

Cold hardiness in diapause and non-diapause larvae of the summer fruit tortrix, *Adoxophyes orana* (Lepidoptera: Tortricidae)

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Abstract. Cold hardiness of larvae of the summer fruit tortrix moth, *Adoxophyes orana* (Fischer von Rosslerstamm) (Lepidoptera: Tortricidae) was examined in the laboratory. Supercooling point of field collected larvae increased significantly from a mean value of -23.9°C in February 1998 to -16.9°C in June 1998. Mean supercooling points for laboratory diapause and non-diapause larvae were -20.7°C and -17.2°C respectively. Short period of acclimation (10 days at 0°C) significantly decreased supercooling point to -24.7°C for laboratory diapause larvae. Acclimation for 12 days at 5°C decreased supercooling point to -19.4°C for non-diapause larvae. Pre-freeze mortality for diapause and non-diapause larvae was also studied. Constant exposure of diapause larvae at -5°C resulted in high mortality (63.1%) after a period of 30 days. In contrast, only 6 days at -5°C were sufficient to cause 100% mortality of non-diapause larvae. Mortality of non-diapause larvae reached 100% after 12 and 18 days at 0 and 5°C respectively. The importance of these findings for the overwintering strategy of *A. orana* is discussed.

INTRODUCTION

Various strategies of adaptations have been evolved by insects to survive the adverse seasons. In temperate regions diapause and cold hardiness are essential for insect survival during winter. Cold hardiness is defined as the capacity of a species to survive long or short-term exposure to low temperatures (Lee, 1991). This capacity is influenced by many factors including developmental stage, genetic potential, season, duration of exposure and nutritional status. Insects are commonly classified as freeze tolerant, those that survive at temperatures which cause extra cellular ice formation and freeze intolerant that avoid freezing by supercooling, being unable to survive at temperatures causing ice formations in the body fluids (Block, 1995). However, Bale (1993) described "new classes" of insect cold-hardiness, arguing that measurement of supercooling point is not a reliable index of cold-hardiness as many insects die before they freeze.

There is conflicting evidence on whether there is or is not a relationship between diapause and cold hardiness (Ring, 1972; Pullin & Bale, 1989). In some species cold hardiness is likely to be an integral part of the diapause syndrome, while in others it can occur completely independent of diapause. A close relation between these adaptive mechanisms implies that they are controlled by the same factors (Denlinger, 1991). Pullin (1996) argued from an evolutionary point of view that diapause-related carbohydrate synthesis could have been an important component of insect colonisation of colder climates

A. orana is a multivoltine species that is distributed from Asia through all Northern Europe. Since 1980's it has spread through southern Europe and become a serious pest of peach, cherry and apple orchards in Greece. *A.*

orana has three to four generations per year. Flight period starts early in May and lasts till mid-November. Third instar larvae of the last generation enter the diapause stage from late October and overwinter in diapause at rough places on trees. Overwintering larvae emerge in spring and feed on flower buds. Although larvae feed mainly on leaves, they also attack fruits superficially when they are close to each other or when they are in touch with leaves. Although several studies have covered various aspects of *A. orana* biology there is not much information concerning its winter biology (Charmillot & Brunner, 1990). The objective of this study was to investigate the cold-hardiness of *A. orana* in the southern part of its distribution area and some aspects of its winter biology.

MATERIAL AND METHODS

Insects

Larvae of *A. orana* were collected from overwintering sites on peach trees in northern Greece in February and June 1998. They were placed in 30 ml glass tubes and held in outdoor conditions shielded from direct sunlight until they were used in the experiment. Larvae collected in February are still in their overwintering sites but whether they are still in diapause is not known. It may have been terminated but low temperatures suppress development.

A laboratory colony of *A. orana* was established from larvae collected from infested peach shoots in 1996. Larvae were maintained on an artificial diet that has been developed for rearing of *Lobesia botrana* (Denis & Schiffermüller) (Lepidoptera: Tortricidae) (Savopoulou-Soultani et al., 1994). Adults were maintained in truncated transparent plastic cups covered with a transparent plastic sheet. A hole in the bottom of cups was punched and plugged with dental roll wick which provided the adults with a 5% sucrose solution. The egg masses collected from the plastic sheet were placed in new cups and pieces of ar-

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tificial diet were provided. Non-diapause larvae were obtained by rearing at 25°C and a 16-h photophase (16L : 8D). Prior to the use in experiments, non-diapausing larvae were removed from the diet for two days in order to discard any food items from their gut. Larval diapause was induced by rearing larvae at 20°C and 8L : 16D. Larvae that after 40 days in the growth chamber were still in the third instar were considered to be in diapause, removed from the diet for a period of 5 days and then used in the experiments.

Determination of supercooling points

Supercooling point (SCP) of individual larva was measured using a cooling rate of 1°C min⁻¹. Each larva was wrapped in small sections of parafilm before placing in contact with a thermoelectric Peltier plate (Marlow Industries Inc). A copper-constantan thermocouple was attached to the surface of each larva to monitor its temperature. The lowest temperature reached before an exothermic event occurred due to release of latent heat was taken as the supercooling point of the individual.

Determination of low temperature survival

Third instar non-diapause larvae were exposed for different periods of time (depending on exposure temperature) to a constant temperature of +5, 0, or -5°C. Following the exposure to the low temperature the insects were kept in the laboratory at 25°C and 16L : 8D and survival was estimated after 24 h. If larvae were able to move after a tactile stimuli they were considered alive. Survival for different periods of time was also estimated for diapaused larvae at -5°C. To test if acclimation could enhance survival of diapause larvae, groups of larvae were placed at 5°C for a period of five days after which they were transferred to -12°C for different time periods.

Determination of lethal temperature

Larvae of *A. orana* were examined for their tolerance to temperatures ranging from -15 to -30°C, depending on their physiological condition. Groups of ten larvae were cooled with the same procedure as for the SCP determination (1°C min⁻¹) until reaching a specific temperature, after which they were removed to ambient conditions. Mortality was recorded 1 and 24 h after exposure. The above experiments were performed using non-diapause third instar larvae, similar larvae acclimated

for 10 days at 5°C, diapause larvae, diapause larvae acclimated for 10 days at 0°C, and field collected diapause larvae in February.

Statistical analysis

Differences between treatment means of SCP were compared by *t*-test. Probit analysis was used to estimate lower lethal temperature of each experimental group and the exposure time needed to kill a given percentage of the population at each exposure temperature as well.

RESULTS

The daily minimum and maximum air temperatures in the study area from October through April are shown in Fig. 1. The minimum air temperature from November through April is between 5 and 0°C and in some days below 0°C. The maximum temperature remains above 0°C throughout the year.

Supercooling points

The distribution of SCPs for both acclimated and non-acclimated diapause and non-diapause larvae, and for field collected larvae are shown in Fig. 2a, b, c. Supercooling points ranged from -12 to -22°C for non-acclimated and acclimated non-diapause larvae while for both groups of diapause larvae it ranged from -17 to -29°C, whereas, the broadest distribution (-15 and -29°C) was observed in field collected larvae on 24 February. Acclimation had a significant effect on mean SCP

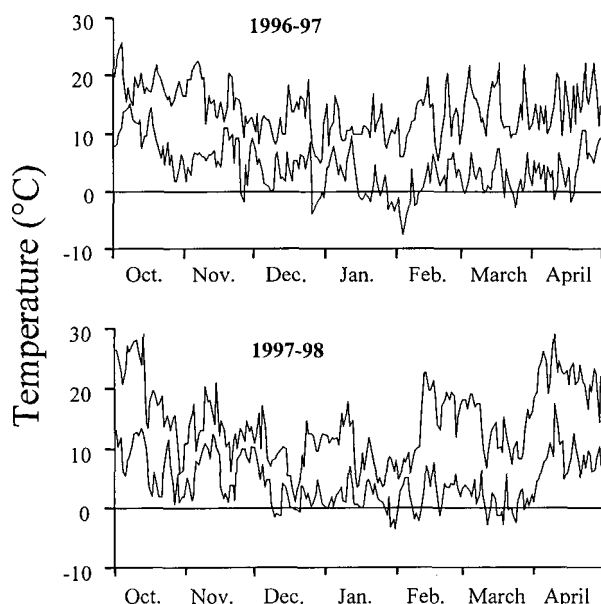


Fig. 1. Records of daily minimum and maximum air temperatures from October through April during 1996-1998.

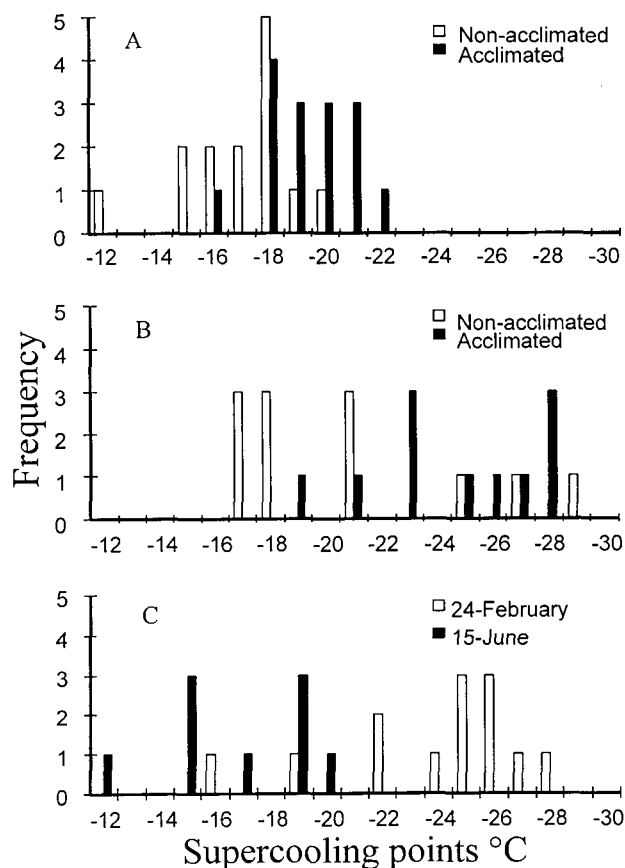


Fig. 2. Frequency distribution of supercooling points of non-diapause third instar larvae of *A. orana* (A), diapause larvae (B) and larvae field collected in February and June (C).

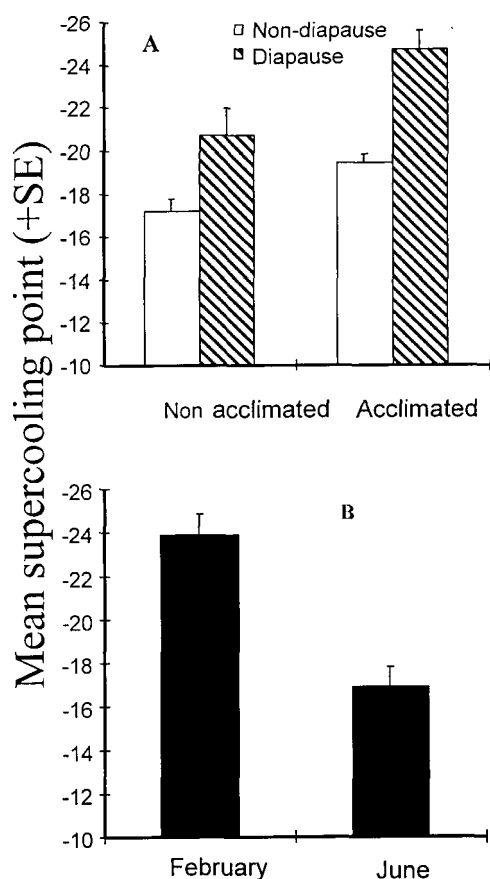


Fig. 3. Mean supercooling point (+ SE) of acclimated and non-acclimated diapause and non-diapause third instar larvae of *A. orana* (A) and field collected larvae in February and June 1998 (B).

($P < 0.01$, t-test) for diapause and non-diapause larvae as well (Fig. 3). A significant difference ($P < 0.01$, t-test) on the mean SCP was also observed between diapause and non-diapause larvae (Fig. 3) for acclimated and non-acclimated groups as well. The mean SCP for larvae collected in June was significantly lower than that for larvae collected in February (Fig. 3). Moreover, the mean SCP of field-collected larvae in February (-23.9°C), was similar to that of acclimated diapause larvae. In all cases insects were found dead after freezing.

Low temperature survival

The exposure time needed to cause (50, 90%) mortality of non-diapause larvae is shown in Fig. 4. It is apparent that significant mortality was observed at all temperatures examined. There was a substantial reduction on the $L_{\text{time}_{50, 90}}$ values at lower temperatures though the differences were not always significant. At -5°C the $L_{\text{time}_{90}}$ was only 5.7 days being significantly lower than that at 5°C which was 23.3 days. The lethal time for diapause larvae was examined at -5°C (Fig. 4B). It is clear that diapause larvae can survive for longer periods of time at -5°C than non-diapause larvae. The estimated $L_{\text{time}_{50, 90}}$ values for diapause larvae were significantly higher than those for non-diapause larvae.

When diapause larvae were transferred directly from 20 to -12°C for 1 h mortality was almost 100%. In contrast,

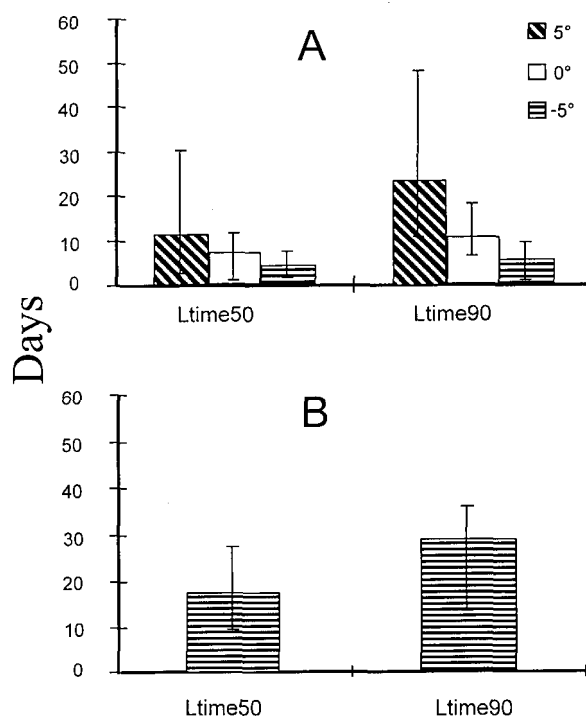


Fig. 4. $L_{\text{time}_{50, 90}}$ ($\pm$ 95% fiducial limits) of non-diapause larvae of *A. orana* at 5, 0 and -5°C (A) and diapause larvae at -5°C (B).

an acclimation period of 5 days at 5°C was sufficient to cause a substantial increase in cold hardiness of diapause larvae. An exposure period longer than 2 days was needed to cause high mortality on acclimated diapause larvae, whereas after one day of exposure at -12°C mortality was just 15.7% (Fig. 5).

Lethal temperature

The estimated temperatures required to cause 10, 50 and 90% mortality of the different experimental groups are shown in Fig. 6. The $L_{\text{temp}_{10, 50, 90}}$ for acclimated diapause and non-diapause larvae as well, was slightly lower than that for non-acclimated larvae of both groups, though the differences were not statistically significant. The $L_{\text{temp}_{10, 50, 90}}$ values for diapause larvae were lower in comparison to those of non-diapause larvae, both for acclimated and non-acclimated groups. The $L_{\text{temp}_{10, 50, 90}}$ values for field collected larvae was similar to those of diapause larvae and though they were lower than the respective values of non-diapause larvae, the difference was significant only for 90% mortality.

DISCUSSION

In many insects, SCPs have been used as an index of cold hardiness. Freeze intolerant species reduce the risk of freezing by enhancing their ability to supercool during harsh environmental conditions (Lee, 1991). Supercooling point of field-collected overwintering larvae was relatively low (about -24°C) but increased significant to -17°C in June. High supercooling ability can be often attributed to the absence of ice nucleating agents or accumulation of cryoprotectant elements or both. The lower supercooling point may be a result of elevated concentra-

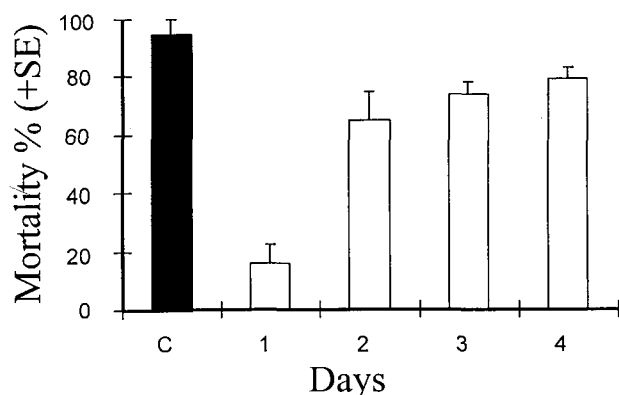


Fig. 5. Mortality of diapause larvae of *A. orana* at -12°C after a prior acclimation of 5 days at 5°C (C – Control).

tions of polyols and sugars in combination with decreased water content. Overwintering larvae of *A. orana* do not feed during winter and are thus free from gut contents which could potentially act as ice-nucleating agents (Watanabe & Tanaka, 1997).

Laboratory diapause larvae had lower SCP than non-diapause larvae indicating a relation between cold hardiness and diapause. If a relation between those two responses is confirmed then they should be controlled by the same regulators (Denlinger, 1991). More detailed experiments are needed in order to clarify if the above assumption is true for *A. orana*. Diapause larvae were able to survive for 20 days at -5°C , which is a much longer extended period that usually occurs in field conditions, but they failed to survive at -12°C for 1 h. Acclimation had a significant effect on survival of diapause larvae at -12°C and on depressing supercooling point both for diapause and non-diapause larvae as well. The most common response to low temperature cues is the synthesis of cryoprotectant substances (Lee, 1991). This has been confirmed for another tortricid species *Choristoneura fumiferana* (Clem.) (Han & Baue, 1995). Whether this is also true for *A. orana* is left to be examined.

Many studies have shown that supercooling is not a reliable index for insect cold hardiness as it does not take account of the mortality caused at sub-zero temperatures above the insect's supercooling point (Bennett & Lee, 1989; Bale, 1993). Increased mortality was observed after prolonged exposure of diapause larvae of *A. orana* at sub-zero temperatures indicating that some pre freeze mortality occurred. A notable pre freeze mortality at temperatures higher than SCPs was observed for non-diapause larvae as well. Many researchers have claimed that the role of cryoprotectant polyols may be other than depressing SCP (Chen et al., 1987; Pullin et al., 1991). However, the exact mechanisms involved remain to be clarified.

Leather et al. (1993) outlined the great influence on population dynamics of some insect species that might be exerted by the temporal and spatial variation in winter temperatures. In northern Greece winter is not characterized by severe frost and the number of air frost days (from 0 to -5°C) are not more than 10 days per year. The results from our study indicated that *A. orana* is well adapted to the local winter conditions as it has the ability

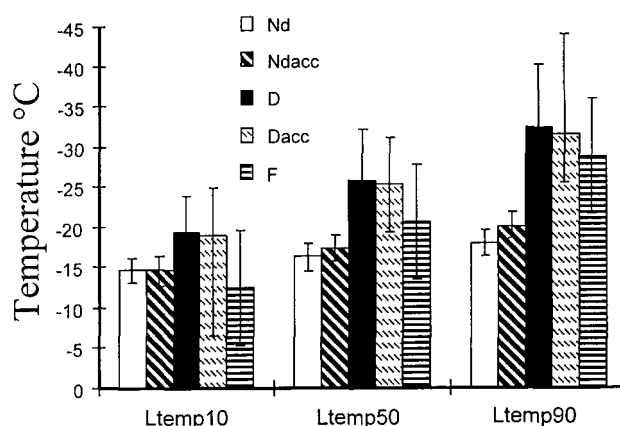


Fig. 6. $Ltemp_{10}$, $Ltemp_{50}$, and $Ltemp_{90}$ ($\pm$ 95% fiducial limits) of non-diapause third instar larvae (Nd), non-diapause acclimated third instar larvae (Ndacc), diapause larvae (D), acclimated diapause larvae (Dacc) and field collected larvae in February (F) of *A. orana*.

to survive for a substantial period of time even at -5°C . This is not unexpected for this species since northern Greece is included in the southern limits of its distribution (Barel, 1973).

Since most of the present results were achieved on laboratory insects it is difficult to extrapolate them to the field situation. However, based on these results an insight could be gained for the winter biology of this species. Overwintering diapause larvae appear in late September, long before the onset of cold weather. After moving to their overwintering sites they cease feeding and their guts contain no food particles; this enables them to increase their supercooling capacity. Insects at this time have the ability to survive an exposure to sub-zero temperatures which might occur. Based on our findings, acclimation has an important effect on non-freezing survival of diapause larvae. The low temperatures that are experienced by larvae as winter proceeds are presumably the trigger for supplemental synthesis of cryoprotectants by overwintering larvae.

The minimum temperatures at the overwintering sites inhabited by larvae are usually much higher than their supercooling ability, indicating that non-freeze mortality should be considered as the crucial factor in the decline of postwinter population. In addition to laboratory experiments concerning the physiological and biochemical changes of diapause larvae of *A. orana* direct observations on field mortality are required to obtain a more comprehensive idea on winter biology of this species.

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