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Cold Tolerance Including Rapid Cold-hardening and Inoculative Freezing of Fall Migrant Monarch Butterflies in Ohio

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Migrants of the eastern North American population of the monarch butterfly, *Danaus plexippus*, are often subjected to subzero temperatures, heavy dews and frost in late September and October during the autumn migration to overwintering sites in Mexico. Adults of this generation had the capacity to rapidly increase their cold-hardiness. A chilling period of 1 h at +4°C before exposure to −4°C for 24 h significantly improved the survival (>80%) of monarchs over those with no chilling prior to −4°C exposure (<40%). This is the first report of rapid cold-hardening in Lepidoptera, and the capacity to rapidly cold-harden may protect monarchs against cold injury during diurnal changes in temperature. An extended period of progressively lower temperature acclimation enhanced cold-hardiness to a greater extent than did the rapid cold-hardening treatment. When exposed to −4°C, migrants collected in the field from Ohio were less tolerant (LT_{50} = 37.5 h) than migrants gradually acclimated over several weeks from 20 to 25°C in the field to overwintering conditions of +4°C in the laboratory (LT_{50} = 85.8 h). External moisture on the exoskeleton significantly raised supercooling points (SCPs) of acclimated monarchs from −8.2 to −4.7°C. In addition, external moisture decreased the supercooling capacity and triggered internal ice formation at subzero temperatures above the SCP of dry monarchs. No monarchs survived internal ice formation associated with SCP determination, evidence that the monarch butterfly is a freeze-susceptible insect.

Supercooling point Cold tolerance Freezing temperatures *Danaus plexippus*

INTRODUCTION

Monarch butterflies, *Danaus plexippus* (L.), from eastern North America migrate each autumn from breeding grounds in the upper U.S. and southern Canada to overwintering sites high in the transvolcanic range of central Mexico (Urquhart and Urquhart, 1976, 1978; Brower, 1977; Calvert and Brower, 1986). Migration is used by monarchs to avoid the harsh winter conditions in temperate regions with prolonged subzero temperatures and lack of their milkweed host plants (Urquhart, 1987; Wells and Wells, 1992; Cockrell *et al.*, 1993; Malcolm *et al.*, 1993). Monarchs, like most other insects, use environmental cues such as shorter days, cooler temperatures, and declining host plant condition (Danks, 1978; Tauber *et al.*, 1984; Brower, 1985) as stimuli to begin their autumn migration from summer breeding grounds. The migrants enter a reproductive diapause which lasts until late January and begin the return migration when suitable milkweed hosts are available the following spring (Barker and Herman, 1976; Herman, 1981).

Cool, humid conditions at the Mexican overwintering sites promote the conservation of lipids, reduced metabolism, and cessation of activity (Calvert and Brower, 1986; Masters *et al.*, 1988). These locations in the fog belt and dense forests moderate climatic conditions and reduce temperature fluctuations to which the overwintering monarch clusters are exposed (Calvert and Brower, 1981; Calvert *et al.*, 1982). However, despite the moderating effect of the forest, the overwintering monarchs are occasionally exposed to subzero temperatures and can suffer significant mortality (Calvert and Brower, 1981; Calvert and Cohen, 1983; Calvert *et al.*, 1983). Winter storms which hit the central highlands of the transvolcanic range often begin with rains, soaking the overwintering monarch clusters followed by snow at these sites (Calvert *et al.*, 1983). As the storms clear, subzero temperatures as low as −5°C occur, usually at night due to radiative cooling (Calvert *et al.*, 1982). Concerns about the ability of monarch butterflies to survive

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these conditions, particularly when faced with excessive deforestation at overwintering sites, has led to recent studies evaluating habit structure and cold-tolerance of monarchs at their overwintering sites (Calvert and Brower, 1981; Weiss *et al.*, 1991; Alonso-Mejia *et al.*, 1992; Anderson and Brower, 1993).

However, migrating monarchs can often be subjected to subzero temperatures while still in their summer breeding range during September and October. In Michigan and Ohio, monarchs have been observed migrating following nights of heavy frost and exposure to subzero temperatures in late September (K. J. Larsen, personal observation). Recent studies of monarch cold-hardiness have been restricted to overwintering sites or southern U.S. migrants (Alonso-Meijia *et al.*, 1992; Anderson and Brower, 1993), therefore the present study examines cold-hardiness in a northern population of migrating monarchs. In particular, we evaluated two distinct types of cold tolerance: the rapid cold-hardening and the winter cold-hardening associated with the gradual long-term acclimation to colder temperatures of migrating monarch butterflies in southern Ohio. We wanted to determine whether short-term versus long-term low temperature acclimation enhanced their cold tolerance. In addition, we tested whether surface moisture, as would be naturally experienced due to rain or dew, would influence their capacity to remain unfrozen by supercooling and, thereby, survive subzero temperatures.

MATERIALS AND METHODS

During the height of the 1992 autumn migration, several hundred monarchs were collected between September 15 and 24 from fields at the Ecology Research Center of Miami University near Oxford, Ohio. They were placed individually in glassine envelopes to prevent them from sustaining physical damage during transport to the laboratory. Adults were held in 30.5 × 61 × 61 cm polyester netting cages set up inside an environmental growth chamber set at 20°C and a 12 h:12 h L:D cycle. A honey solution was provided daily by soaking strips of cloth hanging inside the cages. Cages were misted daily to maintain a RH of 80%.

The monarchs were divided into two cultures: a "field migrant" culture held at 20°C (a daytime temperature commonly experienced in Ohio in late September), and tested as described below for cold tolerance within 1 to 2 weeks of collection from the field, and an "acclimated" culture in which the monarchs were slowly acclimated from "field migrant" conditions (20°C) to simulated overwintering conditions of 4°C by dropping the air temperature gradually at 0.5 to 1°C per day over a 3 week period, then maintained at 4°C for 1 to 2 weeks after which cold tolerance tests were conducted.

Exposure chamber

Cold tolerance was tested by placing individual monarchs in glassine envelopes within a small exposure chamber consisting of an insulated beaker with coolant flowing within the walls. The double-walled Pyrex beaker was constructed from a 1000 ml beaker nested inside a 2000 ml beaker with nozzles attached on opposite ends and sides to ensure an even flow of coolant. The open end of the chamber containing the monarchs was plugged with a styrofoam lid with a small access hole for thermocouples. Air temperature inside the exposure chamber was maintained by a flow of 95% ethanol coolant chilled by a flow-through refrigerated circulating bath (Endocal Model RTE-8, Neslab Instruments, Newington, NH).

Rapid cold-hardening response

To determine whether monarchs have the capacity to rapidly cold-harden as occurs in the flesh fly, *Sarcophaga crassipalpis*, the milkweed bug, *Oncopeltus fasciatus*, and the elm leaf beetle, *Xanthogaleruca luteola* (Chen *et al.*, 1987a; Lee *et al.*, 1987), two groups of monarchs were taken from the 20°C culture within 1 week of field capture. One group ($n = 14$) was placed directly from 20°C into the exposure chamber at −4°C for 24 h (the "no conditioning" group). A second group ($n = 14$) was "prechilled" at +4°C for 1 h immediately prior to exposure to −4°C for 24 h. After exposure, all butterflies were removed and placed in a recovery cage at 20°C for 24 h to evaluate survival. Following the recovery period, monarchs were classified as "normal" (those able to walk, eat, and fly easily), "flight impaired" (those having difficulty flying), "moribund" (those unable to fly), or "dead", following the categories defined by Calvert *et al.* (1983).

Long-term cold-hardening

Tolerance of −4°C, a subfreezing temperature not uncommon following January storms endured by overwintering monarchs in Mexico (Calvert *et al.*, 1983), was tested for both the field migrant and acclimated cultures. Cold tolerance at −4°C was tested by placing groups of 10 monarchs (5 males and 5 females) directly from the cultures into the exposure chamber for various time periods. Groups of field migrant monarchs (20°C culture) were exposed for periods of 0, 3, 6, 12, 18, 24, 30, 36, 48 and 72 h. Groups from the acclimated culture (maintained at +4°C) were exposed for 0, 12, 24, 48, 72, 96, 120 and 144 h. Following exposure to −4°C, the monarchs were placed into 30.5 × 30.5 × 30.5 cm screen cages for 24 h at 20°C to recover. After 24 h, only "normal" monarchs were considered survivors. Individual butterflies were used only once and survivors released in the field. Mean time to 50% mortality at −4°C of the two cultures (LT_{50}) was determined using a MINITAB (Anonymous, 1989) probit analysis macro procedure (PROBIT; unpublished program developed by J. R. Sedcole and revised by L. V. Madden) in the same manner used to measure cold-hardiness in *Diabrotica* spp. (Elsey, 1989). Differences over time between the treatments was evaluated using repeated-measures ANOVA (Anonymous, 1993).

Effect of moisture on supercooling capacity

Supercooling point determination. The supercooling point (SCP) was determined as the lowest temperature recorded prior to the release of the latent heat of fusion when body water freezes (Lee, 1989). The SCP was determined for migrant monarchs acclimated to +4°C and was measured for each butterfly by positioning a 36-gauge copper-constantan thermocouple in contact with the insect cuticle on the dorsal surface of the thorax of the butterfly kept stationary inside a glassine envelope using a paper clip. A cooling rate of ca. 0.36°C/min was maintained in the exposure chamber set to decrease from 0 to −15°C. Insect temperature was monitored every 5 s by a multi-channel data logger (Model OM500, Omega Engineering, Stamford, CT). Survival of monarchs following SCP determination was assessed by placing monarchs in a recovery cage at 20°C for 24 h.

Effect of moisture on SCP. The effect of moisture on monarch SCPs was tested by wetting some acclimated monarchs from the 4°C culture ("wet") with a heavy mist of distilled water. SCPs were obtained from at least 6 wet and 6 dry butterflies of each sex, placed within the exposure chamber. Immediately after the SCP was determined, each monarch was removed from the glassine envelope, weighed "wet" to the nearest 0.1 mg, then dried at 60°C for 48 h and re-weighed "dry" to determine body water content.

Duration of supercooling at −4°C. The purpose of this experiment was to determine whether wetting the surface of monarchs affected their capacity to remain unfrozen in a supercooled state. Monarchs from the 4°C acclimated culture were divided into 2 groups, one wet (heavily misted with distilled water) and one dry, each consisting of 8 males and 8 females. The body temperature of each monarch was monitored with thermocouples as they were placed in the exposure chamber and cooled to −4°C at a cooling rate of 0.36°C/min. Once −4°C was reached, the temperature was held constant at −4°C for 24 h. The formation of internal ice was detected by the release of the latent heat of fusion, and the duration of the supercooled state, measured from the time their temperature dropped below 0°C until the ice nucleation event occurred was recorded. After the 24 h exposure to −4°C, monarchs were placed into a recovery cage and held at 20°C for 24 h and classified as normal, flight impaired, moribund, or dead.

RESULTS

Rapid cold-hardening response

Chilling with short-term (1 h) exposure to a non-lethal temperature of +4°C resulted in a rapid increase in the cold-hardiness of migrating monarchs exposed to subfreezing temperatures of −4°C ($\chi^2 = 5.85$, $P = 0.016$). When migrants were exposed directly to −4°C for 24 h, less than 37% were "normal" following the treatment, able to fly with no apparent ill-effects

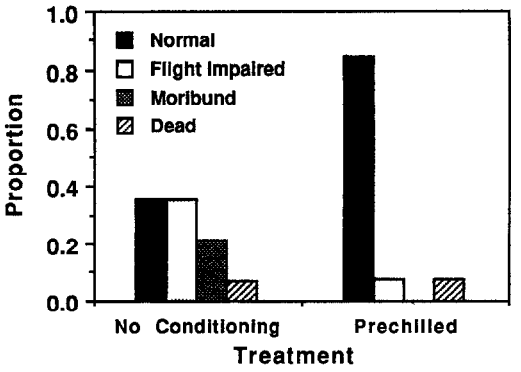

FIGURE 1. Effect of a 1 h chilling at +4°C ("prechilled") prior to exposure to −4°C for 24 h on the survival and flight ability of migrating adult monarch butterflies held at 20°C for 3 to 4 days after field capture.

(Fig. 1). Rather, 37% were "flight impaired" and had difficulty flying, and more than 20% was "moribund", unable to fly at all. In contrast, of the "prechilled" migrants exposed to +4°C for 1 h immediately prior to exposure to −4°C for 24 h, 85% were "normal" and none was "moribund" (Fig. 1).

Long-term cold-hardening

There was a significant increase ($F = 6.16$; $df = 1, 7$; $P = 0.04$) in the cold tolerance of migrant monarch butterflies when they had been gradually acclimated to simulated overwintering conditions of +4°C (Fig. 2). Migrants taken from the field and maintained at 20°C were able to survive exposure to subfreezing temperatures of −4°C less than half as long ($LT_{50} = 37.31$ h) as acclimated migrants ($LT_{50} = 85.82$ h). This long-term period of low temperature acclimation conferred greater protection at −4°C than did the rapid cold-hardening response (Fig. 1).

Effect of moisture on supercooling capacity

Supercooling point. Among monarchs from the overwintering "acclimated" (+4°C) culture, the presence of external body moisture from misting resulted in a signifi-

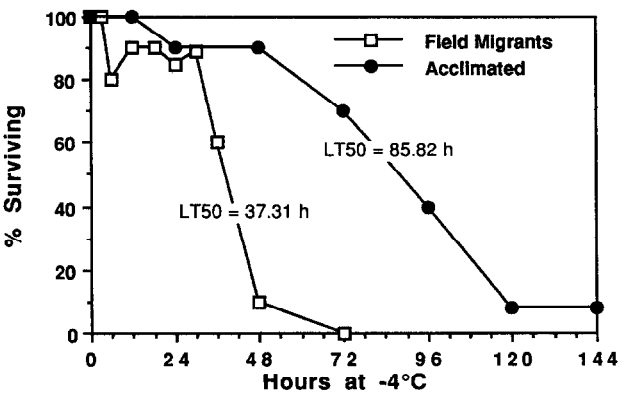

FIGURE 2. Survival of adult monarch butterflies at −4°C of field-collected migrants held at 20°C for less than 2 weeks and migrants cold-acclimated over a 6 to 8 week period to simulated winter conditions of 4°C. For each sample, $n = 10$ butterflies.

TABLE 1. Supercooling points (SCPs; mean ± SE and range) of dry and misted (wet) male and female fall migrant monarch butterflies after a 6 to 8 week acclimation period to simulated winter conditions of 4°C

Body surface	Sex	n	SCP (°C) mean ± SE	Range (°C) Min	Max
Dry	Male	6	−8.5 ± 1.1 a	−13.0	−6.5
	Female	6	−7.9 ± 0.6 a	−10.2	−5.8
Wet	Male	12	−4.3 ± 0.4 b	−6.9	−2.2
	Female	15	−5.0 ± 0.4 b	−8.9	−3.0

Means followed by different letters are significantly different (Fisher's Protected Least Significant Difference; $P = 0.05$).

cant ($F = 37.79$; $df = 1, 35$; $P < 0.001$) elevation of their SCP (Table 1). When dry, the SCP was −8.2°C (±0.6°C SE), while the SCP was elevated to −4.7°C (±0.3°C SE) when monarchs were wet. There was no significant difference in SCPs between the sexes when either wet or dry ($F = 0.005$; $df = 1, 35$; $P = 0.95$). None of the butterflies survived the internal freezing of body water that occurred during SCP determination.

Monarchs which were "dry" had a body water content of 54.1% (±1.1% SE), significantly less ($T = 2.98$; $df = 19$; $P < 0.008$) than "wet" monarchs with a body water content of 59.9% (±1.6 SE). Although there was no apparent correlation ($r = 0.147$; $P = 0.65$) between the body water content of "dry" monarchs and their SCP [Fig. 3(a)], there was a significant correlation ($r = 0.719$; $P = 0.008$) between the body water content of "wet" monarchs and their SCP [Fig. 3(b)].

Duration of supercooling at −4°C. As subzero temperatures in Mexico overwintering sites do not decrease

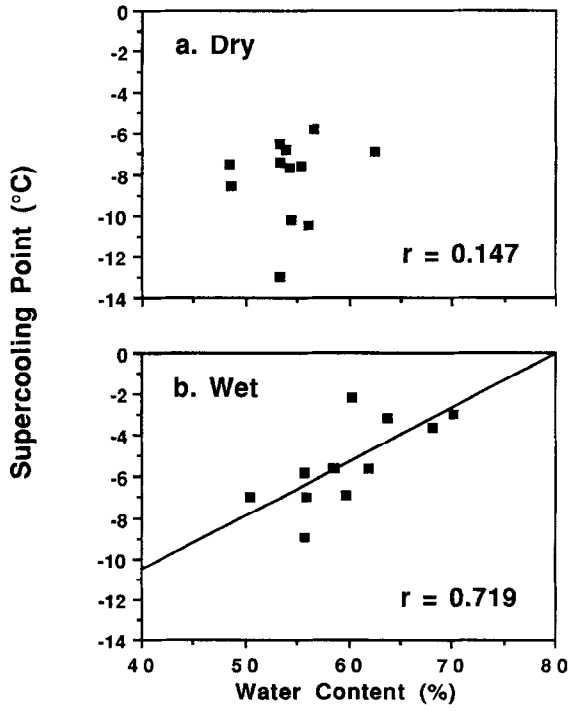

FIGURE 3. Correlation of supercooling point (SCP) and water content of (a) dry, and (b) wet monarch butterflies after acclimation over a 6 to 8 week period to simulated winter conditions of 4°C.

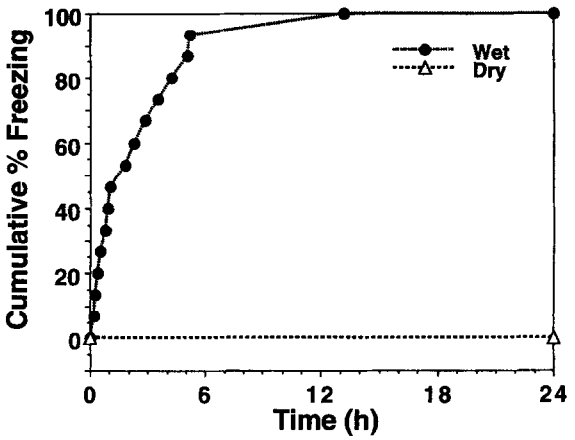

FIGURE 4. Duration of supercooling at −4°C as measured by the cumulative percent freezing and time to ice nucleation of misted (wet) monarch butterflies ($n = 16$) maintained at simulated winter conditions of 4°C. Dry acclimated monarchs ($n = 16$) maintained in winter conditions at 4°C remained supercooled at −4°C and did not experience ice nucleation within 24 h.

to the SCPs recorded for these monarchs, the relationship of external body moisture to internal ice formation at a more commonly occurring subzero temperatures was evaluated. Monarchs with higher surface water content, resulting from external water application, had a smaller capacity to remain supercooled before internal freezing began. External body moisture had a significant effect ($F = 462.0$; $df = 1, 23$; $P < 0.001$) on the ability of "acclimated" overwintering monarchs to survive subfreezing temperatures above their SCP. Of the 16 "dry" monarchs, all survived and were "normal" following 24 h of exposure to −4°C. However, all 16 of the "wet" monarchs were dead after a 24 h exposure to −4°C (Fig. 4) and experienced internal ice formation as indicated by the release of the latent heat of fusion within 3 h (2.81 ± 0.87 h) after the exposure temperature dropped below 0°C.

DISCUSSION

Rapid cold-hardening response

Over 80% of the migrant monarchs prechilled prior to exposure to −4°C were normal and their behavior suggested they would be able to continue the migration, while less than 40% of the unchilled migrants survived direct exposure to −4°C. Because none of these monarchs actually froze, this indicates that monarchs may experience cold-shock, a form of non-freezing injury (Lee, 1991). The monarch is the first Lepidoptera known to have the capacity to rapidly cold-harden. Enhanced survival of subzero temperatures following exposure to a brief chilling period is possibly due, at least in part, to a rapid increase in synthesis of a cryoprotectant (Lee *et al.*, 1987).

This rapid cold-hardening response may allow monarchs to withstand diurnal exposure to low temperatures during the autumn migration, especially on clear nights when radiative cooling is greatest. However, as

shown in this study, the rapid cold-hardening response is distinct because it generally provides less protection against low temperature injury as compared to longer-term winter cold-hardening (Lee, 1991).

Long-term cold-hardening

During the autumn migration, cold hardiness of the monarch butterfly population of eastern North America apparently increases as the population becomes acclimated to winter temperatures. In Ohio, migrating monarchs were less able to survive prolonged periods of subfreezing temperatures of −4°C than were migrants brought into the laboratory and gradually acclimated to overwintering conditions of +4°C. Although the cold-hardiness of many insects is influenced by environmental conditions present during development (Salt, 1953; Chen *et al.*, 1987b), it is evident in monarchs that migrating adults, as is commonly observed in insects, have the capacity to cold-harden in response to environmental cues such as low temperature (Lee, 1991).

The SCP is that subzero temperature at which spontaneous tissue freezing occurs and this freezing is lethal to freeze-susceptible insects (Salt, 1961; Lee, 1989). None of the "field migrant" or "acclimated" monarchs survived the freezing event which occurred at the SCP, indicating the monarch is freeze-susceptible. Our SCP values of approx. −8°C for dry monarchs (Table 1) are similar to those reported for individuals collected from overwintering clusters in Mexico (Anderson and Brower, 1993).

Inoculative freezing

Previous field studies at overwintering sites in Mexico have shown increased mortality of monarchs at subzero temperatures due to moisture from dew or rain on the exoskeleton (Calvert *et al.*, 1983; Alonso-Medjia *et al.*, 1992). Both Calvert *et al.* (1983) and Alonso-Mejia *et al.* (1992) hypothesized that increased mortality of wet monarchs was due to inoculative freezing, which occurs when ice crystals form on the surface of the insect and trigger internal ice nucleation, possibly via the spiracles (Salt, 1961). We found that wetting monarchs, as can occur following a rain or heavy dew, resulted in a significant elevation of the SCP, and thereby reduced cold-hardiness. When the body surface was dry, there was no relationship between total body moisture content and SCP. However, when the body surface was wetted, increased body water content significantly reduced supercooling capacity. This raised SCP and the resulting increase in mortality of wet monarchs thus confirms the speculation by Calvert *et al.* (1983) and Alonso-Mejia *et al.* (1992) that monarchs are susceptible to inoculative freezing. Both Alonso-Mejia *et al.* (1992) and Anderson and Brower (1993) refer to unpublished data by Anderson and Brower who showed a similar response.

Surface moisture also influenced the capacity of the monarchs to remain supercooled at subzero temperatures above their SCP. When dry, monarchs survived and remained supercooled for the 24 h duration of the experiment. When wet, however, monarchs remained supercooled at subzero temperatures above their SCP for only 2 to 3 h before lethal spontaneous freezing occurred. These observations further confirm the role of inoculative freezing in reducing cold tolerance proposed by Calver *et al.* (1983) and Alonso-Mejia *et al.* (1992).

We have identified both freezing and cold shock as forms of cold injury and mortality in migrating monarchs. From our rapid cold-hardening test, it is evident that some injury caused by cold shock rather than freezing can result in mortality to migrating monarchs at temperatures above their SCP. Mortality in other unfrozen, but supercooled insects has been reported by Lee and Denlinger (1985) and Knight *et al.* (1986). In our study, we showed that rapid cold-hardening protected the monarchs from lethal injury due to cold shock. We also have shown that long-term acclimation to progressively lower temperatures increased survival of this northern population of migrating monarchs. Furthermore the northern population of migrants had similar limits of cold tolerance, with SCPs of around −8°C, as do the monarchs at Mexican overwintering sites (Anderson and Brower, 1993), confirming that the monarch butterfly is a freeze-susceptible insect.

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