehalose were present only in the LG sample. A wide range of unidentified carbohydrates was recorded in many of the samples. These were present in smaller amounts than the above carbohydrates and their RRTs are given in Table 6.2.

Table 6.3 lists the carbohydrates identified from the A. fabae samples. The overall range of carbohydrates was smaller with all major peaks identified but with no range of unidentified compounds, comparable with those recorded in M. persicae. All individuals contained glucose, with glycerol and mannitol present in all aphids except laboratory maintained first and second instars. These two instars and virginoparae maintained continuously on beans were the only samples which contained fructose. Trehalose was present only in eggs collected in February and March 1983. Dulcitol was present only in aphids which had fed on spindle whether field collected or laboratory maintained. Sucrose was present only in the adult apterae maintained continuously on bean and spindle.

Many of the aphids analysed had been supercooled and petroleum jelly (used to attach specimens to the thermocouples) constituted part of these samples. An extraction of the jelly was made, as for the aphid samples, and analysed using GLC. A number of small peaks were recorded just after the solvent front (RRTs = 0.08-0.10) indicating that all peaks recorded in the aphid samples were therefore of aphid origin.

Carbohydrate concentrations in aphids

The relative response factors (RRF) calculated for carbohydrate standards are given in Table 6.4.

Table 6.3 Carbohydrate content of *A. fabae* samples, as identified using gas-liquid chromatography

Sample	Glycerol	Fructose	Glucose (α and β)	Mannitol	Dulcitol	Sucrose	Trehalose
20°C CULTURE							
First instar nymphs	-	X	X	-	-	-	-
Second instar nymphs	-	X	X	-	-	-	-
Third instar nymphs	X	-	X	X	-	-	-
Fourth instar nymphs	X	-	X	X	-	-	-
Adult apterae	X	-	X	X	-	-	-
FIELD COLLECTED APHIDS							
Oviparae, October 1982	X	-	X	X	X	-	-
Eggs, sample A (December 1982 and January 1983)	X	-	X	X	-	-	-
Eggs, sample B (February 1983 and March 1983)	X	-	X	X	-	-	X
Fundatrigeniae, May 1983	X	-	X	X	X	-	-
Virginoparae, July 1983 from beans	X	-	X	X	-	-	-
Virginoparae, July 1984 from beans	X	-	X	X	-	-	-
HOST TRANSFER EXPERIMENTS							
Virginoparae, bean, continuous culture	X	X	X	X	-	X	-
Virginoparae, spindle, continuous culture	X	-	X	X	X	X	-

X = present
- = absent

Table 6.4 Relative response factors for carbohydrate standards

Carbohydrate	Relative response factor (RRF)
Arabitol (internal standard)	1.0
Glycerol	0.47
Glucose (α and β anomers)	0.81
Mannitol	0.92
Dulcitol	0.87
Sucrose	0.75
Trehalose	0.81

The carbohydrate concentrations and their percentage of aphid fresh body weight was determined for laboratory maintained M. persicae (Table 6.5). Substances present only in trace amounts were not quantified (NQ) and fructose concentration was therefore disregarded in all stages. Glucose concentrations were highest in first instar nymphs and decreased as development progressed except for a slight increase in fourth instars. Mannitol also occurred in the greatest concentration in first instars with lower levels in subsequent instars. Sucrose was only quantified in third instars where it constituted just over 0.6% of the body weight. Trehalose composed 0.3% of the body weight of the same instar, with trace amounts in fourth instars and in adults composed almost 2% of the body weight. Glycerol made up over 6% of the body weight of first instars, decreased to trace amounts in second instars and then increased again with development to account for almost 4% of adult M. persicae body weight.

Table 6.6 shows the carbohydrate concentrations of adult A. fabae from cultures maintained on bean and spindle. The bean maintained aphids contained more than twice as much glucose as the sample from spindle. There was twice the concentration of mannitol in spindle reared A. fabae as in bean reared individuals, although these concentations were less than 1% of the body weight. Glycerol comprised just over 0.6% of the body weight of bean aphids which was almost twice the amount present in A. fabae from spindle. Sucrose accounted for over 1% of the body weight of bean reared individuals while the concentration of this carbohydrate in spindle reared aphids was too low to be quantified. Only a low concentration of fructose

Table 6.5 Concentrations of carbohydrates in laboratory maintained M. persicae

Stage (sample size)		Carbohydrates (µg/mg fresh weight)					
		Glycerol	Glucose (α and β)	Mannitol	Sucrose	Trehalose	Total carbohydrate content (%)
First instar nymph (n=100)		65.7	50.0	5.8	NQ	NQ	
	% fresh body weight	6.6	5.0	0.58	-	-	12.18
Second instar nymph (n=100)		NQ	24.8	1.5	NQ	NQ	
	% fresh body weight	-	2.48	0.15	-	-	2.63
Third instar nymph (n=50)		2.1	3.0	0.4	6.1	3.0	
	% fresh body weight	0.21	0.3	0.04	0.61	0.3	1.46
Fourth instar nymph (n=20)		8.8	15.7	0.4	NQ	NQ	
	% fresh body weight	0.88	1.57	0.04	-	-	2.49
Adult apterae (n=20)		38.2	4.9	3.0	NQ	19.4	
	% fresh body weight	3.82	0.5	0.3	-	1.94	6.56

Table 6.6 Concentrations of carbohydrates in adult apterae of A. fabae maintained continuously on bean and spindle

Host Plant (sample size)	Carbohydrate (µg/mg fresh weight)						
	Glycerol	Fructose	Glucose (α and β)	Mannitol	Dulcitol	Sucrose	Total carbohydrate content (%)
Bean (n=40)	6.3	NQ	25.5	0.9	none	12.9	
% fresh body weight	0.63	-	2.55	0.09	-	1.3	4.57
Spindle (n=40)	3.7	none	9.1	2.2	2.4	NQ	
% fresh body weight	0.37	-	0.91	0.22	0.24	-	1.74

was present in bean aphids and was therefore not quantified. Dulcitol in spindle derived A. fabae composed just over 0.2% of the body weight of these aphids. The separation of aphid carbohydrates as achieved by GLC of TMS derivatives is represented in Figures 6.2 and 6.3, where the chromatograms obtained for the bean and spindle reared A. fabae are shown.

Figure 6.2 Chromatogram showing carbohydrate content of A. fabae maintained on bean plants, as separated on an SE-30 fused silica capillary column (see Materials and Methods). The peaks correspond to glycerol (1), internal standard, arabitol (2), fructose (3), α glucose (4), mannitol (5), β glucose (6) and sucrose (7).

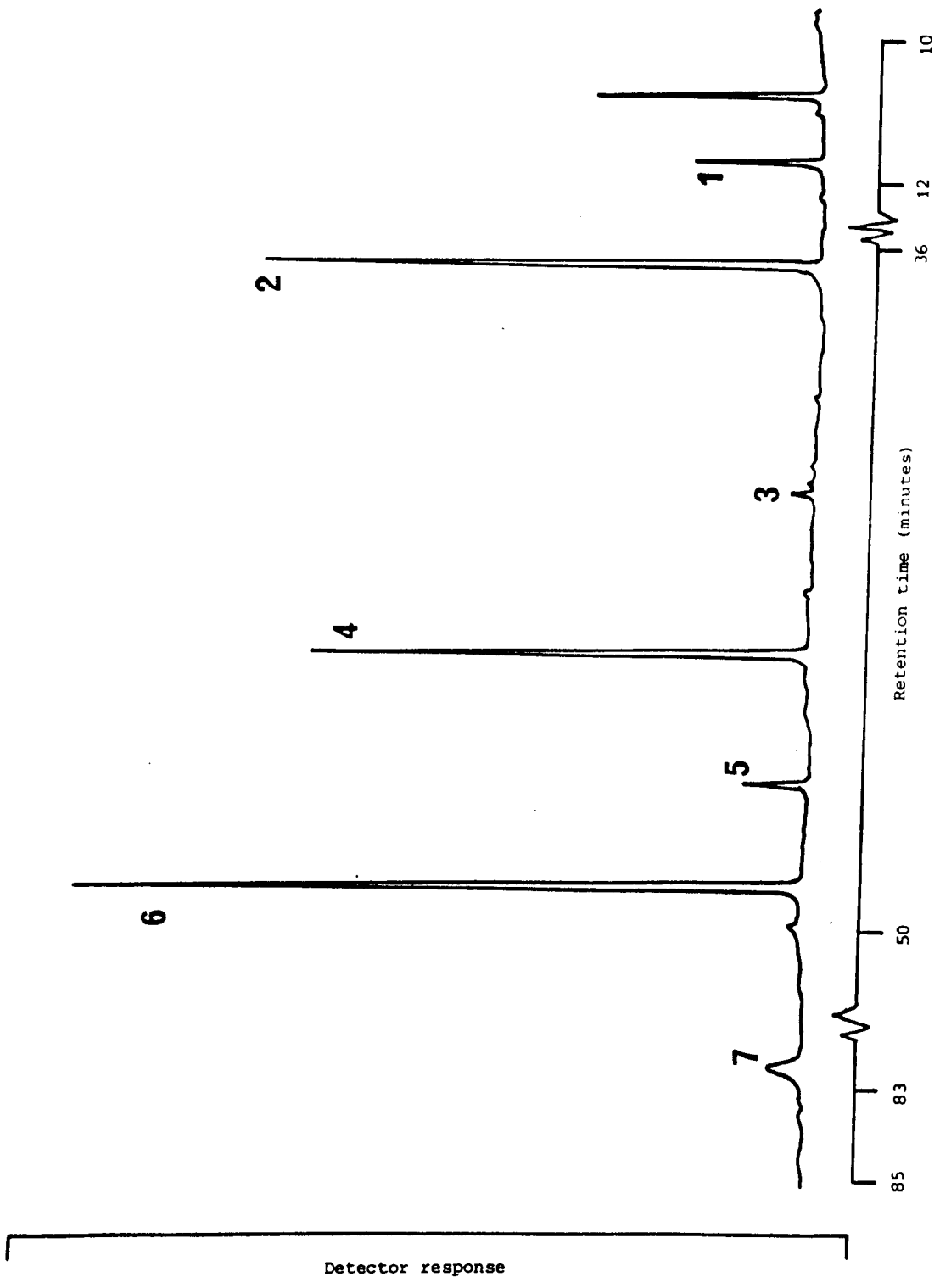
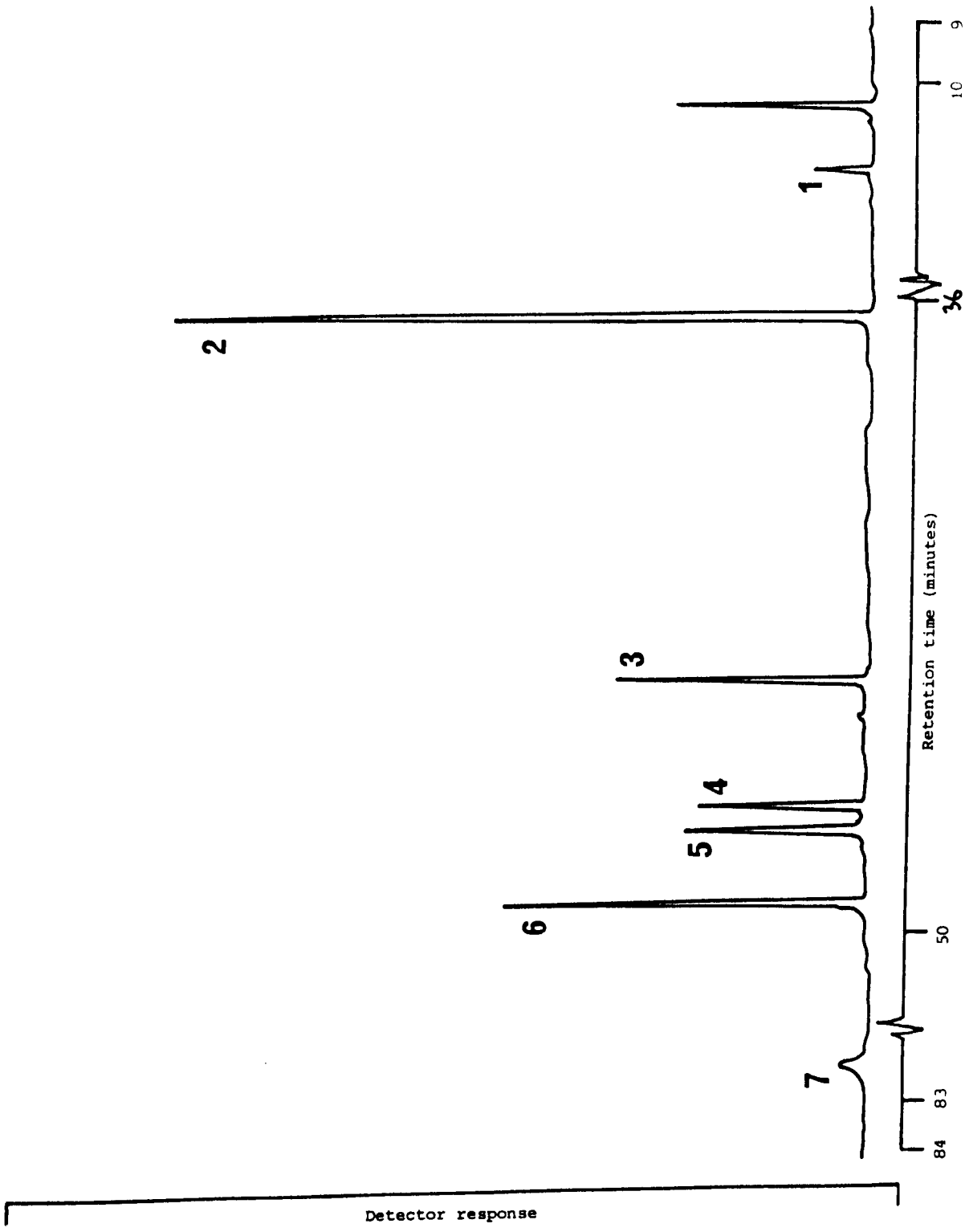

1
2
3
4
5
6
7
Detector response
85
83
50
36
12
10
Retention time (minutes)

Figure 6.3 Chromatogram showing carbohydrate content of *A. fabae* maintained on spindle, as separated on a SE-30 fused silica capillary column (see Materials and Methods). The peaks correspond to glycerol (1), internal standard, arabitol (2), α glucose (3), mannitol (4), dulcitol (5), β glucose (6) and sucrose (7).


1
2
3
4
5
6
7
Detector response
Retention time (minutes)
9
10
36
50
83
84

DISCUSSION

The carbohydrate standard list (see Table 6.1) included all those compounds implicated as having a cryoprotective function in terrestrial arthropods (Duman, 1982; Duman, Horwarth, Tomchaney and Patterson, 1982), including those which have been found in aphids (Sømme, 1964, 1969; Parry, 1979a, 1979b, 1985). In addition, substances associated with sap feeding insects (Auclair, 1963; Wyatt, 1967) were included. Carbohydrates outside this range were not present as major components of the M. persicae and A. fabae samples analysed. The presence of trace amounts or the apparent absence of substances in the qualitative analyses, may to some extent be a reflection of the small amount of material analysed. For example with respect to A. fabae analysis, each represented an extraction of 20 aphids, except for the samples continuously cultured on bean and spindle; some substances were recorded in the older instars but 'absent' from first and second instars (e.g. glycerol, mannitol) and the presence of most of the identified substances in the bean and spindle aphids might imply that concentrations may sometimes have been below the detection limits.

The chromatograms of the separated carbohydrates were not complex (see Figs. 6.2 and 6.3) and all substances in both aphid species could be identified on the basis of actual and relative retention data, particularly since the presence of these carbohydrates in aphids was not unusual. The use of the capillary column assisted in precise identification of aphid components since some compounds with similar elution times (mannitol, sorbitol and dulcitol) are unlikely to be totally resolved using packed columns (Adam and Jennings, 1975; Womersley, 1981).

The presence of glucose in all M. persicae and A. fabae samples was not unexpected since it plays a major part in the carbohydrate metabolism of all insects (Wyatt, 1967), and the decrease in concentration in M. persicae with instar age is probably associated with growth and development (Chippendale, 1978). However the amount of free glucose in insects is generally low (Wyatt, 1967) and thus the concentration in first instars, composing 5% of the total body weight could be considered atypically high. Concentrations of glucose in adults of A. fabae were higher than in M. persicae, and composed over 2.5% of the fresh body weight of bean feeding A. fabae. Apart from its metabolic origin, glucose in aphids may occur as a result of sucrose hydrolysis in the gut (Auclair, 1963, 1964) and therefore the higher glucose concentrations in A. fabae reared continuously on bean plants, may reflect a higher sucrose concentration in these plants compared to that in leaf discs of Chinese cabbage or cut spindle branches.

Sucrose is the principal sugar in plant sap and almost exclusively the only carbohydrate ingested by sap feeders (Auclair, 1963). Its presence was detected only in the larger A. fabae samples (n=40) from bean and spindle, but while the concentration was too low to enable quantification in spindle A. fabae, this sugar constituted over 1% of the body weight of bean aphids. Although detected in all laboratory samples of M. persicae which were quantitatively analysed, sucrose was present in high enough concentrations to do so only in third instars and additionally was absent in many of the other M. persicae samples. This suggests that sucrose concentrations in aphids were in general low and consequently were only above the qualitative detection level in those samples with larger aphid numbers.

Fructose was present only in trace amounts in those aphids in which it was detected and may have occurred as a direct result of sap uptake (Wyatt, 1967) or following sucrose hydrolysis in the gut (Auclair, 1964).

The disaccharide trehalose is the predominant haemolymph sugar and a major carbohydrate energy source in most insects (Wigglesworth, 1972; Florkin and Jeuniaux, 1974). The high level in alate M. persicae (in relation to the other aphid stages since it was not actually quantified) was presumably associated with the role of this carbohydrate as the principal energy source for insect flight (Wyatt, 1967), although during prolonged flight, aphids are more dependent upon fat sources (Richards and Davies, 1977). In M. persicae, the highest trehalose concentrations were in adults; the low levels in the instars may be the result of hydrolysis to release glucose during growth and development. In A. fabae, it was surprising that trehalose occurred only in the eggs. It was recorded from the pre-hatch sample (February/March) only; the eggs overwinter in diapause (Braune, 1973) and trehalose levels may increase during subsequent embryonic development to provide an energy source (Chippendale, 1978) and play a role in the hatching mechanism (Wyatt, 1967).

Glycerol is the most common carbohydrate cryoprotectant associated with arthropod cold hardiness (Danks, 1978; Sømme, 1982) and accumulation during acclimatisation has been correlated with increased supercooling potential in freezing-susceptible arthropods (Sømme, 1982). In the present study it was found in all aphids except first and second instar A. fabae. Its presence in most samples was not unusual since it is a normal insect metabolite and a principal

constituent of lipids (Chippendale, 1978), but the very high concentrations in first instar M. persicae, constituting nearly 7% of the fresh body weight of these individuals was particularly interesting on account of their observed cold hardiness, both under field and laboratory conditions. However, the trace amounts present in the equally cold hardy second instar nymphs suggests that this high glycerol concentration alone did not confer the extensive supercooling potential of first instars. In addition, glycerol composed almost 4% of the body content of adult M. persicae while in bean feeding A. fabae, with comparable supercooling potential, the glycerol concentration was less than 1%.

Mannitol concentrations were low in all aphid samples, always less than 1% of the total body content, which may account for it being undetected in the November field samples of M. persicae and first and second instar A. fabae. It is less widely reported as a cryoprotectant (Sømme, 1982) but its concentration has been shown to be correlated with acclimatisation in the eggs of a number of aphid species (Sømme, 1964, 1969; Parry, 1985). In this study it was present in both samples of A. fabae eggs but the concentration was not quantified. Parry (1979a) reported that in the green spruce aphid Elatobium abietinum mannitol was accumulated by overwintering aphids but disappeared rapidly when these aphids were maintained under laboratory conditions at 15°C. The presence of mannitol in unfed first instars contributed to their supercooling potential but this antifreeze influence was 'masked' in feeding aphids.

Dulcitol was present only in A. fabae which had fed on spindle. Baker (1949) identified the white encrustation on the leaves of

spindle colonised by unidentified aphids as this carbohydrate, and a similar white substance was observed in this study when A. fabae were maintained on spindle in the laboratory. Krog, Zachariassen, Larsen and Smidrød (1979) reported a carbohydrate with nucleator activity in the plant Lobelia telekii. This might suggest that dulcitol may be the sap nucleator proposed to induce poor supercooling ability in spindle-fed A. fabae. However the carbohydrate in L. telekii was a high molecular weight polysaccharide and both the lower molecular weight of dulcitol and its low concentration , comprising just over 0.2% of spindle-fed A. fabae, and therefore probably with an even lower sap concentration, suggests that there is perhaps less likelihood of dulcitol having a similar nucleator activity. The dramatic losses of supercooling potential observed when aphids were fed on spindle implies a very efficient sap nucleator occurring in a high concentration within the sap (Zachariassen, 1982) or with a more complex molecular structure (Duman, 1982; Zachariassen, 1982).

It would appear that no individual substance can be correlated with supercooling potential in the aphid species and stages studied in this work, since levels often fluctuated between individuals of comparable supercooling ability. Ring (1977) observed that in overwintering larvae of the freezing-susceptible bark beetle Scolytus ratzeburgi which were capable of considerable supercooling, glycerol accounted for 9% of the fresh body weight. However in the other developmental stages which possessed quite considerable supercooling ability, glycerol was absent but these individuals contained trehalose and sorbitol as part of the multifactorial cryoprotectant system in this species. Sømme and Block (1982) found the main substances in a

comparable system within a freezing-susceptible collembolan to include glycerol together with trehalose, mannitol and possibly fructose. The advantage of this type of cryoprotection is that it reduces the toxic effect of any one antifreeze agent (Ring, 1980; Sømme, 1982). A similar cryoprotective effect may be produced in M. persicae and A. fabae as a result of the observed carbohydrate range; however there can be little doubt these aphid substances also have major metabolic functions in addition to any cryoprotective influence they may confer (Duman, Horwarth, Tomchaney and Patterson, 1982).

In M. persicae the total carbohydrate content was high in a number of stages, but particularly so in first instars where over 12% of the body weight was accounted for by the identified carbohydrates, with almost half of this attributable to glycerol alone. This level of glycerol is almost comparable with that observed in cold hardy insects which actively accumulate this cryoprotectant (Ring, 1977). As development proceeded, carbohydrate levels fell but then rose so that adult M. persicae were almost 7% carbohydrate. The total sugar content of 8.1% in the haemolymph of the aphid Megoura viciae is apparently one of the highest sugar content levels recorded for an insect (Wyatt, 1967). Although concentrations in this study were based on whole body sugar measurements it is likely that the method used to prepare aphid samples would extract mainly 'free carbohydrates' (Womersley, 1981) and thus these carbohydrate concentrations in M. persicae may be considered comparatively high. It seems likely that such high carbohydrate levels in aphids is related to plant sap feeding, since the phloem sap will probably contribute to the levels of body carbohydrate either by the intake of such substances as a direct result of feeding or following subsequent

digestion/metabolism. Thus, extending this relationship, it may be that the intake of phloem sap contributes to the potential to supercool extensively by conferring these high total body carbohydrate contents.

In spindle-fed A. fabae it is likely that the dominant influence on supercooling potential would be the presence of the sap nucleator thought to be associated with this host plant (Chapter Five). High cryoprotectant and solute concentrations do not influence the activity of nucleators so that supercooling ability is not depressed below the level at which the nucleator induces freezing (Lee, Zachariassen and Baust; 1981; Zachariassen, 1982). Thus the carbohydrate content of A. fabae which had fed continuously on bean plants which, composing almost 5% of the total body weight can again be considered a high level, would be of little consequence when the aphids were transferred to spindle, since uptake of the sap nucleator would be a more influential factor on subsequent supercooling potential.

While protein correlates of cold hardiness were not studied in this work, Sømme (1966, 1967) has found that supercooling point depression in some insect larvae is related to an increase in the concentrations of amino acids. The phloem sap on which aphids feed contains a range of amino acids (Auclair, 1963); Parry (1978) however found that amino acid concentration has no effect on the supercooling potential of M. persicae.

Much greater information on the role of plant sap as an influence on aphid supercooling ability may be obtained by combining phloem sap analysis with the results of this study. The information available on the intermediary metabolism within aphids once carbohydrate-rich sap

is imbibed is limited and it is only recently that the established view that carbohydrates pass through the body largely unchanged, has given way to the theory that there is some degree of subsequent carbohydrate digestion/ metabolism (Auclair, 1963, 1964; Fisher, Wright and Mittler, 1984). Such studies may further assist in determining how feeding status in M. persicae affects supercooling ability.

CHAPTER SEVEN

COMPARATIVE STUDIES ON THE COLD HARDINESS OF THE BLACK BEAN APHID *APHIS FABAE*

SUMMARY

All stages of Aphis fabae maintained parthenogenetically at 20°C on broad bean were capable of extensive supercooling ability to below -20°C. First and second instar nymphs were the most cold hardy individuals.

First instars and adult apterae of A. fabae collected from natural summer populations on broad bean and sugar beet showed similar levels of supercooling potential to laboratory maintained samples. All aphids associated with the primary host, spindle, showed a substantially poorer ability to supercool, normally to less than -15°C, both in autumn (oviparae) and spring (fundatrices and fundatrigeniae). All samples of eggs supercooled to below -30°C, becoming fully acclimatised in mid winter and losing supercooling potential prior to hatching in spring. Eggs and oviparae were not susceptible to inoculative nucleation when wet.

Differential mortalities, during preliminary experiments in which aphids were subjected to prolonged exposure at -20°C, were demonstrated between A. fabae samples originating from bean (good supercoolers) and those from spindle (poor supercoolers), with increased survival in the former group. At -5°C aphids experienced mortality more rapidly than if induced by a freezing death alone and this may have been attributable to temperature shock or desiccation.

INTRODUCTION

The black bean aphid Aphis fabae Scopoli overwinters in Britain almost entirely as sexually produced eggs (holocyclic life cycle - see Fig. 1.1), although in areas of south-west Britain where winter conditions tend to be milder, overwintering as anholocyclic individuals is quite frequent (Anon, 1974). Spindle tree (Euonymus europaeus) is almost exclusively the primary host on which overwintering eggs are laid (Way and Cammell, 1982), although some egg laying may occur on Viburnum and Philadelphus (Eastop, 1983).

A wide range of herbaceous hosts are colonised following egg hatch and spring migration, including bean and beet crops, and weeds such as fat hen, dock and poppies (Anon, 1974; Eastop, 1981). Damage is caused by the feeding of large colonies which can build up on host plants (see Plate 1), reducing vigour and yield. A. fabae is also a vector of over 35 virus diseases (Eastop, 1983), of which beet yellows and beet mosaic are the most important.

The holocyclic overwintering of this species contrasts with the predominantly anholocyclic nature of M. persicae and studies on the cold hardiness of A. fabae were therefore carried out to provide comparative data for these two aphid species with different overwintering strategies. This chapter describes studies on the supercooling ability of laboratory maintained individuals and natural populations of A. fabae, and preliminary experiments to relate supercooling potential of the aphids to mortality at sub-zero temperatures.

MATERIALS AND METHODS

Aphids and laboratory culture

In October 1982, two sources of A. fabae on spindle were located; one at the University experimental garden (Ridgeway) (Plate 22), and the other within the University precinct (Plate 23). The identification of these aphids as fabae, from the complex of closely related Aphis species also found on spindle, was confirmed by transferring individuals collected in autumn and the following spring to broad bean (Vicia faba) plants on which it was then observed that they fed and reproduced (Blackman, 1974a).

A single virginopara collected from the precinct spindle in autumn 1982 was used to establish an experimental clone in the laboratory. The aphids were maintained at 20 ± 2°C with a 18h/6h photoperiod in an 'aphid bay' as described in Chapter 2. The aphids were fed on 4-week old whole broad bean plants (var. Dreadnought) within individual plant cages.

Supercooling point studies

Supercooling point determinations were carried out as detailed in Chapter Three and the results analysed statistically as outlined in Chapter Four.

(a) Inherent supercooling ability

Samples of all the developmental instars, apterous (newly moulted) and alate virginoparae were collected from the laboratory culture and their supercooling potential assessed.

(b) Seasonal supercooling ability

From October 1982 until the summer of 1984, the supercooling potential of A. fabae individuals associated with both the primary woody host, spindle, and herbaceous summer hosts was assessed.

Plate 22 Spindle bushes (background) at the University experimental garden, Leeds, from which samples of Aphis fabae were collected.

Plate 23 Spindle bushes (left hand side) in the University of Leeds precinct, from which samples of Aphis fabae were collected.

In October 1982, oviparae were collected from the precinct spindle and tested, as were samples of eggs at monthly intervals from November 1982 until March 1983; eggs became increasingly difficult to locate on the precinct spindle and the egg samples collected in February and March were taken from the bushes at Ridgeway. Fundatrices and fundatrigeniae were also collected from this latter source in April, May and June 1983, following egg hatch in March. Migration from these bushes subsequently reduced the population on the primary host and samples of adult apterae and first instar nymphs were collected from broad beans at Ridgeway in July 1983, and from sugar beet plants on the department rooftop in August 1983.

Oviparae were sampled from the precinct spindle in November 1983. Eggs were not collected in this winter but following egg hatch, fundatrigeniae and spring migrants were collected in May 1984. These samples also provided the aphids for the host plant transfer experiments described earlier in Chapter Five. Finally, a sample of adult A. fabae was collected from broad beans at the University farm (Tadcaster) in July 1984.

(c) Contact moisture

The influence of contact with surface moisture on the supercooling potential of oviparae and eggs of A. fabae, two of the stages most likely to experience low temperatures when wet, was examined by thoroughly wetting individuals with rainwater using a fine paintbrush, after which their supercooling points were determined.

Low temperature mortality experiments

It was known from the host plant transfer experiments (Chapter Five) that the mean supercooling points of laboratory reared cultures

of A. fabae on bean and spindle differed by over 12°C (-23.8°C and -11.5°C respectively), and this provided an opportunity to study the relationship between supercooling ability and mortality at less severe temperatures (i.e. above the supercooling point).

The following experiments were carried out;

(i) In a preliminary experiment, samples of adult apterae of A. fabae were taken from the cultures on bean and spindle and 'acclimated' at 5°C for 1.5h, then at 0°C for a further 1.5h. The aphids were then placed in crystallising dishes on dry filter paper with no food, in a cold cabinet at -20°C for 15, 20, 40 and 60 minutes. At the end of these exposure times, the aphids were kept at room temperature for 24h, after which the percentage mortality was assessed. Aphids were recorded as either dead or alive, and the former category included those individuals which showed some movement but did not fully recover. In these experiments, only a single sample of 10 aphids from each culture was tested at the different exposure times.

(ii) Further experiments were carried out involving the same procedure as above but in which aphids were tested at -20°C and with longer exposures at -5°C. The same sample size (n=10) was used, but 5 replicates of each were tested (but see below).

RESULTS

Inherent cold hardiness

All stages of A. fabae maintained at 20°C possessed considerable supercooling potential (Table 7.1). There was a trend of decreased supercooling ability with increasing aphid age, with first and second instar nymphs the most cold hardy ($P<0.05$) although there was an increase in supercooling ability during the fourth instar which was lost after moulting to the adult.

Seasonal cold hardiness

The supercooling ability of the aphid samples studied during autumn 1982 to summer 1984 is shown in Table 7.2. Oviparae in 1982 and 1983 had similar supercooling abilities which were very poor compared to the subsequent egg stage. All egg samples except those collected in March had mean supercooling points below -30°C. There was an increase in egg cold hardiness in December and January ($P<0.05$) followed by a loss of about 6°C of supercooling potential by February ($P<0.05$) and a further decrease in March just prior to hatching. Fundatrices and fundatrigeniae in 1983 and 1984 were less cold hardy than the oviparae ($P<0.001$), with mean supercooling points of around -10°C or less. This level of cold hardiness was maintained through to the alate fundatrigeniae in late spring. Aphids samples from summer hosts in 1983 and 1984 all showed extensive supercooling, similar to laboratory maintained aphids.

A. fabae therefore shows a distinct seasonal pattern of cold hardiness. Aphids associated with spindle in autumn and spring have much reduced supercooling ability compared with populations feeding on summer hosts. Eggs are the most cold hardy stage in the life cycle.

Table 7.1 Inherent supercooling ability (°C) of A. fabae maintained at 20 ± 2°C on broad bean

	Mean ± S.E. (n=20)				
	Range				
First instar	Second instar	Third instar	Fourth instar	Adult apterae (newly moulted)	Adult alatae
-26.9 ± 0.22	-26.5 ± 0.24	-23.8 ± 0.33	-25.0 ± 0.19	-23.6 ± 0.37	-23.9 ± 0.33
-24.6/-28.4	-23.2/-27.9	-21.1/-26.2	-22.5/-26.1	-19.9/-25.5	-19.8/-25.4

Table 7.2 Supercooling ability (°C) of seasonally collected A. fabae (adults, unless stated otherwise)

Sampling date	Mean ± S.E. (n=20) Range				
	Oviparae	Eggs	Fundatrices	Fundatrigeniae	Virginoparae
22 October 1982	-14.6 ± 0.29 -12.2/-16.0				
	-14.5 ± 0.36 -10.5/-16/6 (wet with rainwater)				
18 November 1982		-34.1 ± 0.73 -26.0/-38.3			
20 December 1982		-37.5 ± 0.36 -33.2/-40.0			
17 January 1983		-36.6 ± 0.60 -31.8/-39.6			

Table 7.2 (Continued)

Sampling date	Mean ± S.E. (n=20) Range			
	Oviparae	Eggs	Fundatrices	Fundatrigeniae	Virginoparae
20 February 1983		-30.9 ± 0.53 -23.2/-34.5 -30.4 ± 0.34 -27.9/-32.7 (wet with rainwater)			
6 March 1983		-29.1 ± 0.40 -24.3/-32.3			
4 April 1983			-12.2 ± 0.44 -10.1/-16.0 (second/third instars)		
3 May 1983				-10.4 ± 0.11 -9.4/-11.0	

Table 7.2 (Continued)

Sampling date	Mean ± S.E. (n=20) Range				
	Oviparae	Eggs	Fundatrices	Fundatrigeniae	Virginoparae
7 June 1983				-9.9 ± 0.14 -8.9/-11.0	
6 July 1983					-23.4 ± 0.64 -14.9/-26.1 -25.8 ± 0.43 -20.4/-27.7 (first instars)
5 August 1983					-24.2 ± 0.28 -21.2/-26.1 -27.5 ± 0.17 (first instars)
13 November 1983	-15.7 ± 0.29 -11.2/-17.6				

Table 7.2 (Continued)

Sampling date	Mean ± S.E. (n=20) Range				
	Oviparae	Eggs	Fundatrices	Fundatrigeniae	Virginoparae
13 May 1984				-9.8 ± 0.08 -9.5/-10.4 (n=10)	
17 May 1984				-10.2 ± 0.14 -9.7/-10.9 (alate; n=10)	
3 July 1984					-23.8 ± 0.50 -17.1/-25.8

Contact with surface moisture

Neither oviparae nor eggs of A. fabae appear to be subject to instantaneous inoculative nucleation when wet (Table 7.2); the range of supercooling points was very similar to those of the corresponding dry (control) specimens.

Mortality at sub-zero temperatures

In the preliminary experiments, A. fabae from spindle showed 100% mortality after 40 minutes at -20°C, while bean-reared samples did not show this mortality level until exposed at this temperature for 60 minutes (Table 7.3). Mortality levels of over 70% were observed in the spindle aphids after 20 minutes, but 40 minutes exposure was required to achieve a similar level of mortality in the bean aphids.

In subsequent experiments mortalities of 62% after 40 minutes and 100% after 60 minutes at -20°C were recorded for bean aphids, being comparable to the preliminary experiment results (Table 7.4). However, spindle-reared aphids showed much lower mortalities with only 4% killed after 40 minutes, although 100% mortality was still observed after an exposure of 60 minutes (Table 7.5); these results were therefore very different to those of the preliminary experiment.

In the experiments conducted at -5°C, 5% mortality was observed in the spindle aphids after exposure for 240 minutes, with no mortality in the bean aphids by this time. After 720 minutes, there was 30% mortality in the spindle-derived aphids, with 50% mortality in the bean aphids. Two samples of bean-derived aphids experienced 100% mortality after only 300 minutes at the same temperature.

Table 7.3 Mortality (%) of A. fabae exposed to -20°C for varying periods of time

Host source of aphids	Period of exposure (minutes)			
	15	20	40	60
Bean	0	0	70	100
Spindle	0	71.4	100	100

Table 7.4 Mortality (% per sample and mean (underlined)) of A. fabae originating from bean plants and exposed to various time-temperature treatments

	Period of exposure (minutes)						
Temperature	15	20	40	60	240	300	720
-5°C					0;0; <u>0</u>	100;100; <u>100</u>	50;
-20°C	0;0;20; 40;50 <u>24</u>	20;20;20; 30;50; <u>28</u>	30;60;70; 70;80; <u>62</u>	100;100;100; 100;100; <u>100</u>			

Table 7.5 Mortality (% per sample and mean (underlined)) of A. fabae originating from spindle and exposed to various time-temperature treatments

	Period of exposure (minutes)						
Temperature	15	20	40	60	240	300	720
-5°C					0;10; <u>5</u>		30;
-20°C	0;0;10; 10;60 <u>16</u>	0;10;10; 40;50; <u>22</u>	0;0;0; 0;20; <u>4</u>	100;100;100; 100;100; <u>100</u>			

These latter results were very variable and while based on very few samples, it appeared that aphids were dying at the higher sub-zero temperatures after shorter exposures than might be expected if mortality was caused by freezing death alone. Based on these preliminary results it was concluded that other factors were influencing mortality levels and these experiments were therefore terminated because insufficient time was available for the development of the work. Hence these experiments did not progress beyond the preliminary stage.

It was noted that in the second set of experiments during exposure to -20°C for 15 minutes, A. fabae derived from bean produced nymphs which survived upon removal; spindle aphids did not produce any nymphs under the same conditions. So few samples were studied at the higher sub-zero temperatures that it was not possible to comment on nymphal production under these conditions.

DISCUSSION

The levels of inherent supercooling ability in all stages of Aphis fabae maintained under laboratory conditions were extensive and very similar to that observed for Myzus persicae when under the same conditions. First instar nymphs were also the most cold hardy stage and the same trend of decreasing supercooling potential with increasing age existed in this aphid species. In addition, A. fabae collected in summer from different herbaceous hosts exhibited comparable levels of supercooling potential to laboratory maintained individuals. However when associated with the primary host, spindle, all stages of the aphid except the eggs showed poor supercooling ability, with levels approximately half of that achieved by laboratory specimens or summer collected individuals. In view of the fact that overwintering occurs as the egg stage which was capable of supercooling to below -30°C, acclimatising and becoming most cold hardy in midwinter, the poor supercooling ability of the oviparae and fundatrices/fundatrigeniae is of little consequence ecologically since these individuals are unlikely to experience frosts of -10°C in autumn or spring (see Appendix 4) and are therefore adequately protected.

The poor levels of supercooling under these conditions might appear to detract from the 'efficient vessel' theory of inherent aphid cold hardiness; however the host plant transfer experiments with A. fabae make it clear that it is feeding on spindle which markedly reduces their inherent supercooling ability (Chapter Five). Various reasons for this loss of supercooling potential were discussed and it was suggested that a nucleating agent in spindle sap might account for the dramatic reduction in supercooling potential when aphids are

feeding on this woody host. This in turn suggests that if fed on herbaceous hosts, oviparae and fundatrices/fundatrigeniae should exhibit considerable supercooling potential and indeed, M. persicae oviparae and males when produced on Chinese cabbage in the laboratory, demonstrated extensive supercooling ability similar to that of non-sexual morphs. Unfortunately during this study neither A. fabae holocyclic morphs were induced on secondary hosts nor comparable M. persicae morphs collected naturally from the primary woody host, peach. In addition there are apparently no published accounts of the supercooling ability of aphid oviparae.

The supercooling potential of A. fabae eggs was comparable to those found for other aphid species (Sømme, 1964; 1969; Parry, 1979b; James and Luff, 1982; Parry, 1985). Acclimatisation of aphid eggs overwinter has been observed by other workers (Sømme, 1969; Parry, 1979b, 1985; James and Luff, 1982), with maximum cold hardiness achieved in December or January; both this increase in supercooling potential in winter and the subsequent loss prior to hatch has been correlated with cryoprotectant and particularly glycerol levels (Sømme, 1964; 1969; Parry, 1985). It has already been reported in Chapter Six that both mannitol and glycerol were found in A. fabae eggs but the concentrations in the various monthly samples were not quantified. However the supercooling trends observed in this study in A. fabae eggs may also be accounted for by changes in the levels of these cryoprotectants. The eggs were also resistant to instantaneous inoculative nucleation when in contact with surface moisture. Parry (1985) suggested that ice around aphid eggs would act as an insulating layer and that because of the size of the cuticular pores they would

be unlikely to experience inoculative nucleation; eggs of A. fabae are apparently well adapted for overwinter survival although factors other than cold can induce natural mortality during winter (Way and Banks, 1964). The supercooling point temperatures of M. persicae eggs (Chapter Four) were similar to those of A. fabae and this might suggest that even though the former were produced under laboratory conditions, they represent at least the true extent of supercooling of non-acclimatised eggs. The loss in supercooling potential when the eggs of A. fabae hatch to produce the fundatrices is apparently related to the intake of spindle sap.

Oviparae of A. fabae were also not susceptible to inoculative nucleation when wet, although the inherent supercooling potential of these aphids was possibly above the temperature at which the water might induce nucleation; when M. persicae individuals were wet, the first rebound which corresponded to the water freezing took place at around -15°C (Chapter Four). Under field conditions however the water might freeze at higher sub-zero temperatures but A. fabae individuals are like M. persicae, apparently resistant to nucleation in this way (E.J. Major, personal communication) and despite their waxy bloom, do not seem to obtain any additional protection against surface moisture effects.

During the low temperature mortality experiments there was a lag between placing specimens in the cold cabinets and the test temperature being re-established. In the preliminary experiments mortality of the spindle-reared A. fabae reached 100% after 40 minutes exposure, but an exposure of 60 minutes was required to induce 100% mortality of bean-reared aphids. This might suggest that it took

these periods of time respectively to achieve the sub-zero temperatures equivalent to the mean supercooling points of the two groups. This lag was not measured in these experiments since one of the aims of the study was to compare the two groups, and therefore if the lag period remained fairly constant it should not influence this comparison. There was a differential mortality in the two groups with the good supercoolers (bean) surviving longer than the poor supercoolers (spindle) which initially suggested that supercooling ability was related to survival at low temperature.

However while similar results were obtained for the bean aphids when more samples were tested at -20°C, the results for the spindle aphids were quite different. The substantial increase in mortality between the 40 and 60 minute exposure periods of this group (spindle) might suggest an increase in the lag period during the 40 minute experiment, so that it took longer to achieve a temperature of -20°C. This suggests that in future experiments the lag period would have to be closely monitored.

As the test temperature became less severe (-5°C) not only was percentage mortality within the same aphid group variable, but aphids were dying after comparatively shorter exposures, which implied that other factors were confounding the results. M. persicae for example has been reported to survive for between 10-14 days at -5°C (Pozarowska, 1983; S.D.J. Smith, personal communication) and in this study, M. persicae starved at 0°C survived for 24 days. A. fabae individuals might be expected to show similar survival times.

Two reasons which may have accounted for this apparent premature mortality are temperature shock or desiccation. Pozarowska (1983)

reported that the only aphids (M. persicae) which survived the exposure to -5°C were individuals which had been 'acclimated' at 10°C for a month prior to exposure. The short 'acclimation' allowed in this study, followed by rapid exposure to sub-zero temperatures may have induced a 'temperature shock'. No free moisture was included in the test dishes, to remove any possibility of inoculative nucleation. Aphids are known to experience mortality more rapidly when starved without a water source (Maltais, 1952). This has also been a contributing factor in the low temperature survival of other phloem feeders (Decker and Cunningham, 1967). These facts suggest that the unavailability of moisture during the experiments described here might also have contributed to the observed mortality.

Whatever the cause, the results of the sub-zero mortality experiments as presented in this chapter are not conclusive. A decision was made to terminate the experiments after collecting these results, which were based on a minimum of replicates; time was not available to continue this study. It was however interesting that the nymphs produced during exposure to -20°C by bean-reared aphids survived upon removal, while there was no nymphal production in the spindle-reared aphids. While this may be a cold hardiness advantage to anholocyclically overwintering aphids, it is more likely that the bean A. fabae were about to give birth when the experiments commenced.

A. fabae thus possesses some supercooling characteristics which are comparable with M. persicae, such as extensive inherent cold hardiness and resistance to instantaneous inoculative nucleation. Of

greater interest however is the dramatic influence which the host plant exerts upon the supercooling potential of this species. The overall influence of this and other factors on aphid cold hardiness and overwintering survival is discussed in the concluding chapter.

CHAPTER EIGHT

GENERAL DISCUSSION

GENERAL DISCUSSION

Supercooling point experiments provide a useful method by which to assess the cold hardiness of freezing-susceptible arthropods and allow comparisons to be made between different species, experimental groups, or strains and populations within the same species, provided a standard technique is used (Ring, 1980). Prior to supercooling point determination the culture conditions and experimental history of samples should be controlled, so that as far as possible the only variable between experimental groups is the factor being studied (Salt, 1961). With field specimens however, there is inevitably a level of uncontrollable variation.

Salt (1966b) recommended a cooling rate of 1°C/min as a biological standard for supercooling point measurements, since from a practical viewpoint this rate would also provide convenient experiment times. This rate is however far in excess of that which would be experienced by organisms in the field and thus the observed supercooling potential will be overestimated under such experimental conditions; this is because spontaneous nucleation which terminates supercooling, is largely a function of the time-temperature relationship of cooling. During supercooling point experiments the time spent at any sub-zero temperature is minimal and supercooling therefore continues until the temperature at which spontaneous nucleation occurs (Salt, 1966b). Consequently, under field conditions, at sub-zero temperatures above the supercooling point the probability of freezing increases with longer exposure; thus supercooling point temperatures are regarded primarily as an index of cold hardiness and in this thesis therefore the terms 'supercooling ability' and 'cold hardiness' have been used synonymously.

The automatic cooling method and computer-based recording system developed during the research for supercooling point determination, provided the facility to process many aphid samples from a range of experimental conditions and seasonal studies. Standard culturing conditions were used for all the aphid clones studied and a cooling rate of 1°C/min was used in all supercooling point experiments. On this basis the results described in this thesis can be compared directly, and comparisons made with studies on other freezing-susceptible species.

Sømme (1982) reviewed the main features of freezing-susceptible arthropods which include, (a) a seasonal pattern of cold hardiness which is at a maximum in winter, (b) an acclimation response induced in the laboratory during exposure to low temperature, (c) inoculative freezing above the inherent supercooling point when in contact with external surface moisture and (d) reduced cold hardiness when feeding due to nucleators in the gut. The main finding of this thesis is that Myzus persicae is atypical with respect to all these features.

When feeding under favourable conditions the inherent supercooling levels of M. persicae were as extensive as that of many polar species (Sømme, 1981, 1982). This feature was unexpected in a temperate species which overwinters successfully as active individuals only in mild winters (van Emden, Eastop, Hughes and Way, 1969). However as the study continued the pattern which emerged suggested that aphid cold hardiness was related to a number of biological features, and particularly that of 'feeding'. The inability of individuals other than the younger instars to acclimate in laboratory experiments was also reflected in the seasonal patterns of

supercooling in which younger instars underwent natural acclimatisation, thus possessing considerable levels of cold hardiness throughout the year, while adults lost cold hardiness overwinter. Of the factors which might account for these seasonal variations, the feeding status of adult aphids in winter appears the most likely to account for the observed losses of supercooling potential; clonal differences, adult age, reproductive status, the likelihood of instantaneous inoculative nucleation when in contact with surface moisture and insecticide resistant-type appear less influential.

Previous studies on M. persicae had suggested a relationship between insecticide resistance and cold hardiness on the basis of the geographical distribution within Britain of insecticide resistant-types, or their ability to overwinter in certain years (Devonshire and Needham, 1975; Baker, 1977a, 1977b, 1978). Biological performance of susceptible and resistant aphids had implied lowered 'fitness' in the latter individuals (Banks and Needham, 1970; Rose O'Doherty, 1981, unpublished B.Sc. Honours thesis, University of Glasgow; A Comparative Study of Two Clones of Myzus persicae (Sulz.) (Homoptera: Aphididae)). However, Pozarowska (1983) concluded from a study of several clones of M. persicae that there was no relationship between insecticide resistance and low temperature performance including survival, and the direct assessment of cold hardiness levels in this work, further suggests that there is little difference between insecticide resistant and susceptible M. persicae. While R_2 adults, unlike S strain individuals, lost supercooling potential when acclimated, the loss was small. Moreover, S strain adults when starved lost levels of supercooling capacity comparable with that observed in natural

populations overwinter, and it would appear that the influence of feeding on cold hardiness suggested from the present work would affect all aphids equally, irrespective of resistant-type.

Unlike the majority of freezing-susceptible arthropods, aphids overwintering anholocyclically continue to feed. That feeding during winter is actually a requirement is suggested by the association of overwintering aphids with suitable host plants and the mortality observed when aphids were experimentally starved; there appears to be very little published information on the feeding behaviour of overwintering aphids. However, from the results described in this thesis it is proposed that the inability to obtain food, or food of adequate 'quality', affects the cold hardiness of adult M. persicae by reducing supercooling potential. In natural overwintering populations while a proportion of a sample showed substantial losses of supercooling potential (greater than 10°C) the remainder showed only a slight reduction in cold hardiness. Similar distributions were obtained when adult M. persicae were starved experimentally at 5°C. These results and the inability to reproduce this LG/HG distribution in subsequent starvation experiments indicates that a complex of factors may act to modify the magnitude of the feeding influence on cold hardiness when aphids are starved at low temperatures. The starvation influence seems more important than a low temperature stress since adults maintained at 5°C with food, showed supercooling potential consistently in excess of -20°C. In addition Parry (1978) observed a loss of supercooling potential in adult M. persicae when starved at 20°C, and similar losses of supercooling potential to those recorded in this study have been reported following experimental

starvation of this species at low temperatures (S.D.J. Smith, personal communication).

The Aphis fabae studies have provided convincing evidence that food source can dramatically influence the supercooling ability of aphids. When transferred from bean plants to spindle, adult A. fabae lost over 10°C supercooling potential after feeding for a few days, while natural populations of A. fabae feeding on the woody host always showed poor levels of supercooling ability. The ability to shift experimentally the supercooling potential of whole groups of aphids by feeding them on spindle implied the presence of a nucleating agent in the sap. It is interesting to note that in insects (freezing-tolerant) which synthesise nucleating agents for winter survival, supercooling potential is subsequently reduced to around -10°C (Zachariassen, 1982). Powell (1976) suggested that changes in the nucleator concentration of the phloem sap ingested by the aphids Elatobium abietinum, Eucallipterus tiliae and Drepanosiphum platanoides could influence the supercooling potential of these aphids; thus when nucleator concentrations were greatest, adult supercooling points were approximately -15°C, -17°C and -15°C respectively. These species are all tree feeders and since supercooling levels are very similar to that of A. fabae when feeding on spindle, might suggest a more general association of sap nucleators with woody hosts.

A. fabae, E. tiliae and D. platanoides all overwinter as eggs while E. abietinum, unlike the majority of tree aphids, survives in Britain as active aphids (Carter, 1972; Powell, 1973) and therefore continues to feed during this period. Powell (1974) and Parry and

Powell (1977) proposed that mortality during winter in E. abietinum could occur as a result of inoculative nucleation via the mouthparts when ice formed in spruce needles. Since plants are freezing-tolerant, they supercool only a few degrees below their true freezing point before freezing extracellularly and so avoid lethal intracellular ice formation (Levitt, 1980; Li and Sakai, 1982). Ice nucleation in the sap would therefore be more likely to occur in plant species in which there is a nucleator and aphids feeding on such plants may then experience inoculative nucleation. Overwintering M. persicae are associated with cold hardy herbaceous plant species, many of which also acclimatise (Burke et al, 1976). These plants may not possess the sap nucleators thought to be present in the woody hosts of other aphid species, which implies that there is a reduced likelihood of inoculative nucleation occurring in this aphid species during winter.

Powell (1976) stated that the selection of different feeding sites by adults and first instar nymphs of E. tiliae and D. platanoides resulted in adults obtaining poorer quality sap from the larger veins on which they fed, and might account for their poorer supercooling potential compared to first instars which preferred to feed on the minor veins in which the sap is more concentrated. Parry (1978) proposed that this differential selection of feeding sites contributed to the greater supercooling potential of first instars, compared with adults, of M. persicae. In the present study while it was observed that this variation in feeding site did occur, and first instars possessed greater supercooling potential, the phloem sap obtained from leaf discs would be more uniform in quality (Adams and

van Emden, 1972) suggesting that nymphs are inherently capable of supercooling more extensively. The preference shown by younger instars for the minor veins is thought to be related to the smaller stylet size of these individuals (Wearing, 1967; Powell, 1976), and this may account for the similarity of the supercooling potential of first instars allowed access only to main veins with that of unfed individuals, as described earlier.

The relationship between aphids and their host plants and particularly the inability to separate the two experimentally has led to the suggestion that any demonstrated aphid acclimation/ acclimatisation may in fact be a reflection of plant cold hardening which is 'transferred' to feeding aphids (Powell, 1974; Parry, 1978, 1979a). Leaf discs would cease to be capable of an acclimation response soon after excision, although there would be physiological changes associated with premature senescence (Adams and van Emden, 1972). The acclimation ability observed in the younger instars of M. persicae when maintained on leaf discs in this study suggests that it is a direct aphid response to low temperature exposure. In addition, seasonal acclimatisation of younger instars on field hosts was of the same magnitude (2-3°C) again suggesting little plant influence.

As laboratory acclimation or natural acclimatisation increased supercooling potential to such a small extent, it would appear that M. persicae does not accumulate cryoprotective substances in winter as do many other freezing-susceptible arthropods (Sømme, 1982), although the levels of the carbohydrate compounds identified in acclimated individuals was not quantified. The use of gas-liquid chromatography to investigate the carbohydrate content of aphid samples has enabled

the entire spectrum of separated carbohydrates to be analysed simultaneously, rather than a few selected components, which is a limitation imposed by the use of more specific techniques such as thin-layer or paper chromatography, (Danks, 1978; Duman, Horwarth, Tomchaney and Patterson, 1982; Storey, 1984). It has been suggested that the range of carbohydrates detected in M. persicae and particularly the high total percentages of the body content which they composed, may contribute to the extensive supercooling potential of laboratory maintained and summer individuals, and thus unacclimated aphids. Low molecular weight antifreezes such as those found in freezing-susceptible arthropods act to increase supercooling potential by depressing the true freezing point in a colligative manner, that is, by an increase in solute concentration (Ring, 1980; Duman, 1982). The combined concentrations of the various carbohydrates in M. persicae may act to produce a type of multifactorial cryoprotective system (Ring, 1977, 1980; Sømme, 1982) and confer the ability to supercool extensively by their additive solute effect. Certainly in first instars of M. persicae the glycerol concentration, composing almost 7% of the total body weight would increase the viscosity of these individuals and reduce the probability of freezing (Lee and Lewis, 1985). However other instars with comparable supercooling potential did not possess such high carbohydrate levels and might suggest that an additional influencing factor in first instars is their small size which reduces the likelihood of ice nucleation; inherent supercooling ability in M. persicae was observed to decrease with increasing size. The total carbohydrate body content decreased with aphid development but in adults was observed to rise to almost 7%

and was thus the second highest concentration among the developmental stages. It is possible that continued feeding is required to maintain such high carbohydrate levels. First instar nymphs are unlikely to have acquired such carbohydrate levels by feeding alone and following the moult to second instars, levels were comparatively low; however the high initial concentration within first instars might decrease more slowly if feeding ceased, particularly under low temperatures, and indeed following experimental starvation at 5°C, first instar supercooling was unaffected.

Parry (1978) found that by feeding adult M. persicae on a 25% sucrose solution, supercooling potential increased and suggested that host plants may therefore exert a similar influence via the phloem sap. The results of the present study, in which starved adult M. persicae lost supercooling potential further implies that in aphids, 'feeding status' has the opposite effect to that observed in other freezing-susceptible species (Salt, 1953, 1961; Sømme, 1976, 1982; Block, 1982a). Further and more detailed analysis of the concentrations of the carbohydrate spectrum in poor (LG) supercoolers of M. persicae would provide more information to clarify the relationship between feeding activity, carbohydrate concentration and supercooling potential in aphids.

In A. fabae the presence of a sap nucleator is thought to account for the poor supercooling potential of aphids when feeding on the woody host, spindle. Nucleators within phloem sap have been shown to influence the supercooling ability of other aphid species (Powell, 1976). The identification of such substances is unknown; most biological nucleating agents have been identified as proteinaceous

substances (Duman, 1982; Zachariassen, 1982) or high molecular weight polysaccharides (Krog, Zachariassen, Larsen and Smidrød, 1979) which might imply a relationship between complex molecular structure and nucleator activity. The presence of dulcitol in A. fabae which had only fed on spindle indicates that it is likely to be of plant origin, but further work is required to assess whether it posseses nucleator activity within these aphids.

The A. fabae studies demonstrated that this aphid species has very similar inherent supercooling capacities to M. persicae and provides further data to support the theory that aphids maintained under favourable feeding conditions, provide 'vessels' in which supercooling is enhanced. For this reason their ability to supercool extensively can, in cold adaptive terms, be considered to represent an 'artifact' since it is a consequence of obtaining phloem sap and unrelated to any specific adaptation to tolerate low temperatures. That they are extremely cold hardy at the same time can be regarded as superfluous since they do not encounter such 'freezing' temperatures either in summer or under laboratory conditions. The supercooling potential of A. fabae was dramatically influenced by the host plant on which they were feeding and while the mechanism acting in this species, involving a sap nucleator, may be different from that in M. persicae, the A. fabae results support the overall hypothesis proposed following the M. persicae studies, that the inability of aphids to obtain sufficient food and/or of a certain quality during winter may markedly reduce their inherent supercooling potential. This being so, it can be further proposed that aphids in general may conform to a similar pattern of cold hardiness, and may as a group be

atypical to other freezing-susceptible arthropods. Cold hardiness studies of all species of aphids have shown them to be freezing-susceptible, with many possessing supercooling abilities very similar to that of the good supercoolers described in this thesis (MacPhee, 1964; Sømme and Zachariassen, 1981; Williams, 1984). Others however, even though actively feeding, possess poorer supercooling potential such as the aphid species studied by Powell (1973, 1974, 1976) which is probably attributable to the presence of nucleators in the sap of the host trees and their uptake by feeding aphids (Powell, 1976). Thus first instar E. abietinum had mean supercooling points below -20°C until they imbibed plant sap, following which supercooling potential was reduced by up to 10°C (Powell, 1974). In the present study, unfed and fed first instar M. persicae showed only a slight difference in supercooling ability (2-4°C) suggesting an absence of such nucleating agents in their food. Parry (1978) has noted a number of similar trends of supercooling potential in M. persicae to those described in the present study, but the extent of supercooling was less extensive; it seems likely that this variation may be attributable to the use of different supercooling point determination methods, aphid material or host plants.

Extensive supercooling ability in phloem feeders other than aphids has also been reported and attributed to their specialised feeding habit (MacPhee, 1964; Cannon, 1983). Eguagie (1969, 1972, 1974) recorded extensive levels of supercooling potential in overwintering, non-feeding adults of the lacebug, Tingis ampliata; there was a loss of supercooling potential when these individuals fed, although the majority still supercooled to below -20°C. Decker and

Maddox (1967) found the mean supercooling points of field collected specimens of four species of the leafhopper Empoasca to be between -11.9°C and -19.4°C. It was also noted that the most cold hardy species (E. recurvata) exhibited a bimodal distribution of supercooling points, with peaks at approximately -15°C and -20°C and this was attributed to a heterozygous conditions for cold hardiness in this leafhopper. Thus the suggestion of a general pattern of supercooling ability and potential influences in aphids may extend to other phloem feeders.

These studies have concentrated primarily on the ability of aphids to supercool in order to avoid death overwinter from freezing. Low temperatures also impose another major constraint on freezing-susceptible organisms by a general lowering of metabolism which in turn influences a number of processes (Block, 1980) requiring regulation or acclimatisation at the metabolic level (Bullock, 1955; Prosser, 1975; Block and Young, 1978). This reduced metabolism at low temperatures influences body functions such as activity, growth and performance in general. Studies on the overwintering survival of aphids in terms of their biological performance at low temperature, is an approach that has been adopted by a number of investigators. Differences were observed in the rate of nymphal development at low temperature of the different M. persicae strains studied in this work. Pozarowska (1983) studied the developmental and reproductive characteristics of different clones of M. persicae and Aulacorthum solani in detail and suggested that they could influence the overwintering survival of these aphid species. The higher reproductive rate of an anholocyclic strain of M. persicae at low

temperature compared with that of a holocyclic strain, was suggested to indicate a cold tolerance adaptation in the former (Tamaki et al, 1982).

The above studies illustrate that low temperatures during winter can affect aphids other than by the induction of a freezing death, and suggests that an important influence in aphids, which continue to give birth to nymphs at low temperatures and thus throughout winter (Barlow, 1962; Whalon and Smilowitz, 1977; Pozarowska, 1983), is the effect on reproduction rates and therefore recruitment to the population as observed for example in cereal aphid populations by Smith (1981).

There was no evidence to suggest that anholocyclic M. persicae or parthenogenetically reproducing A. fabae were more cold adapted than the holocyclic morphs of these species; levels of supercooling in androcyclic (R_1, R_2) and holocyclic (H, S) clones of M. persicae were comparable, either as holocyclic morphs or parthenogenetically reproducing individuals from holocyclic clones, as was the supercooling potential of A. fabae maintained as parthenogenetic individuals within the laboratory. In addition, when maintained as parthenogenetic cultures within the laboratory, the field collected clones, which would all have been anholocyclic/androcyclic, possessed comparable and extensive levels of supercooling ability to each other and to the above clones. The poor supercooling ability observed in holocyclic morphs of A. fabae under field conditions has been attributed to a nucleator in the sap of the primary host, spindle. Even so this level of supercooling ability would appear to be adequate for this species since overwintering occurs as cold hardy eggs.

Pozarowska (1983) found no relationship between life cycle type and adaptation to low temperatures in clones of M. persicae and A. solani. Powell (1973) suggested that overwintering virginoparae of E. abietinum in Britain were unlikely to be cold adapted since the ability to do so as active individuals represented an opportunistic strategy rather than any pre-adaptation for winter survival (Dixon and Wellings, 1982). All aphid eggs including those examined in this research, in common with the eggs of all freezing-susceptible arthropods, are capable of extensive supercooling (Sømme, 1982); holocylic aphid species or populations within a species which overwinter as eggs can therefore be considered to be cold hardy and well adapted for winter survival (Dixon, 1985).

For species which overwinter as active individuals, the same cold hardiness requirement is necessary to ensure survival. In Britain, the majority of populations of M. persicae overwinter anholocyclically since the sexually produced egg stage of holocyclic populations is much less common in this country (Blackman, 1971). It is widely recognised that successful overwintering of this aphid species is associated with mild winters (van Emden, Eastop, Hughes and Way, 1969; Turl, 1978; Eastop, 1983; Dixon, 1985). However, despite much interest in the ecology and overwintering biology of M. persicae, and the relationships established between meteorological data and predictions of virus epidemics in crop plants in those years when anholocycly has been favoured, no theories have been proposed to suggest how low temperatures overwinter act on aphid populations (Taylor, 1977, 1983; Watson, Heathcote, Lauckner and Sowray, 1975; Turl, 1977, 1978, 1980; Harrington, 1984; Harrington and Xia-Nian,

1984; Dixon, 1985). It is proposed that the results described in this thesis may go some way to providing a possible explanation for the association of successful anholocyclic overwintering with mild winters.

Mild winters would permit continued aphid feeding, maintaining adult cold hardiness, and therefore survival in turn would result in increased recruitment of younger instars to the overwintering population. In severe winters, a number of factors (see Chapter Five) may act to disturb aphid feeding and so induce the losses of adult cold hardiness observed in natural populations in this work. The loss of adults from the population in such winters would also mean an associated loss of reproduction and thus an overall reduction in the number of winter survivors. However it is likely that these 'fated' adults would have already commenced reproduction and there would be at least a residual population of younger instars. This research has shown first instar nymphs to possess extensive levels of cold hardiness throughout the year and to be less susceptible to those factors including starvation, which influence adult cold hardiness; first instars will also have a lower requirement for food to maintain basal metabolism. In addition, first instars acclimatise in winter and consequently extensive cold hardiness is maintained until at least the third instar stage; low temperatures overwinter would reduce nymphal development, maintaining the nymphal population in the more cold hardy early stages, further enhancing their chance of winter survival. Thus it is possible by the above proposed mechanism, that even in the spring following a severe winter, anholocyclic populations may still retain the capacity to contribute a reservoir of survivors,

albeit much reduced, to the subsequent production of spring migrants. Prosser (1975) stated that "at the level of populations, survival fails when reproduction is no longer effective"; in cold hardiness terms, it would appear that it may be the potential of M. persicae adults to produce cold hardy progeny which may ensure the survival of anholocyclically overwintering populations of this aphid species. In very severe winters when even the survival of younger instars is threatened, the anholocyclic nature of this aphid species would still be maintained by the ability of most anholocyclic clones to produce males which may contribute to the sexual reproduction of a holocyclic clone (Blackman, 1972, 1974b). Additionally, sheltered sites such as glasshouses and potato chitting stores provide for continued parthenogenesis in most years (van Emden, Eastop, Hughes and Way, 1969; Anon, 1980).

The use of supercooling point measurements as an indicator of the cold tolerance of freezing-susceptible species is a widely used technique and significant differences in supercooling potential are considered to represent real differences in cold hardiness (Sømme, 1982). Thus, the substantial losses of cold hardiness observed in naturally overwintering populations of M. persicae would suggest that the exposure of these LG/poor supercoolers to frosts comparable with their supercooling point temperatures would result in an instantaneous freezing death (Salt, 1961). In Britain frosts of -5°C to -10°C would be encountered in most winters by overwintering aphids. In the seasonal studies the proportion of LG individuals was never greater than 25% of the sample implying that in such samples the majority of overwintering adults would not be susceptible to a freezing death.

However, the winters of 1982-83 and 1983-84 were comparatively mild and in some of the field studies, the full extent of winter temperature was not experienced by aphids (see Figs. 4.1 and 4.2). Therefore winters in which aphids were to encounter more severe temperatures may show an increase in the proportion of LG individuals within samples; this is suggested by the population of M. persicae on Cineraria in 1982-83 which appeared to lose cold hardiness in two stages, with firstly an overall loss in all adults as winter progressed, followed by the appearance of individuals with much poorer supercooling potential. Attempts in this work to establish the relationship between supercooling potential and mortality at sub-zero temperatures in A. fabae did not prove conclusive and further work is necessary to ascertain that reduced supercooling ability overwinter does represent real losses of cold tolerance in aphids. Such relationships may subsequently provide the basis for the incorporation of supercooling point data in, for example, aphid forecasting systems.

Williams (1984) stated that cereal aphids could not be described as entirely freezing-susceptible since he observed that mortality above the supercooling point was attributable to a chilling rather than a freezing factor and suggested that 'chill-susceptible' may be a more appropriate description. There can be little doubt that natural mortality does occur above the supercooling point due to the effects of chilling temperatures, and this topic is little researched. Aphids experience chill-coma at fairly high sub-zero temperatures (Powell, 1973; Smith, 1981). In winter active freezing-susceptible insects, Sømme and Østbye (1969) and Sømme (1976) proposed that

continued activity was important in maintaining the ability of such species to escape a freezing death by movement to a more favourable habitat. In overwintering aphids, the ability to feed may cease when chill-coma temperatures are experienced. In addition, when dislodged from host plants overwinter inhibited movement at the chill-coma temperature may prevent M. persicae from being able to colonise new feeding positions (Harrington, 1984). These influences may not only induce starvation but considering the proposal of this thesis, may ultimately reduce adult cold hardiness, increase the likelihood of a freezing death and ultimately reduce overwintering survival.

The ability to propose the above theory which suggests a mechanism to account for the observed overwintering epidemiology of M. persicae is intimately linked with the requirement of aphids to feed during winter; this is not only a feature in contrast to many other overwintering arthropods which may cease feeding overwinter, but is also one for which there appears to be little experimental data. Little is known of the nutritional requirements of aphids at low temperature (T.E. Mittler, personal communication). In addition, anholocyclically overwintering aphids are often stated to do so as older nymphs or apterous adults (Jones and Jones, 1974). There appears to be little field confirmation of this; Pozarowska (1983) found all stages of M. persicae to overwinter with no one stage apparently dominating the population structure. Such gaps in our knowledge of the basic overwintering biology and ecology of M. persicae and other aphids have to some extent facilitated the above theory; future research must tackle such questions.

Recent studies in this laboratory however have shown similar cold hardiness characteristics as those described for M. persicae to exist in cereal aphids. In particular the presence of high numbers of LG individuals in winter samples under 'true' field conditions suggests that the pattern of aphid cold hardiness described in this thesis may be a general one.

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APPENDICES

APPENDIX 1

Abbreviations

ADAS	Agricultural Development and Advisory Service
BYDV	Barley yellow dwarf virus
°C	degrees Centigrade
CJC	cold junction compensation
cm	centimetre
emf	electromotive force
EHS	Experimental Horticulture Station
FID	flame ionisation detection
Fig.	Figure
g	gramme
GLC	gas-liquid chromatography
h	hour
HG	high group (in supercooling point distribution)
IS	internal standard
l	litre
LG	low group (in supercooling point distribution)
m	metre
mA	milliampere
mg	milligramme
min	minute
ml	millilitre
mm	millimetre
msec	millisecond
P	probability
PAGE	polyacrylamide gel electrophoresis
PEG	polyethylene glycol
PLRV	Potato leaf roll virus

Appendix 1

Abbreviations (continued)

PROM	programmable read-only memory
RES	Rothamsted Experimental Station
R.H.	relative humidity
RRF	relative response factor
RRT	relative retention time
RT	retention time
s	second
SCP	supercooling point
S.E.	standard error (of the mean)
THP	thermal hysteresis protein
TMS	trimethylsilyl
$\bar{u}$	average linear gas velocity
V	volt
µg	microgramme
µg	microlitre
%	percent

APPENDIX 2

Origin and previous cultural history of *Myzus persicae* clones

Clone	Origin	Previous culture
S	sugar beet, Bottisham, Cambs., 31.5.74 clone USIL from RES	day:16h light at 25°C night 8h dark at 18°C on *Brassica pekinesis*
R_1	potato, ADAS field trial, Cambs., 12.7.77 clone 405D from RES	"
R_2	sugar beet, Eaton Socon, Beds., 10.6.75 clone TIV from RES	"
OH(4)	*Cineraria*, University Gardens, Oxley Hall, Leeds, 3.11.82	-
Savoy	savoy cabbage, ADAS field trial, High Mowthorpe EHS, Yorkshire, December 1982	-
OSR	oil seed rape, Swillington, near Leeds, December 1982	-
Kale	kale, Ridgeway experimental gardens, Leeds, July 1983	-
H	original host plant unknown, Gosfield, Essex, collection date unknown	potato, laboratory culture conditions

APPENDIX 3

A. Nymphal development data at 20°C

S strain — Instar developmental periods (h)

NUMBER	FIRST	SECOND	THIRD	FOURTH	TOTAL DEVELOPMENTAL PERIOD (h)
1	44	28.5	31	42	145.5
2	44	28.5	31	42	145.5
3	44	28.5	31	39	142.5
4	44	28.5	31	39	142.5
5	44	28.5	31	39	142.5
6	33.5	39.0	31	39	142.5
7	31.5	41	31	39	142.5
8	44.5	24.5	31	39	139.0
9	44.5	24.5	31	39	139.0
10	44.5	24.5	42.5	30	141.5
11	44.5	24.5	31	39	139.0
12	44.5	24.5	31	39	139.0
13	44.5	24.5	51	30	150.0
14	44.5	24.5	42.5	30	141.5
15	44.5	24.5	31	39	139.0
16	44.5	24.5	31	39	139.0
17	44.5	24.5	31	39	139.0
18	41.5	24.5	31	39	136.0
19	41.5	24.5	31	39	136.0
20	41.5	24.5	31	39	136.0

Appendix 3

R_1 strain	Instar developmental periods (h)				
NUMBER	FIRST	SECOND	THIRD	FOURTH	TOTAL DEVELOPMENTAL PERIOD (h)
1	31.5	43.5	42.7	33.2	150.9
2	38.5	45.5	32.5	46	162.5
3	26	43.5	34.5	36	140.0
4	33	36.5	34.7	50.5	154.7
5	33	36.5	34.6	40.5	144.7
6	33	45.2	47	48.2	173.4
7	33	36.5	34.5	36	140.0
8	38.7	18.2	34.5	45.7	137.1
9	38.9	27	34	51.2	150.9
10	38.7	27	34	51.2	150.9
11	38.7	48.2	50.5	26	163.4
12	38.7	27	47.2	46.5	159.4
13	31.5	36.7	37.5	38	143.7
14	31.5	18.2	34.5	41.5	125.7
15	31.5	36.7	37.5	38	143.7
16	31.5	26	34	61.5	153.0
17	31.5	26	47.2	50.5	155.2
18	36.5	20.	32.5	44	133.0
19	36.5	8.5	37.5	46.5	129.0
20	36.5	34.5	36	38	145.0

Appendix 3

R_2 strain	Instar developmental periods (h)				
NUMBER	FIRST	SECOND	THIRD	FOURTH	TOTAL DEVELOPMENTAL PERIOD (h)
1	36.5	35.2	33.0	46.2	151.0
2	32.7	39.0	33.0	46.2	151.0
3	27.5	33.0	43.2	46.2	150.0
4	32.7	27.7	39.7	50.7	151.0
5	32.7	27.7	39.7	50.7	151.0
6	32.7	39.0	28.5	50.7	151.0
7	25.5	57.5	34.7	55.7	173.5
8	28.0	31.2	41.0	55.7	156.0
9	32.7	39.0	28.5	50.7	151.0
10	32.7	39.0	28.5	50.7	151.0
11	27.5	33.0	34.7	55.7	151.0
12	42.5	28.	20.	53.	143.5
13	27.5	33.0	34.7	55.7	151.0
14	32.7	27.7	39.7	50.7	151.0
15	48.5	23.5	20.	53.	145.0
16	27.5	33.0	64.2	26.2	151.0
17	32.7	39.0	28.5	50.7	151.0
18	32.7	39.0	28.5	50.7	151.0
19	41.5	28.	32.	48.	149.5
20	32.7	39.0	28.5	50.7	151.0

Appendix 3

B. Adult fecundity and longevity data at 20°C

S strain

a) FECUNDITY

1.	84	11.	54
2.	92	12.	101
3.	91	13.	93
4.	100	14.	86
5.	72	15.	75
6.	105	16.	71
7.	94	17.	88
8.	97	18.	106
9.	100	19.	94
10.	105	20.	86

b) LONGEVITY (days)

1.	27	11.	31
2.	32	12.	31
3.	34	13.	30
4.	27	14.	35
5.	32	15.	26
6.	32	16.	26
7.	32	17.	35
8.	38	18.	31
9.	36	19.	32
10.	35	20.	26

c) REPRODUCTIVE PERIOD (days)

1.	20	11.	19
2.	17	12.	17
3.	18	13.	17
4.	29	14.	18
5.	19	15.	18
6.	18	16.	18
7.	17	17.	16
8.	17	18.	18
9.	20	19.	18
10.	21	20.	19

d) POST-REPRODUCTIVE PERIOD (days)

1.	7	11.	12
2.	15	12.	14
3.	16	13.	13
4.	7	14.	17
5.	13	15.	8
6.	14	16.	7
7.	15	17.	19
8.	21	18.	13
9.	16	19.	14
10.	14	20.	6

Appendix 3

R_2 strain

a) FECUNDITY

1.	91	11.	86
2.	87	12.	91
3.	75	13.	98
4.	97	14.	94
5.	105	15.	81
6.	88	16.	94
7.	86	17.	90
8.	95	18.	86
9.	97	19.	96
10.	90	20.	89

b) LONGEVITY (days)

1.	34	11.	32
2.	29	12.	31
3.	29	13.	25
4.	36	14.	30
5.	30	15.	31
6.	30	16.	26
7.	30	17.	30
8.	31	18.	25
9.	29	19.	30
10.	30	20.	31

c) REPRODUCTIVE PERIOD (days)

1.	21	11.	19
2.	20	12.	23
3.	23	13.	20
4.	21	14.	22
5.	17	15.	24
6.	22	16.	18
7.	19	17.	17
8.	19	18.	17
9.	18	19.	24
10.	20	20.	19

d) POST-REPRODUCTIVE PERIOD (days)

1.	13	11.	13
2.	9	12.	8
3.	6	13.	5
4.	15	14.	8
5.	13	15.	7
6.	8	16.	8
7.	11	17.	13
8.	12	18.	8
9.	11	19.	6
10.	10	20.	11

Appendix 3

C. Nymphal development data at 5°C

S strain Instar developmental periods (h)

NUMBER	FIRST	SECOND	THIRD	FOURTH	TOTAL DEVELOPMENTAL PERIOD (h)
1	314	312	264	-	-
2	336.5	250	288	264	1138.5
3	270.5	261	288	270	1089.5
4	270.5	261	-	-	1089.5
5	314	260.5	170	383	1127.5
6	283.5	288	170	383	1124.5
7	283.5	288	-	-	1055.5
8	224	-	-	429	1010.0
9	224	-	-	429	1062.0
10	198.5	258	224	309	989.5
11	283.5	288	170	314	1055.5
12	456	-	-	309	989.0
13	283.5	288	170	314	1055.5
14	270.5	261	288	218	1037.0
15	225	306	-	-	989.0
16	311	260.5	-	-	-
17	220.5	384	-	-	-
18	268	300	240	312	1120.0

Appendix 3

R_2 strain — Instar developmental periods (h)

NUMBER	FIRST	SECOND	THIRD	FOURTH	TOTAL DEVELOPMENTAL PERIOD (h)
1	275	310	294	375	1254.0
2	278	290	288	360	1216.0
3	278	290	288	360	1216.0
4	287	296	314	368	1265.0
5	287	296	314	368	1265.0
6	168	264	367	432	1231.0
7	168	264	294	456	1182.0
8	275	-	-	504	1109.0
9	168	316	314	-	-
10	240	-	-	312	1008.0
11	270.5	316	288	355	1229.5
12	245.5	286	-	-	1179.5
13	336.5	293	-	-	1301.5
14	287	340	316	198	1141.0
15	168	-	-	-	1224.0
16	168	-	-	-	1111.0

APPENDIX 4

Temperature records

1982-83

a) Glasshouse temperature

Date	Daily (°C)	
	Maximum	Minimum
10 November	14	11
17 November	10	4
24 November	11	4
6 December	8	1
14 December	6	-1
21 December	6	3
31 December	9	6
9 January	13	4
13 January	11	2
25 January	10	-2
4 February	3	-4
9 February	2	-2
16 February	6	1
28 February	10	-3
8 March	13	4
24 March	11	1
3 April	14	0
12 April	21	2
3 May	22	6

Appendix 4

1982-83

b) Field temperature

Date	Daily (°C)	
	Maximum	Minimum
10 November	12	11
17 November	10	3
24 November	10	3
6 December	6	0
14 December	5	-1
21 December	5	3
31 December	7	6
9 January	10	2
13 January	10	2
25 January	9	-3
4 February	2	-4
9 February	2	-3
16 February	5	0
28 February	9	-3
8 March	11	3
24 March	13	0
3 April	10	-1
12 April	17	2
3 May	9	5

Appendix 4

1983-84

a) Glasshouse temperature

Date	Daily (°C)	
	Maximum	Minimum
26 October	12	8
1 November	14	9
7 November	12	9
26 November	9	4
5 December	8	5
2 January	13	4
15 January	11	0
20 February	8	0
15 March	14	1

1983-84

b) Field cage temperature

Date	Daily (°C)	
	Maximum	Minimum
9 November	15	8
13 November	12	4
26 November	11	-4
5 December	10	5
20 December	7	5
31 December	10	5
6 January	8	0
13 January	6	0

Appendix 4

1983-84

c) Field temperature

Date	Daily (°C)	
	Maximum	Minimum
1 October	15	13
8 October	14	9
15 October	12	7
30 October	11	3
7 November	11	9
16 November	9	2
26 November	11	-3
16 December	8	4
3 January	10	0
15 January	2	-2
16 February	10	-4
15 March	12	-1
17 April	19	0

Cryo-Letters (1982) 3

SUPERCOOLING ABILITY AND ACCLIMATION IN THE PEACH-POTATO APHID *MYZUS PERSICAE* (SULZ.) (HOMOPTERA: APHIDIDAE)

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The peach-potato aphid, *Myzus persicae*, is a species of world-wide distribution but is particularly important as a major pest of European agriculture through its ability to transmit virus diseases of crop plants such as potatoes and sugar beet. Throughout its range the aphid may overwinter either as sexually produced eggs on *Prunus* species, particularly peach (the primary host) in a holocyclic life cycle or as parthenogenetic active stages on summer herbaceous hosts (crop plants and weeds) where such plants continue to be available in winter (anholocyclic life cycle). Where winter conditions are severe, the former strategy predominates but the survival of anholocyclic clones is particularly favoured by mild winters and increases the economic importance of *M. persicae* since it results in earlier spring migration by winged forms (alatae), compared to holocyclic lines of development, and in turn, the earlier infection of susceptible young plants with virus diseases. Additionally in recent years, the appearance of strains of the aphid resistant to organophosphorus and carbamate insecticides, which are physiologically distinct from susceptible strains, has intensified the agricultural problem that this species represents.

Clones of *M. persicae* classified as susceptible (S), moderately resistant (R_1) and highly resistant (R_2) to organophosphorus insecticides were maintained on leaf discs of Chinese cabbage (*Brassica pekinensis*) at 20 ± 1°C and under a long photoperiod.

Supercooling points were determined for the four instars and adult apterae (wingless adults) of each clone; freezing was found to be fatal to all aphids (freezing susceptible). Supercooling point determinations were made using a thermoelectric method in which the aphids were cooled at $1^{\circ}C/min.^{-1}$.

There were no significant differences in mean supercooling point within the same age group between clones but there was a consistent and significant difference between the age groups of the aphid which was observed in all clones. All age groups of the three clones had mean supercooling points below -20°C and first instars were the most inherently cold hardy stage with mean supercooling points of -25.04 ± 0.23, -24.52 ± 0.47 and -24.99 ± 0.23 °C for the S, R_1 and R_2 clones respectively. There was no significant difference in supercooling ability between newly moulted and reproductive apterae nor between apterae and alate morphs of the S and R_2 clones.

First and fourth instar and adult apterae of the S and R_2 clones were examined in acclimation experiments at 5°C for 2, 7 and 14 days. There was a significant depression of the supercooling point in R_2 first instars after 2 days and in the S strain after 7 days. Nymphal development continued at 5°C in both clones to second instars by 14 days and these aphids were significantly more cold hardy than second instars maintained continuously at 20°C ($P < 0.01$). Fourth instars and adults showed no increase in supercooling ability under any of the acclimation experiments.

The results will be discussed with regard to the resistant-type and overwintering reproductive strategy of the aphid and in relation to other potential influences on its cold hardiness.

COLD HARDINESS OF THE PEACH-POTATO APHID RESISTANT AND SUSCEPTIBLE TO INSECTICIDES

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Summary. The cold hardiness of the peach-potato aphid (Myzus persicae) was assessed by studies on the supercooling ability of 3 strains of the aphid, differing in insecticide resistance; susceptible (S), moderately resistant (R_1) and highly resistant (R_2). The inherent supercooling ability of all age groups maintained at a constant temperature of 20°C was below -20°C, with no difference in cold hardiness between strains. First instars in all strains were the most cold hardy stage. First, second and third instars of the S and R_2 strains increased their cold hardiness when acclimated at 5°C. Differences in the cold hardiness of the two strains were found in fourth instars and adults, R_2 individuals being less cold hardy. In addition, R_2 adults completing their development at 20°C and then acclimated at 5°C lost cold hardiness while S individuals showed no change. The results indicate a marked variation in overwintering survival abilities between different age groups of the aphid.

INTRODUCTION

The relationship between successful anholocyclic overwintering of the peach-potato aphid (Myzus persicae)and economically important levels of virus disease the following year in a range of crops is widely recognised (Watson et al, 1975; Turl, 1981). Such overwintering allows earlier spring migration to younger and thus more virus susceptible plants with an increased probability of virus disease transmission. This was perhaps most dramatically appreciated during the potato leaf roll virus epidemic in Scottish seed potato growing areas in the mid-seventies following a series of mild winters which facilitated increased levels of anholocycly (Howell, 1977).

Recently, there has been much interest in the possiblity of predicting dates of spring migration particularly by analysis of aphid catches against meteorological data (Turl, 1981). A potentially limiting aspect is the lack of knowledge on the cold hardiness of the aphid itself, which appears to have been surprisingly neglected.

Prediction work is particularly concerned with the need to confine use of granular insecticides at seed potato planting to 'early aphid years'. Limited insecticide use is desired to avoid the selection of insecticide resistant aphids, particularly in Scotland where such aphids exist but are at present of limited distribution (Woodford et al, 1983).

This paper reports the results of studies on the cold hardiness of susceptible and resistant M. persicae by assessment of the supercooling ability of individual aphids. Being freezing-susceptible, aphids are killed at their supercooling point (SCP), the temperature at which ice forms in the body of the aphid as it is cooled below its true freezing point. Thus supercooling point data is widely used as a means of assessing and comparing the cold hardiness of freezing-susceptible insects (Sømme, 1982)

METHODS AND MATERIALS

Three laboratory-reared strains of M. persicae were studied; susceptible (S), moderately resistant (R_1) and highly resistant (R_2) to organophosphorus and also, in the latter strain, carbamate insecticides.

All aphids were maintained at 20 ± 1°C on leaf discs of Chinese cabbage (Brassica pekinenesis) under a long photoperiod (18 hours).

Supercooling points of individual aphids were determined by the method described by Bale et al (in press); aphids were cooled thermoelectrically at 1°C/min^{-1} and their body temperature was sensed and recorded by thermocouples interfaced to a microcomputer. The SCP is recognised as a temporary rise in body temperature associated with the release of latent heat when the insect freezes.

The inherent supercooling ability of the three strains was determined by taking samples of all the developmental stages, newly moulted adults, reproductive adults, and alate (winged) aphids from the cultures as described above.

Further experiments to examine acclimation ability were carried out by:

a) placing first instars, taken from the cultures at 20°C, at 5 ± 1°C on leaf discs and subsequently sampling them at each instar as they developed, including newly moulted adults.

b) newly moulted adults from the 20°C cultures were placed under the same conditions and sampled after 2, 7, 14, 21, and 28 days. Only the S and R_2 strains were considered in these experiments.

RESULTS

Inherent supercooling ability

The mean SCP of all the life cycle stages of the S, R_1 and R_2 strains are shown in Table 1. All stages in each strain had a mean SCP below -20°C with little variation within each stage. There was no difference in cold hardiness between strains.

Within the same strain there was a progressive loss of supercooling ability with increasing age and in all three strains, the first instar was the most cold hardy, although only significantly so in the S and R_2 strains ($p < 0.05$).

Table 1

Supercooling ability ($\bar{x} \pm$ S.E. and range (n = 20); °C) of insecticide resistant and susceptible *M. persicae* maintained continuously at 20°

Strain	Stage of aphid						
LT_{50}	First instar	Second instar	Third instar	Fourth instar	Newly moulted adult	Reproductive adult	Alate adult
S	-26 ± .25	-25.1 ± .25	-23.9 ± .36	-24.3 ± .30	-24.3 ± .40	-24.2 ± .29	-23.8 ± .27
	-23.1/-27.4	-23.6/-27.6	-21.1/-27	-22/-27.1	-18.8/-27.1	-21.3/-26.1	-21.1/-25.6
R_1	-25.4 ± .47	-25 ± .27	-24.1 ± .29	-23.3 ± .40	-24.7 ± .21	-23.2 ± .49	-23.9 ± .25
	-21.1/-27.9	-22.9/-26.9	-22/-27.1	-19.1/-25.4	-23.3/-26.2	-18.2/-26.3	-21.8/-25.8
R_2	-25.9 ± .25	-24.4 ± .34	-23.5 ± .30	-24.3 ± .29	-23.5 ± .42	-23.8 ± .20	-23.9 ± .38
	-23.7/-28.1	-21.3/-26.3	-20.2/-26	-21.5/-27.1	-17.7/-25.5	-22.3/-26.1	-20.5/- 26.2

Acclimation ability

Loss of cold hardiness with increasing age was also demonstrated during the acclimation experiments (Table 2). First instars of both the S and R_2

Table 2

Supercooling ability ($\bar{x} \pm$ S.E. and range (n=20); °C) of M. persicae acclimated at 5°C

Strain	Stage of aphid				
	First instar	Second instar	Third instar	Fourth instar	Adult (newly moulted)
S	-26.9 ± .23	-26.6 ± .22	-26.3 ± .15	-25.3 ± .33	-23.8 ± .25
	-24.9/-28.5	-23.7/-28.0	-24.1/-27.2	-21.3/-27	-22.2/-25.5
R_2	-26.5 ± .20	-25.8 ± .26	-25.4 ± .42	-21.6 ± .18	-21.9 ± .31
	-25/-28.3	-23.6/-27.3	-20.1/-27.2	-20.4/-23.4	-19.6/-24.8

strains kept at 5°C were more cold hardy than first instars at 20°C ($p < 0.05$) This increase in supercooling ability was maintained into the second and third instar stages within both strains so that these stages were more cold hardy than the corresponding instars reared continuously at 20°C ($p < 0.001$). However, in the fourth instar while S strain individuals remained more cold hardy than those at 20°C ($p < 0.05$) in the R_2 strain this instar was less cold hardy ($p < 0.001$). The difference in cold hardiness between strains was more apparent in the adult stage when S individuals did not differ in cold hardiness compared to samples at 20°C, and R_2 adults were less cold hardy than their counterparts at 20°C ($p < 0.01$).

Differences were further demonstrated by a loss in supercooling ability by R_2 adults after 14 days at 5°C ($p < 0.05$; Table 3) although this loss was regained with longer exposures to 21 and 28 days. S strain adults did not show any changes in cold hardiness under any of the exposure times.

Table 3

Supercooling ability ($\bar{x}$ ± S.E. and range (n=20);°C) of adult S and R_2 strain *M. persicae* acclimated at 5°C

Strain	Period of acclimation				
	2	7	14	21	28
S	-24.6 ± .27	-23.8 ± .29	-23.7 ± .30	-24.9 ± .32	-24.5 ± .29
	-22.2/-26.8	-20.6/-25.7	-20.4/-26.3	-21.8/-26.5	-22.5/-26.1
R_2	-22.8 ± .37	-22.7 ± .21	-21.6 ± .33	-23.4 ± .36	-22.4 ± .49
	-19.4/-25.2	-20.4/-24.3	-17.1/-24.5	-19.5/-26	-14.2/-25.9

DISCUSSION

Supercooling point (SCP) experiments provide a method of assessment of cold hardiness in freezing-susceptible insects. Since freezing is fatal in such animals the SCP of any individual is a direct measure of its instantaneous low temperature death point and this allows comparison in terms of cold hardiness to be made either between different individuals within the same species or between different species, provided a standard SCP determination technique is used (Sømme, 1982).

In all experiments, all life stages of the aphid demonstrated good supercooling ability, considering its temperate distribution, although apparently more extensive than that described by Parry (1978).

The strains studied in this work have been laboratory-reared for a number of years. However, SCP data of field samples of *M. persicae* corresponded very closely with that of laboratory strains (O'Doherty, unpublished results), and thus it would seem valid to relate the results of this research to the cold hardiness of the aphid in the field.

Many insects extend their supercooling ability as they enter winter (acclimatise) or can be induced to do so experimentally when exposed to low temperatures (Sømme, 1982). The increase in supercooling ability during acclimation at 5°C in first, second and third instars, although statistically significant, never exceeded 2.4°C. Therefore, although biologically interesting that the younger instars possess some acclimation ability it is unlikely to be important in terms of overwintering survival.

Baker (1977) discussed the possibility of differential cold hardiness in susceptible and resistant *M. persicae* with respect to their overwintering sites in Scotland, if the mutation for resistance were also to confer lowered fitness (cold tolerance). Although the strains did not differ in their inherent cold hardiness, the results from the acclimation experiments might imply inferior cold hardiness in the R_2 strain. SCP data from field experiments during the winter of 1982/83 and laboratory data collected from

S strain individuals infer that both the availability and quality of food during periods of low temperatures overwinter is in general, irrespective of resistance status, detrimental to adult aphid cold hardiness (O'Doherty, unpublished results). First instars, however, remain very cold hardy under all similarly stressful conditions.

In conclusion, a picture is emerging in which severe winter conditions make adult aphid mortality more likely while young instars born in winter maintain their cold hardiness to survive through to spring and complete development.

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An Automatic Thermoelectric Cooling Method and Computer-Based Recording System for Supercooling Point Studies on Small Invertebrates

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An automatic controller is described for use with fluid-cooled thermoelectric assemblies and compatible bipolar controllers in supercooling point studies on small invertebrates. The automatic controller provides constant and repeatable cooling rates in the range of 0.4 to 3°C min^{-1} from +25 to −50°C; under manual control this range can be extended from less than 1 to 15°C min^{-1}. The controller includes a range of preset lower-limit temperatures and a reset function to heat the system between experiments to the start temperature (+15°C) in less than 2 min. Supercooling points were sensed, observed, and recorded by a 12-channel, cold-junction-compensated, thermocouple convertor interfaced to a microcomputer, color monitor, and dot-matrix printer. Thermocouples were calibrated against a platinum resistance thermometer to derive polynomial descriptors which were incorporated into a microcomputer program (BASIC, Applesoft) to match thermocouples to ±0.2°C. Each thermocouple was sampled at 3-sec intervals to ±0.1°C and temperatures of insect-bearing sensors were plotted against time in distinguishing colors on the monitor, using a second computer program. On completion of an experiment all supercooling points for each thermocouple were printed on the dot-matrix printer; the graphical display could be stored on disk or output to a printer. The computer program can be readily modified to implement individual protocol requirements such as the range of temperatures and related information displayed on the monitor, and the thresholds (time and °C) for the recognition of supercooling points. The thermocouple convertor/microcomputer system could be used with any method of cooling.

In terms of cold hardiness, the majority of insects and other arthropods are "freezing-susceptible" and as such they avoid fatal ice formation in their bodies by supercooling; experimentally, the inherent capacity of supercooling in such species is assessed by supercooling point determinations (1), and this is a direct measure of their instantaneous low-temperature death point (2).

Equipment for measuring supercooling ability should cool the specimen at a constant and controllable rate, and detect the latent heat emitted when ice forms in the animal. Thermocouples are the preferred temperature sensors for most applications because they are available in sufficiently small size to detect the heat output of insects on freezing. The "alcohol bath" system has been used by a number of workers to supercool insects and mites (3–5); specimens, attached to thermocouples, are placed in small glass tubes which are suspended in a programmable alcohol bath to achieve a defined rate of cooling for the arthropods. Other sources of cooling have been used to supercool insects such as freezers (6, 7), cryostats (8), and the cooling bath of a cryostat (9), although the rates of cooling with these methods may be less controllable than with the programmable alcohol bath. The thermocouples are connected to cold-junction-compensated, multichannel potentiometric recorders which provide a continuous trace of decreasing temperature, and the supercooling points are readily observed.

Another system of cooling using a frigistor (based on the Peltier effect) was described by Luff (10) and modified by Bale (11) to utilize a freezing microtome stage. In this system the thermocouple and single speci-

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men are placed in a capillary tube which is secured to the cold surface of the stage and insulated from the ambient temperature. The stage is cooled thermoelectrically by manual control of a variable voltage power supply (Pelcool unit) and waste heat generated on the hot surface is removed by a circulating fluid. The manual voltage controller requires constant attention in order to achieve a uniform rate of cooling; furthermore, when the limited cooling capacity of the stage and controller is augmented by circulating fluids at zero or subzero temperatures, the initial rate of cooling of specimens (ambient to 0°C) may be rapid. The system has no heating capacity and the stage returns to ambient by gradual heat gain from the atmosphere.

The limitations of the thermoelectric system as described became apparent in the course of studies on the cold hardiness and overwintering survival of the peach–potato aphid *Myzus persicae* (Sulz.). This paper describes the development of an automatic system that overcomes the problems and disadvantages of previous thermoelectric methods by the use of modern, fluid-cooled Peltier stages and compatible bipolar controllers connected to a prototype automatic temperature controller. Further information is provided on the use of a thermocouple convertor and microcomputer to detect and record supercooling points; this temperature-sensing system could be used to advantage with most methods of supercooling small invertebrates.

MATERIALS AND METHODS

Thermoelectric Cooling System

Thermoelectric units. Modern thermoelectric systems consist of a fluid-cooled stage and compatible bipolar controller. Single-stage systems can produce a maximum temperature differential between the hot and cold surfaces of 60°C; with a hot surface temperature of +20°C, the lowest attainable cold surface temperature would be −40°C. In this research, where lower minimum temperatures were required (−50°C), a two-stage assembly was selected (Cambion 806-1008-01). This module with a square, cold surface area of 1024 mm^2 requires a maximum of 7.5 A at 18 V, supplied by a bipolar controller (Cambion 809-3040-01) in conjunction with a range extender (Cambion 809-1009-01) to achieve a temperature span of +25 to −50°C under manual control. An aluminium four-chamber specimen holder was mounted on to the surface of the cold stage (Pl. 1) to accommodate insects and thermocouples, and covered by an aluminium lid. Thermocouples (Type T) were made from a pair of twisted, PTFE-insulated, 0.2-mm-diameter wires with a junction response time of less than 15 msec. The whole assembly was surrounded by expanded polystyrene within a purpose-built metal container.

Automatic control. An automatic temperature controller (Pl. 2, Fig. 1) was designed to provide cooling rates of 0.4 to 3°C min^{-1}. The thermistor in the cold stage was replaced by a linear sensor, a silicon diode, which in its forward conduction region has a temperature coefficient of about −2 mV $°C^{-1}$; the maximum departure from linearity of the system did not exceed ±0.25% (0.15°C) over the normal operational range of +15 to −45°C at a cooling rate of 1°C min^{-1}. The sensor voltage was amplified in a two-stage amplifier to 100 mV $°C^{-1}$ and supplied to a differential amplifier. A second input to this amplifier was provided by a negative ramp voltage using a variable frequency oscillator (0.06 to 0.5 Hz) to drive a 10-bit counter which, in turn, was coupled to a digital-to-analog convertor to give a staircase ramp with steps equivalent to 0.1°C; different cooling rates are therefore obtained by changing the time interval (2–15 sec for rates of 3–0.4°C min^{-1}) between successive decrements in temperature. A digital technique was adopted in order to generate the very low rate of voltage change required (100 mV min^{-1}). The difference between the ramp and sensor voltages is

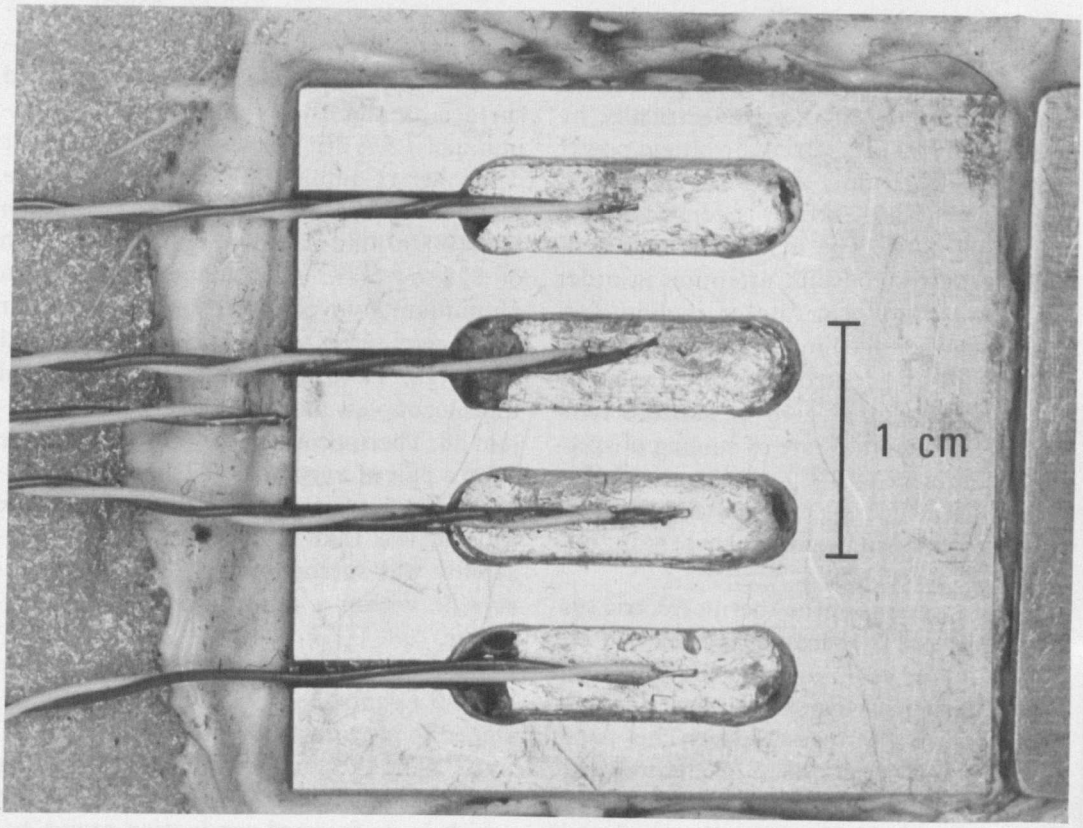

PLATE 1. Aluminium four-chamber specimen holder mounted on to the cold stage. Each chamber holds a thermocouple which is removable for the attachment of specimens. The "block" thermocouple (central) is permanently secured into the cold stage.

compared by the differential amplifier, and an error signal is fed to the power amplifier in the bipolar controller so that the cold stage temperature tracks the ramp voltage.

When the system is switched on or when the reset switch is used, a logic control presets the counter and, consequently, the ramp voltage, to a value equivalent to +15°C. The subsequent falling ramp voltage can be interrupted during cooling, using a stop switch, or automatically when the ramp reaches a preset lower temperature limit. Optional manual control of cooling rate is selected by connecting a multiturn potentiometer (range of ±5 V) to the differential amplifier. A center-zero meter indicates the magnitude and polarity of the error signal during both manual and automatic control; this is useful in the reset phase to show when the stage has reached the start temperature.

Computer-Based Visualization and Recording of Supercooling Points

The thermocouple convertor (CIL Microsystems 1002) includes a programable read-only memory (PROM) routine that measures sequentially the millivoltage of up to 12 experimental type-T thermocouples (channels 4 to 15) and transforms each to a digital value (12 bit); channels 1 and 2 are general-purpose millivolt inputs, channel 3 is a cold-junction compensation signal for the experimental channels, and channel 16 is connected internally to a 1 V reference source. The digital values from the PROM routine were placed in the scratch pad ran-

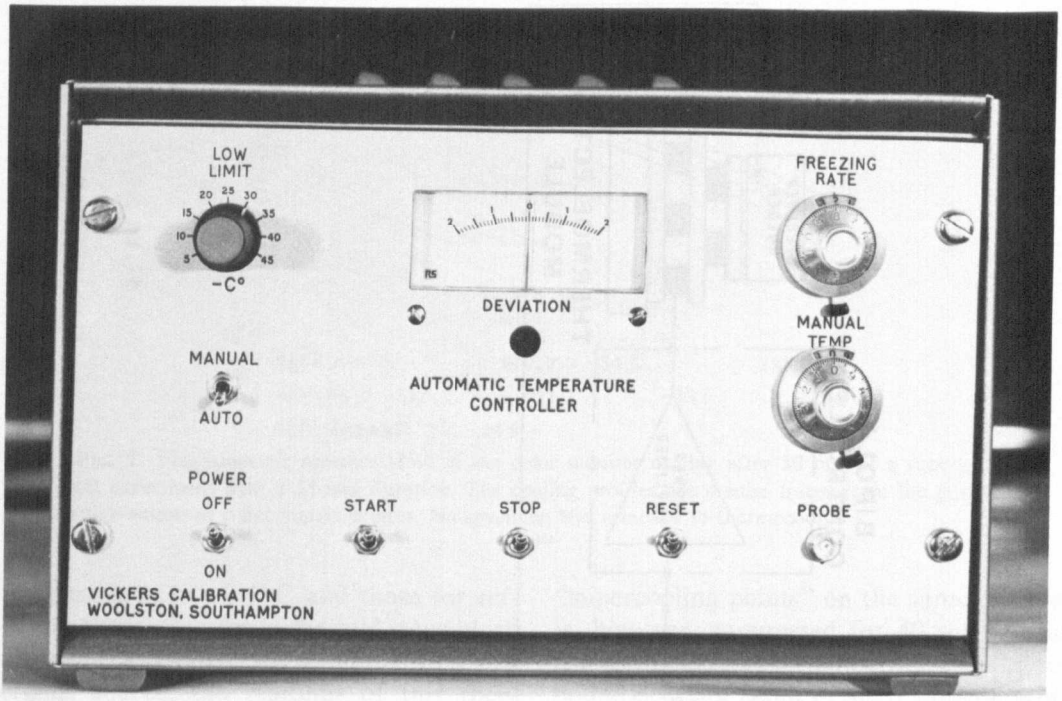

PLATE 2. Automatic temperature controller.

dom-access memory of an appropriate microcomputer (Apple II Europlus) for subsequent analysis. A program in the BASIC language (Applesoft) provided with the convertor was used to calibrate the millivoltage of each thermocouple against temperature in the range of +20 to −50°C as measured by a platinum resistance thermometer (Ancom PTE 361) connected via an adaptor (Ancom RTC-1, resistance thermometer/dvm adaptor) to a digital multimeter (Thurlby 1503 high-resolution multimeter). These values were fitted to a fifth-order polynomial regression using a main-frame computer (Amdahl V7) and an appropriate program (12).

A second program was written in the BASIC language to convert digital values to temperatures as before and detect the supercooling points of insects; the polynomial terms for each thermocouple were included in this program. A cooling rate and lower limit temperature are selected on the automatic controller; the lower limit is set at 5°C below the most extreme supercooling point expected. The computer program requests a string variable to define the experiment and the required duration of the recording period, after which the automatic controller and program are started simultaneously. The program then provides a temperature for each thermocouple after reference to the appropriate polynomial terms and the cold-junction compensation channel within the convertor. The convertor has a sensitivity of ±0.1°C and each thermocouple is sampled at 3-sec intervals. Under the present system of operation, when the temperature of the cold stage reaches 5°C, the temperature of the four insect-bearing thermocouples is plotted against time in distinguishing colors on the monitor. The program allows for the plotting to continue over more than one screen width when supercooling continues for more than 35 min, to −20°C and below (Fig. 2). There is also a continuous digital display on the monitor of the experimental thermocouple

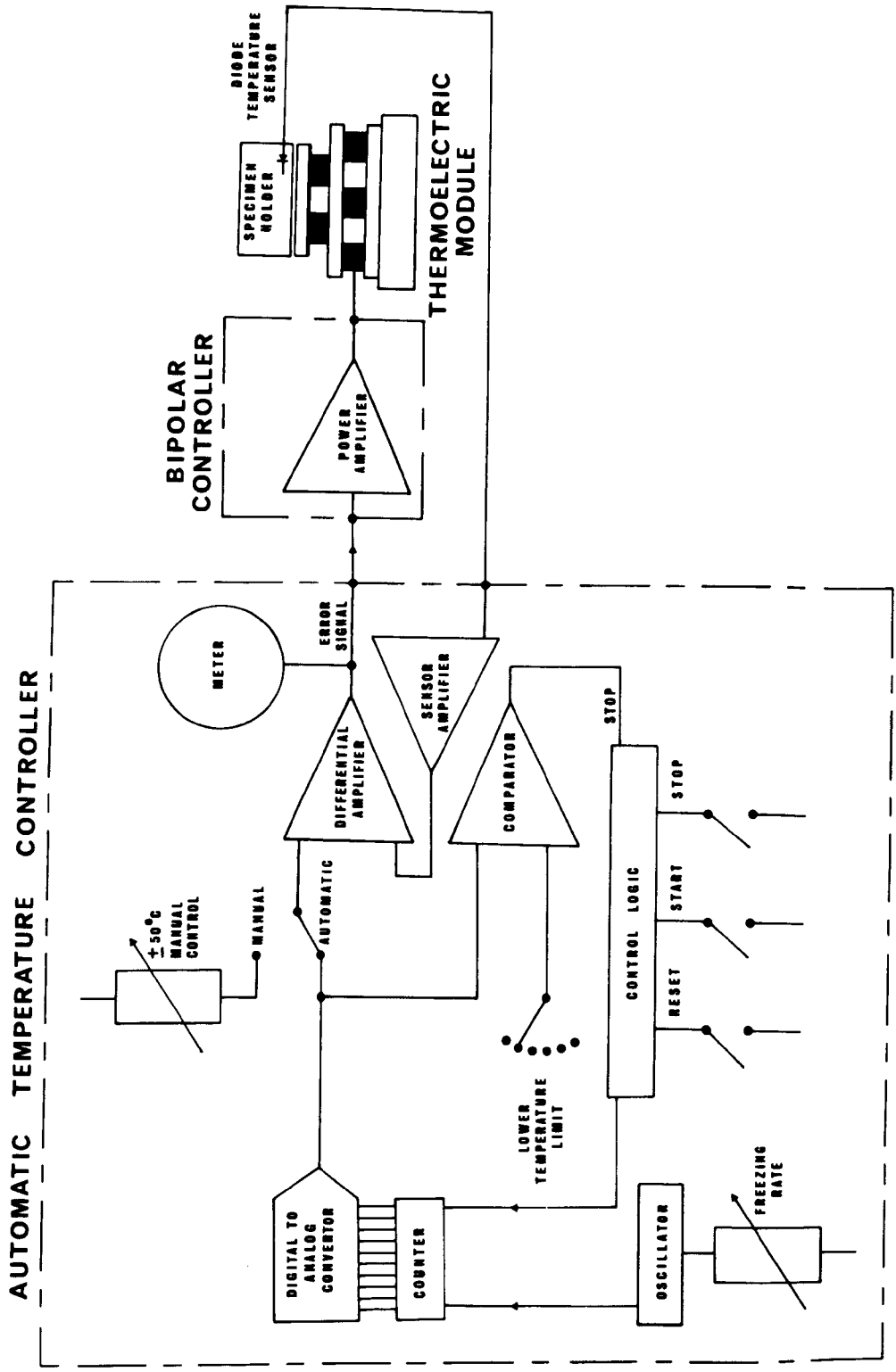

FIG. 1. Block diagram of the automatic temperature controller.

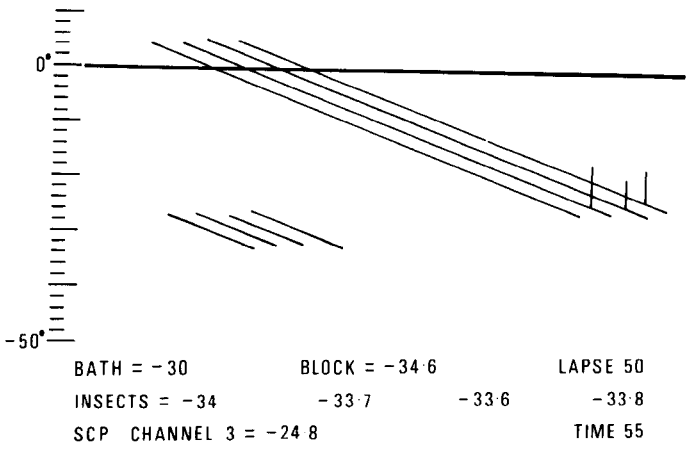

FIG. 2. Diagrammatic representation of the color monitor display after 50 min of a supercooling point experiment with a 55-min duration. The cooling profiles are precise traces from the graphical display output of a dot-matrix printer. No specimen was attached to thermocouple 1.

temperatures to ±0.1°C and those for reference thermocouples in the cold stage block and, where required, in some external reference source; the readings of this thermocouple are compared to those of the platinum resistance thermometer when placed in a low-temperature bath containing ethylene glycol at −30°C as a daily accuracy check on the system. The display also shows the lapsed time since the beginning of the experiment.

Supercooling points are recognized as an increase in temperature in excess of a preset value (0.2°C) above the previous reading for that thermocouple. This preset value prevents the recording of spurious "supercooling points" arising from the slight variations in temperature conversion (mV to °C) which is superimposed on the downward trend of the readings during cooling. When a rise in temperature is detected, the lowest value before the increase, the supercooling point, is stored in a dimensional array together with the temperatures of the cold stage and, where appropriate, the low-temperature bath; the supercooling point is also displayed digitally on the monitor (Fig. 2) and held on the screen until a subsequent supercooling point is detected on the same or a different channel. Detection of further "supercooling points" on the same channel is, however, suppressed for 40 sec so that the continued rise in temperature at the rebound does not result in the reading of multiple, false supercooling points. The suppression time can be modified in the microcomputer program according to the latent heat emission characteristics of different species. Up to 10 supercooling points can be stored for each thermocouple to allow more than one specimen to be cooled on each sensor. The program provides an option for the duration of an experiment to be extended by intervals of 5 min in situations where the low-temperature limit has been set above the supercooling point of one or more specimens. At a cooling rate of 1°C min^{-1}, supercooling points are accurate to ±0.2°C. On completion of an experiment, a dot-matrix printer (Epson MX 80F/T III) prints out the string variable giving the experimental heading and all the supercooling points and accompanying reference temperatures for each thermocouple. It is also possible to store the graphical display on disk or output it to the printer.

The graphical display could be adjusted to show the supercooling points of smaller organisms, and the microcomputer program would require modification if the super-

cooling point rebound was less than 0.2°C or for less than 3 sec. The period between successive "samplings" of the same thermocouple could be markedly reduced by using machine language if this was necessary for particular studies.

DISCUSSION

The automatic thermoelectric system has certain advantages over comparable supercooling point methods. The electronic basis of the automatic and bipolar controllers provide constant and repeatable rates of cooling in the range of 0.4 to 3°C min^{-1}. The manual mode of operation extends this range from less than 1 to 15°C min^{-1}, and, in future designs, the automatic controller could be modified to control any cooling rate within this range and offer considerable flexibility in cooling regimes for the experimentor; such rates would be far in excess of the maximum provided by low-temperature circulating baths. The thermoelectric system includes a "rapid reset" switch so that the cold stage can be heated from −50°C to its start temperature (+15°C) in less than 2 min and thus ensure a minimum time interval between successive experiments.

The thermocouple convertor is available for specified thermocouple types (J, K, T, S, R, and E) and, with the microcomputer, could be used with any cooling system for small invertebrates. The convertor provides a maximum of 12 experimental channels which are cold-junction compensated, and the error associated with changes in ambient state does not exceed 1°C for a 50°C shift in external temperature; by comparison, most multichannel, cold-junction-compensated potentiometric recorders are based on a maximum of six modules and vary by 1°C for a 15°C change in the ambient temperature. The convertor as supplied uses a quartic polynomial to linearize the experimental channels; the accuracy of temperature sensing was however enhanced by the use of fourth (or fifth)-stage polynomial descriptors to calibrate individual thermocouples against a platinum resistance thermometer. The microcomputer program essentially matches the thermocouples to ±0.2°C, and this high level of accuracy may be a particular advantage in long-term studies where absolute temperatures are of interest and experiments cannot be based on controlled concurrent comparisons. The microcomputer confers a flexibility upon the system with the capacity to design programs to the requirements of individual experimentors, such as the range of temperatures and related information displayed upon the monitor. The program described in this paper measured the temperature of each thermocouple at 3-sec intervals, but a machine language could be adopted to double the frequency of temperature sensing if this was required for a particular investigation. The program recognizes supercooling points as rises in temperature of more than 0.2°C lasting for more than 3 sec, and, with these thresholds, readily detects the supercooling points of eggs of the aphid *Aphis fabae* (Scop.) and nymphs and adults of the aphid *Myzus persicae* (Sulz.), with fresh weights of 25, 40, and 550 μg, respectively.

The system has been found to be accurate and reliable and has now replaced a manually controlled cold stage that had been used in all previous work in this laboratory.

ACKNOWLEDGMENTS

The research described in this paper was financed in part by a SERC postgraduate studentship award (No. 8132319) to Rose O'Doherty. The automatic controller was designed in conjunction with Vickers Calibration, Woolston, Southampton, who also produced the prototype model. The thermoelectric modules were supplied by Cambion (U.K.) Castleton, Sheffield.

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crystals resulting in good conservation of ultrastructure, even with unprotected cells. We have developed an ultrahigh speed freezing technique which allows continuous cooling of large quantities of cells. A special silver-coated copper wheel rotating at 3000 rpm is cooled at 20 °K in a LHe bath cryostat. Aqueous cell suspensions are continuously jet sprayed on the wheel, forming an ice band of 10–20 μm thickness. High cooling rates are achieved through centrifugal force enhanced metal to specimen contact and maximal heat conduction of silver in the region of 20° K. First estimates of cooling rates measured by a fast spectroscopic method (F. Aurich and Th. Förster, to be published) give 10^4 °K sec^{-1}. Preliminary results with preservation of unprotected yeast show better viability than formerly reported in literature for rapid freezing methods.

62. *An Automatic Thermoelectric Cooling System with Computer-Based Recording of Insect Supercooling Points.* ROSE O'DOHERTY, J. S. BALE, H. J. ATKINSON, AND R. A. STEVENSON (Department of Pure and Applied Zoology, University of Leeds, Leeds, United Kingdom).

An automatic thermoelectric cooling system with computer-based recording of supercooling points (SCP) was developed during research on the cold hardiness of the aphid *Myzus persicae* to overcome limitations associated with the manual system using potentiometric recorders. An automatic controller was designed to be used with a bipolar controller and fluid-cooled Peltier stage to provide cooling rates between 0.4 to 3°C min^{-1} with a lower limit temperature of −50°C; it includes a range of lower limit temperatures, a reset function to heat the system between experiments to the start temperature (15°C), and optional manual control extending the cooling rates up to 15°C min^{-1}. Temperature is sensed by thermocouples, individually calibrated against a platinum resistance thermometer and is recorded by a 12 channel cold junction compensated thermocouple convertor interfaced to a microcomputer. A program was written in Basic to sample each thermocouple at 3-sec intervals and produce a continuous digital/graphical display of cooling on a color monitor and to recognize SCP as a rise in temperature in excess of a preset value over the previous reading. SCP are accurate to ±0.2°C and up to 10 SCP can be stored per thermocouple during each experiment. Upon completion of an experiment all SCP are printed out by a dot matrix printer and the graphical display can be stored on disc or output onto the printer. The recording system confers a flexibility to design programs to specific experimental requirements and can be used with any cooling system. (Supported by SERC 8132319.)

63. *A Microprocessor-Controlled Temperature-Measurement and -Recording System.* P. D. COLERIDGE SMITH, D. J. MORTLOCK, AND K. E. F. HOBBS, (Academic Department of Surgery, Royal Free Hospital, London NW3, United Kingdom)

A portable multichannel temperature-measuring and -recording system has been developed to overcome the difficulty in providing sufficient resolution, accuracy, and portability associated with conventional chart recorders. The equipment has been designed so that it may be taken to the laboratory in order that measurements can be made, then subsequently removed to the office where the results can be analyzed using a microcomputer system. (Thermocouples (type T) have been used as the thermosensitive element in the system. A signal conditioner unit employing monolithic CMOS integrated circuits allows up to eight thermocouples to be used simultaneously. The low-level amplifiers incorporate ambient temperature sensing in order to provide cold junction correction. Separate amplifiers have been used for each channel to avoid errors due to low-level multiplexing. The amplified signal is carried by multiway cable to the digital recording system. This microprocessor-based system uses a single chip microprocessor (8035, Intel) to control digital conversion, tape storage, asynchronous data transmission, keyboard scanning, eight-digit liquid crystal display, and arithmetic processes. The system samples each channel every 200 msec and has an overall accuracy of ±0.2°C with a resolution of 0.1°. The design temperature range was 0–100°C, although this is easily extended to −150–+200°C.

64. *Mutation Induction in Escherichia coli by Desiccation and Freeze-Drying.* M. J. ASHWOOD-SMITH AND M. LIU (University of Victoria, Victoria, British Columbia, Canada).

It has been reported previously that both desiccation and freeze-drying produced mutations to try^+ from try^- in *Escherichia coli*. This auxotroph to prototroph reversion is associated with the probable suppression of a nonsense codon in the tryptophan synthetase pathway. Reversion rates of this backward mutation are affected by tryptophan levels in the medium and thus an increased number of dead cells could, in theory, affect the observed mutation values. Work to be reported indicates this to be most unlikely as a possible influence on the observed mutations as both desiccation procedures and freeze-drying have now been shown to produce mutations in two strains of *E. coli* (*B* and K_{12}) and the DNA gyrase operon (ecotopoisomerase II) as measured by resistance to the antibiotic nalidix acid.

65. *Recovery of Human Lymphocytes after Freezing under the Microscope in the Presence of Di-*