



Phenology of Cold Hardiness in Reproductive and Migrant Monarch Butterflies (*Danaus plexippus*) in Southwest Ohio

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We examined seasonal changes in the cold hardiness of summer reproductive and fall migrant cohorts of the monarch butterfly in southwest Ohio in 1994. Reproductive and migrant butterflies were distinguished on the basis of lipid content and whether the female had mated. We compared crystallization temperatures (the temperature at which ice forms inside the body) and the capacity to resist chilling injury (i.e. injury due to subzero chilling in animals which supercooled but did not freeze) between reproductives and migrants. Very low crystallization temperatures ($< -10^{\circ}\text{C}$) were found only in the migrants. Among reproductives there was a significant positive correlation between lipid content and crystallization temperature. Chilling injury occurred frequently among reproductives, in contrast to migrants which survived subzero exposure until ice began to form in their tissues. Records of microhabitat and ambient temperatures from field data loggers and meteorological stations indicate that the monarch migrants in this region infrequently encounter temperatures low enough to promote extensive cold acclimation, and that subzero temperatures are very rare during the time we observed migrants. Data from previous studies of monarchs at the overwintering site or acclimated in a laboratory cold room indicate that this species is capable of greater cold hardiness than we observed in freshly-caught field specimens. We conclude that the increased cold hardiness we observed in migrants was due almost entirely to physiological changes accompanying reproductive diapause and migration, and that cold acclimation played little if any role. Copyright © 1996 Elsevier Science Ltd

Cold hardiness Monarch *Danaus plexippus* Chilling injury Supercooling Trehalose

INTRODUCTION

Each autumn a cohort of monarch butterflies enters reproductive diapause and migrates from the eastern and central regions of the U.S. and Canada to a small region of high-altitude fir forests in central Mexico. Each spring this overwintering generation flies north to reproduce in the southern U.S., and the progeny of the migrants repopulate eastern North America (Malcolm *et al.*, 1993). Physiological characteristics distinguishing fall migrants from reproductives are well established. Migrants are reproductively inactive (Herman, 1989), and accumulate two to three times more lipid than reproductive butterflies (Beall, 1948; Brown and Chippendale, 1974).

Aspects of cold hardiness of monarch butterflies have

also been examined. Several studies have correlated survival of butterflies at the overwintering site with environmental parameters such as temperature, moisture, and solar radiation (e.g. Calvert *et al.*, 1983; Weiss *et al.*, 1991; Alonso-Mejia and Arellano-Guillermo, 1992). Laboratory studies have documented decreased crystallization temperature — the temperature at which internal ice formation occurs — in the migrating cohort of monarch butterflies at the overwintering site (Anderson and Brower, 1993) or in laboratory-acclimated butterflies captured in Ohio during the migration (Larsen and Lee, 1994). These laboratory studies indicate that monarchs do not survive freezing of their internal body fluids — i.e. they are freeze intolerant. Cold-induced mortality in freeze-intolerant insects can occur due to either actual freezing of internal body water, or to injury from subzero chilling while in the unfrozen, supercooled state (Lee, 1991). For this report we refer to the latter as chilling injury, and to the ability to avoid chilling injury as chill tolerance.

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Migrating monarchs undergo a reproductive diapause, and are exposed to successively colder temperatures over the fall and winter. Either factor can independently increase cold hardiness in some insects. Cold acclimation — i.e. increased cold hardiness as a result of exposure to low but non-freezing temperatures — often involves the production of low molecular weight cryoprotectants. This occurs due to increased glycogenolysis as a result of exposure to temperatures of 0 to +5°C (Lee, 1991). In some insects mechanisms other than accumulation of low molecular weight cryoprotectants may also promote chill tolerance (e.g. stress proteins, Denlinger *et al.*, 1991). Little is known about the relative roles of these factors in cold hardiness in the monarch, in part because cold hardiness has not been examined in the northern reproductive populations.

In this study, we document when the change-over between reproductive and migrant butterflies occurs in southwest Ohio. We report the physiological differences in supercooling and chill tolerance in freshly-caught reproductive and migrant monarchs. We examine changes in potential low molecular weight cryoprotectants in the hemolymph, and compare the crystallization temperatures of migrants with temperatures experienced by butterflies in this region in the late summer and fall.

MATERIALS AND METHODS

Field observations and collection

Monarch butterflies were observed and collected in a large field of goldenrods at the eastern side of the Miami University Ecological Research Center (ERC, 39.5°N, 84.7°W) between 28 June and 29 Sep of 1994. We were unable to capture butterflies any later in the season, although isolated individuals were observed in the area as late as 15 Oct. Animals were collected on at least weekly intervals and brought into the laboratory. Sex and wet weight were recorded, and the animals were then used immediately for experiments. Following supercooling or chill tolerance assays or flushing hemolymph, the bursa copulatrix was dissected to determine the presence or absence of spermatophores.

Determination of crystallization temperature

Crystallization temperature — i.e. the onset of internal ice formation — was determined by placing male and female butterflies in a glassine paper envelope with a 30 or 36 gauge copper-constantan thermocouple held adjacent to the thorax. The envelope was then pinned to the side of a chilling chamber formed by placing a foam-lined 2,000 ml beaker in the chamber of an ethanol-filled refrigerated bath. The chamber and the bath were cooled from room temperature to -20°C providing a cooling rate of ca. -0.13°C per min. The thermocouples were monitored every 5 s by a multi-channel data logger (CM500, Omega Engineering, Stamford, Connecticut). The crystallization temperature was recorded as the low-

est temperature prior to a transient rise in temperature, indicating release of latent heat concomitant with freezing.

Chill tolerance assays

Animals used in chill tolerance assays were continuously monitored with thermocouples. Male and female butterflies were placed in a glassine envelope with a thermocouple as described above, cooled to -4 to -6°C, and incubated at these temperatures for 24 h. While the temperature within the chamber varied, the continuous temperature readings during the chill exposure allowed us to know the precise temperature exposure for each animal, and whether each animal froze. Following chill exposure the data logger was turned off and the butterflies were returned to room temperature (ca. 23°C), allowed 24 hours to recover, and then categorized as 'alive' (fully recovered), 'moribund' (mildly to severely impaired), or 'dead'.

Determination of whole-body water and lipid content

Wet weight, dry weight, and lipid content as a proportion of dry weight was determined for all animals used in crystallization temperature or chill tolerance assays — both males and females. The animals were placed in a drying oven and held at 65°C until no further weight loss was observed (ca. 24 h). The dry weight was then recorded. The animals were then pulverized with a mortar and pestle, and approximately 100 mg of the resulting homogenate was extracted with 15 ml of 1:1 chloroform:methanol as described by Tuskes and Brower (1978). A 5 ml aliquot was placed on a tared pan, allowed to completely evaporate, and the pan was again weighed. The difference in weight was presumed to be lipid. This provided us with the concentration of lipid in the extraction solvent, and by calculation, the proportion of dry weight of the extracted sample which was lipid.

Hemolymph volume, collection, and metabolite assays

Hemolymph composition was analyzed by injecting an excess amount of buffer in the thorax, clipping the tip of the abdomen, briefly centrifuging the whole animal at low speed, and collected the flushed hemolymph from the bottom of the centrifuge tube. In order to determine the amount of flushing buffer to use, hemolymph volume was determined for several butterflies by the amaranth red dye dilution method (Weinberg, 1980). We estimated the hemolymph volume to be 0.062 ml (std. error = 0.008, $n = 5$).

For the remaining hemolymph analysis, the hemoceol was flushed with 0.2 ml flushing buffer comprising phosphate-buffered saline (PBS: 10 mM sodium phosphate, 150 mM sodium chloride, pH 7.4) containing 10 mM thio-urea, 2 mM ethylenediaminetetraacetic acid, and 1 mM phenylmethylsulfonyl fluoride. The wings and the tip of the abdomen were clipped off and the butterfly was then placed in a disposable plastic 1.0 ml pipetter tip with the tip cut a few millimeters from the bottom. The pipetter

tip was then placed in a 15 ml centrifuge tube, the butterfly was injected in the prothorax with 0.2 ml flushing buffer, and the assembly was centrifuged at ca. 300 rpm for 5 min. The flushed hemolymph was removed from the bottom of the centrifuge tube, placed in a microcentrifuge tube, and centrifuged for 2 min at 16,000 rpm to remove hemocytes. The volume of the resulting plasma was adjusted to 0.2 ml with PBS, and a 0.05 ml aliquot was removed.

The remaining plasma was subjected to perchloric acid deproteination (Passonneau and Lowry, 1993). We added 0.015 ml of 3 N perchloric acid to the plasma, allowed it to incubate on the bench for 2 min, and then centrifuged 2 min at 16,000 rpm to remove the precipitate. The supernatant was removed and the volume was estimated. To neutralize the deproteinated plasma, 0.35 ml 2 M potassium bicarbonate per ml supernatant was added. The supernatant with sodium bicarbonate was incubated on ice for 15 min, and then the neutralized, deproteinated plasma was pipetted off of the precipitated potassium perchloride. The recovered volume was noted, and the deproteinated plasma was divided into 50 μ l aliquots. Deproteinated plasma was stored at -20°C prior to analysis.

Trehalose was measured using the anthrone reagent following the use of hydrochloric acid and sodium hydroxide to destroy reducing sugars and polysaccharides (Van Handel, 1985). Glucose was measured using a Sigma kit for the dianisidine assay (Barham and Trinder, 1972). Glycerol was measured using components from a Sigma kit for determination of triglycerides based on the glycerol phosphate oxidase/peroxidase reaction (Trinder, 1969). In order to validate our deproteination and assay technique with the glycerol test, we confirmed that we could quantify glycerol in the plasma of the flesh fly *Sarcophaga crassipalpis*.

Plasma osmolality was determined using a Wescor 5500 vapor pressure osmometer (Wescor Inc., Logan, Utah). Plasma for osmolality determinations was obtained as previously described except that no flushing buffer was used, and the determinations were made immediately after bleeding.

Temperature data

Microhabitat data were collected using thermistors attached to Hobotemp® data loggers (Onset Computer Corp., Pocasset, Massachusetts). One thermistor was placed on the under-side of a branch at approximately 2 m on the south side a tree which had, in previous seasons, been observed to serve as a monarch roost. The other was placed on a goldenrod stem in a field. Neither of these thermistors were shielded. Temperature was recorded every 48 min, and these data were downloaded periodically onto a Macintosh notebook computer. An hourly record of temperature was obtained from a field meteorological station located in a shaded, ventilated container 2 meters off the ground, at the ERC approximately a kilometer from the study cite. A 48-year data

set (1945–1993) was obtained from a hygrothermograph located 2–3 kilometers from the study cite, maintained by the Miami Conservancy District (MCD). For comparison, temperature records taken at 3h intervals at Cincinnati, Dayton, and Toledo (southwest, west-central, and northwest Ohio, respectively) were obtained from the Ohio State Climatologist, and normal daily minimum temperatures based on data from 1961 to 1990 were obtained for 22 recording stations in Ohio and the Lower Peninsula of Michigan south of 45°N latitude (National Oceanic and Atmospheric Administration, 1994). The names and location of these stations were obtained from data downloaded from U.S. National Oceanic and Atmospheric Administration FTP archives (<ftp://ftp.ncdc.noaa.gov/pub/data/inventories/coopfull.txt>).

Data analysis and statistical procedures

For purposes of analysis, observations were grouped into 7-day intervals. These 'weeks' were based on Julian day rather than calendar weeks [i.e. integer of (day of the year/7) + 1]. The data were analyzed and statistical comparisons were made using SAS (SAS, 1990). Because of unequal sample sizes, analysis of variance (ANOVA) was performed using PROC GLM and pairwise comparisons were made using LSMEANS and PDIF. The proportional lipid content of reproductive and migrant monarchs was compared using CONTRAST under GLM. Nonparametric comparisons were made using rank means provided by the ALL or CMH options for PROC FREQ statistics (Stokes *et al.*, 1995). Temperatures experienced by reproductive and migrant monarchs in chill tolerance assays were compared using either Student's t-test or a 1-way ANOVA with a Bonferroni post-test. For comparison of ERC and logger data, SAS programming instructions were used to change the time stamps on the Hobotemp data to the nearest whole hour and PROC MEANS was used to provide an average for cases of two observations with the same time stamp.

RESULTS

Field observations and physiological status of butterflies

Observations of physical and physiological parameters were initially grouped by week. Females were categorized as mated or not based on presence or absence of a spermatophore, and lipid content was determined for both males and females used in crystallization temperature determinations and chill tolerance assays (Fig. 1). No spermatophore was found in any female collected in the final 4 wk of observations (i.e. after 4 Sep 1995).

Proportional lipid content was compared among weeks and between males and females using 2-way ANOVA (Table 2, Fig. 1). Lipid differed significantly among weeks but not between males and females. The lipid content of butterflies collected during the last four weeks of the study was significantly greater than that of butterflies collected during the preceding 10 weeks ($F = 139.4$, df

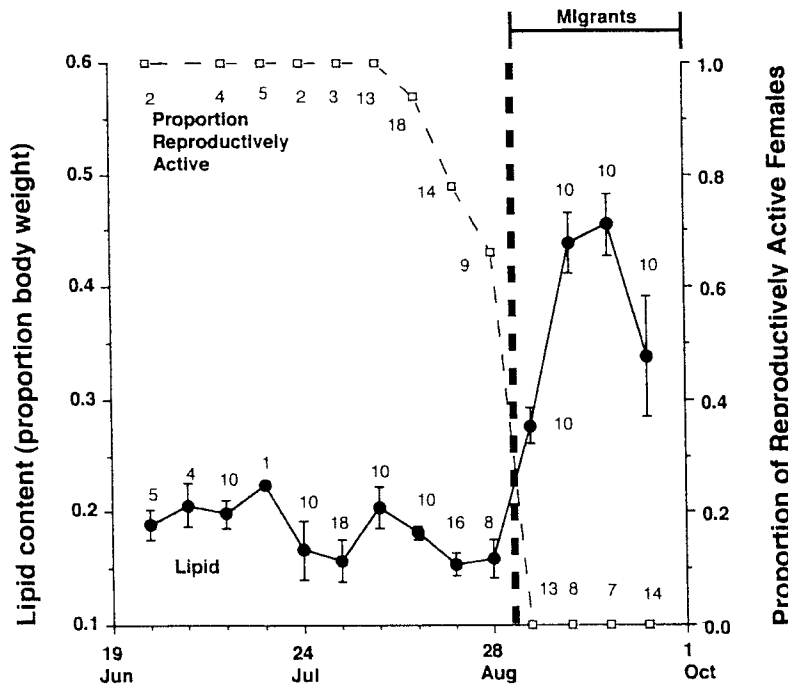


FIGURE 1. Weekly summary of the proportional lipid content of monarch butterflies (both sexes) in southwest Ohio in 1994, and of the proportion of females mated. The markers for lipid content represent the mean, and the error bars represent standard error of the mean. Sample size is given adjacent to the marker.

= 1, 113, $P = 0.0001$). Based on the observations of reproductive status and lipid content, butterflies captured during the first 10 wk (26 Jun–28 Aug) were categorized as ‘reproductives’ for subsequent analysis, whereas those from the final 4 wk (4–25 Sep) were categorized as ‘migrants.’ Our data indicate departure from a 1:1 sex ratio among mating populations, as has been observed in other studies of monarchs (Frey and Leong, 1993). There was no significant departure from a 1:1 sex ratio among the migrants.

Water content of butterflies

The proportional water content, lean dry mass, and total body water of butterflies were compared among weeks (Fig. 2). Proportional water content was significantly lower after 11 Sep than during previous weeks [Fig. 2(A)]. Lean dry mass decreased significantly in late reproductives (31 Jul–21 Aug), and increased significantly at the height of the migration [11 and 18 Sep, Fig. 2(B)]. Late reproductives (14 and 21 Aug) had significantly less water than earlier reproductives. Despite an apparent increase in total body water in migrants from the week of 18 Sep, no migrants had significantly different total water content from the early reproductives [Fig. 2(C)]. Sex was not a significant factor in variation of proportional water content.

Supercooling and chilling injury

The range of crystallization temperatures was greater in the migrants (-12.4 to -2.1°C , $n = 21$) than in the reproductives (-8.6 to -3.4°C , $n = 36$). Relatively small

sample sizes and different variances in the reproductive and migrant populations required the use of nonparametric statistical comparisons. Comparison of frequency distributions between reproductives and migrants reveals a marginally significant difference in the mean rank scores (Fig. 3). Comparisons of crystallization temperature by week using nonparametric techniques revealed significant differences between rank means among reproductives ($Q_S = 15.474$, $df = 6$, $P = 0.017$; data not shown), but not migrants (data not shown). The lowest crystallization temperatures among reproductives were observed on 10 Jul rather than at the time of transition of the population to migrant status. Crystallization temperatures were not distributed differently between males and females, neither among reproductives nor migrants (data not shown).

We tested for possible correlations between the crystallization temperature and mg water, proportional body lipid, and lean dry mass. When data for all butterflies or migrants only were examined, none of these correlations was significantly different from zero. Among reproductives, however, there was a significant positive correlation between proportional lipid content and crystallization temperature ($r^2 = 0.355$, $P = 0.002$).

Chill tolerance was examined by exposing animals to temperatures of -6 to -4°C for 24 h, and then taking them immediately back to room temperature and allowing them to recover for 24 h. By continuously monitoring the animals’ body temperatures during the chill exposure, we were able to make precise comparisons between chill exposure and outcome, and to exclude

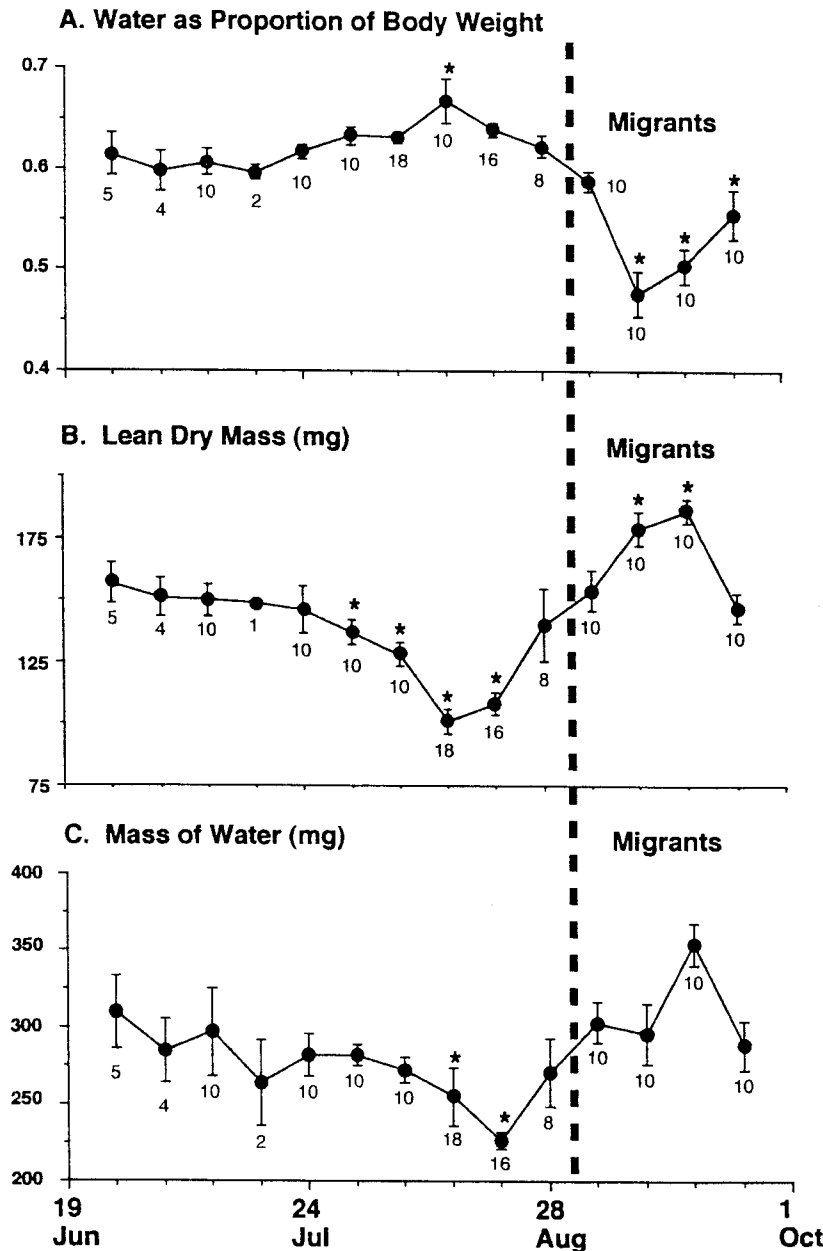


FIGURE 2. Weekly summary of proportional water content, lean dry mass, and total water mass in monarch butterflies in southwest Ohio in 1994. Markers and error bars represent the mean and standard error, with sample size given below the marker. Asterisks above the marker indicate means significantly different from the first week ($\alpha = 0.05$). A. Water content as a proportion of the body weight. B. Lean dry mass (dry weight minus the weight of the lipid extracted). C. Total mass of water per animal.

from the study animals which froze internally. Reproductive ($n = 51$) and migrant ($n = 20$) butterflies were tested in this manner.

Of all butterflies chilled at subzero temperatures for 24 h, 20 froze and 51 did not. All butterflies which froze died. Of the butterflies which did not freeze, 6 died and 7 were moribund following the 24 h recovery period. Fewer migrants than reproductives froze (2/20 vs 17/51) despite the fact that the migrants experienced significantly lower temperatures (-5.4 ± 0.1 vs $-4.7 \pm 0.1^\circ\text{C}$, $t = -4.49$, $df = 62.9$, $P = 0.0001$). Of those butterflies which avoided freezing, significantly more reproductives

than migrants experienced chilling injury (Table 1). The temperature experienced by reproductives demonstrating chilling injury ($-5.1 \pm 0.1^\circ\text{C}$, $n = 12$) did not differ significantly from that experienced by reproductives which froze ($-5.2 \pm 0.2^\circ\text{C}$, $n = 17$). However, the mean of the temperatures experienced by reproductives which fully recovered ($-4.1 \pm 0.1^\circ\text{C}$, $n = 22$) was significantly higher ($\alpha = 0.05$) than that experienced by reproductives which froze or suffered chilling injury, thus suggesting that these data understate the susceptibility of reproductive migrants to chilling injury at temperatures close to the crystallization temperature.

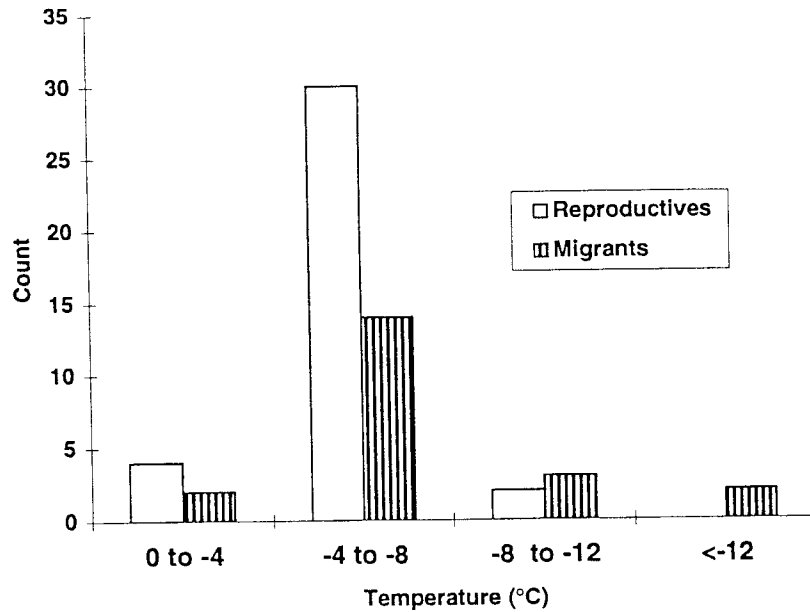


FIGURE 3. Distribution of crystallization temperatures (aka. supercooling point) of reproductive and migrant monarchs captured during the 1994 season. (Difference in mean rank scores: $Q_s = 3.999$, 1 df, $P = 0.065$.)

Osmolarity and potential low molecular weight cryoprotectants

The amount of trehalose detected ranged from 1.0 to 3.5 μ moles per animal (Table 2). No significant differences in plasma trehalose were detected between weeks (1-way ANOVA). Glucose was below our threshold of detection in most butterflies tested, and glycerol was undetectable in all butterflies. Male and female reproductives collected during the week of 14 Aug had an osmolality of 338 ± 8 mOsm (mean \pm standard error), and migrant males and females collected during the weeks of 18 Sep and 25 Sep had plasma osmolalities of 361 ± 30 and 324 ± 24 mOsm. Sample sizes were 6, 5, and 6, respectively, and no significant difference was found between males and females (data not shown).

Temperature data

Microhabitat and ambient temperatures recorded during the 1994 field season were compared to historical records and the developmental status of the butterflies. Temperatures for the 1994 season were obtained hourly, although for clarity only daily high and low temperatures

are plotted on Fig. 4. These were compared to historic record of daily high and low temperatures taken from a more remote location (MCD). Temperatures recorded at the MCD site have tended to be slightly higher than those collected at Miami's ERC (J. Klink, pers. comm.). The hourly data set shows that subzero temperatures were not recorded during the time of our study (ca. 15 Jun–1 Oct), and the historical data set shows that subzero temperatures have not been recorded until 21 Sep and that the average nightly low does not fall below 0°C until after 15 Oct.

We also used thermistors attached to two field data loggers to record microhabitat temperatures between 6 Aug and 11 Oct. One of these loggers was placed on a

TABLE 1. Chill tolerance of reproductive and migrant monarch butterflies

Physiological status*	n	Chilling injury		
		Alive	Moribund	Dead
Reproductive	34	22	6	6
Migrant	18	17	1	0

* Difference in rank mean between rows: $Q_s = 3.992$, 1 df, $P = 0.046$. Butterflies were exposed to sub-zero temperature for 24 h, allowed 24 h to recover, and categorized as 'alive', 'moribund', or 'dead'.

TABLE 2. Seasonal changes in sex ratio and plasma trehalose and osmolality in monarch butterflies

Week	Total plasma trehalose (μ moles)		Plasma osmolality (mOsm)	
	n	mean \pm sem	n	mean \pm sem
Reproductives				
24 Jul	5	2.47 \pm 0.57	–	–
31 Jul	10	2.10 \pm 0.43	–	–
7 Aug	5	2.54 \pm 0.55	–	–
14 Aug	5	2.64 \pm 0.82	6	333 \pm 8
21 Aug	6	3.43 \pm 0.68	–	–
28 Aug	3	0.99 \pm 0.22	–	–
Migrants				
4 Sep	5	2.32 \pm 0.50	–	–
11 Sep	5	2.61 \pm 0.43	–	–
18 Sep	5	–	5	361 \pm 30
25 Sep	5	1.24 \pm 0.22	6	324 \pm 24

Data are pooled observations for animals captured for 7 days starting from the date shown. Dashes indicate that data were not obtained.

50-year and 1994 temperature records, Butler County, Ohio

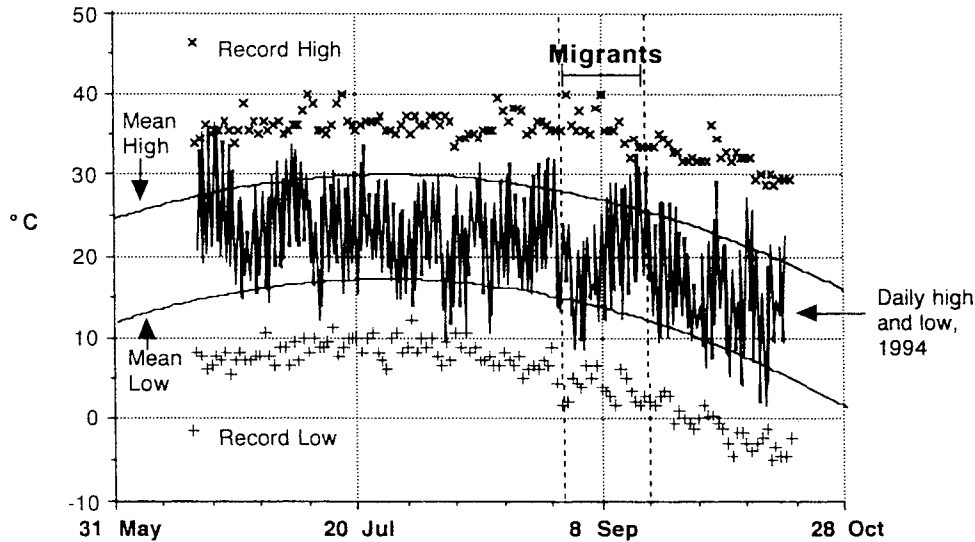


FIGURE 4. Temperatures at the study site during 1994. The \times and $+$ symbols represent the record highs and lows from a 49-year data set for the Miami Conservancy District. The two curved lines are second order polynomials describing mean highs and lows from this data set as a function of julian day (jd) ($^{\circ}\text{C} = -39.20 + 0.68\text{jd} - 0.0016\text{jd}^2$, $r^2 = 0.96$, and $^{\circ}\text{C} = -55.53 + 0.71\text{jd} - 0.0017\text{jd}^2$, $r^2 = 0.98$, respectively). The central line represents daily highs and lows which were extracted from an hourly temperature record from a field meteorology station at the Miami University Ecological Research Center.

goldenrod stalk in a field where foraging was observed, and the other was placed in a tree where nocturnal roosting had been observed in previous seasons. For the sake of analysis, all readings which occurred between 30 minutes before and 29 minutes after the top of the hour were considered to be simultaneous. The temperatures recorded at the tree location averaged $0.8 \pm 0.1^{\circ}\text{C}$ less than those at the ERC ($n = 1588$), and the field location averaged $0.6 \pm 0.1^{\circ}\text{C}$ less ($n = 1588$). The daily lows averaged $0.9 \pm 0.4^{\circ}\text{C}$ less at the tree locations ($n = 67$) and $4.8 \pm 0.5^{\circ}\text{C}$ less at the field location ($n = 67$). The first subzero temperature was recorded in the field on 6 Oct, and in the tree on 10 Oct. The lowest temperature observed from any instrument was -2.3°C , collected from the field logger on 10 Oct.

DISCUSSION

This is the first study comparing cold hardiness between reproductive and migrant monarch butterflies in their summer range in eastern North America. Previous studies have examined cold hardiness in overwintering and reproductive butterflies in Mexico and Florida (Anderson and Brower, 1993), or laboratory acclimated migrant butterflies captured in southwest Ohio (Larsen and Lee, 1994). In this study we compared crystallization temperatures of reproductive and migrant monarchs immediately after capture in southwest Ohio. We also compared chill tolerance by exposing reproductive and migrant monarchs to constant, extended subzero temperatures while monitoring their body temperatures with thermocouples. In this way we were able to positively determine whether each animal had frozen internally, and

to compare the actual temperature experienced by each animal. Using this approach, we showed that reproductives which do not freeze may show chilling injury, but that chilling injury is extremely rare in migrants. A previous study in which chilling injury was examined in overwintering monarchs in Mexico concluded that freezing, not chilling, is the lethal event (Anderson and Brower, 1993). This was also observed in laboratory-acclimated migrants captured in Southwest Ohio (Larsen and Lee, 1994). The present study indicates that, while this is true for the freshly captured migrants in our region, the summer reproductive monarchs in southwest Ohio are potentially susceptible to chilling injury.

As in other studies, we found lower crystallization temperatures and greater supercooling in migrants than in reproductives. However, the distribution of crystallization temperatures we observed differed from those published for overwintering migrants from Mexico. Data from the Mexican overwintering site indicate an approximately normal distribution with a mean of around -8°C (Anderson and Brower, 1993). This was also the approximate mean crystallization temperature of Ohio migrants held in a 4°C cold room for 6–8 weeks prior to testing in a previous study (Larsen and Lee, 1994). We found a higher mean crystallization temperature in freshly captured Ohio migrants ($-6.7 \pm 0.6^{\circ}\text{C}$, $n = 21$), similar to crystallization temperatures observed in spring migrants and slightly higher than those observed in autumn migrants in Florida (Anderson and Brower, 1993). In contrast, no sub- 0°C temperatures were recorded during the period butterflies were captured, and the lowest reading measured was -2.3°C , on 10 Oct. And this low reading came from an unshielded thermistor in an open field,

which would presumably be more exposed to radiational cooling than monarchs, which would usually roost in trees or shrubs. Thus comparison of the laboratory and field data suggest that few of the butterflies we observed in late summer/early autumn 1994 experienced temperatures likely to cause internal freezing, even in reproductives.

One might question whether the higher crystallization temperature (compared to previous studies) we observed in migrants was due to testing a mixed population of reproductive and migrant butterflies. Our distinction between reproductive and migrant monarch butterflies was based on whole-body lipid content of both sexes, and on the mated status of females as indicated by the presence or absence of spermatophores in the bursa copulatrix. Previous studies of the monarch have shown that migrants have far more lipid than reproductives (Beall, 1948; Brown and Chippendale, 1974). Investigations of other Lepidoptera have indicated that virgin females, as indicated by the lack of a spermatophore in the bursa, are extremely rare in reproductively active populations (Burns, 1968), and this finding has been used to examine reproductive activity in previous monarch studies (e.g. Leong *et al.*, 1995). We showed that, after a brief transitional period in late August all females captured in September lacked a spermatophore, and that the proportional lipid content of the migrants as a group was significantly greater than that of the reproductives. These observations indicate that the animals we tested in September had the physiological attributes of migrants, and that the variability in our crystallization temperature data was not due to inadvertently testing a mixed group of reproductives and migrants.

One-third of the unfrozen reproductives subjected to subzero temperatures for 24 h exhibited chilling injury, whereas only 1 of the 17 unfrozen migrants was injured. This increased chill tolerance in migrants compared to reproductives raises two questions: 1) were changes in levels of low molecular weight cryoprotectants involved? and 2) was this difference due to difference in physiological status between migrants and reproductives, to cold acclimation induced by early fall cooling, or a combination of these factors?

We were unable to show a significant difference between the plasma concentration of reproductive and migrant monarchs for any specific low weight cryoprotectant for which we tested for, or for plasma osmolality. However, the trend we observed in total trehalose was consistent with that observed for lipid content and total dry weight (i.e. amounts dropped among late reproductives and increased again among migrants), and the trehalose concentration suggested by our data (average = $2.3 \mu\text{moles}/62 \mu\text{l} = 37.1 \text{ mM}$) is consistent with estimates of trehalose concentration in other insects (Wyatt, 1967). Thus the lack of significant differences among trehalose measurements probably reflect actual population variability rather than technical imprecision.

Trehalose, as well as glucose, glycerol, and other pro-

ducts or intermediates in the glycolytic pathway, can serve as low molecular weight cryoprotectants (Lee, 1991). But this does not appear to have been a factor in the difference in chill tolerance we observed between reproductive and migrant butterflies. Relatively small increases in low molecular weight cryoprotectants have been associated with increased chill tolerance in some insects; e.g. in the case of the flesh fly *Sarcophaga crassipalpis*, a substantial increase in chill tolerance was accompanied by a ca. 40 mM increase in plasma glycerol (Chen *et al.*, 1987). Any substantial increase in the concentration of low molecular weight cryoprotectants would be accompanied by a corresponding increase in osmolality. Given the variances in our data there was indeed a 40 mM difference in trehalose and in plasma osmolality among individuals. However, these differences were as great within reproductive or migrant butterflies as between the two cohorts. It seems unlikely that low molecular weight cryoprotectants are involved in the difference we observed in chill tolerance between reproductive and migrant butterflies, although we cannot rule out the possibility.

Two lines of evidence indicate that cold acclimation probably had little to do with the increased cold hardiness we observed with the changeover from reproductive to migrant cohorts. One of these arguments is that the butterflies we observed weren't exposed to temperatures which have been shown to cause cold acclimation in insects in the laboratory. A variety of *in vitro* and *in vivo* studies show that the activity of insect glycogen phosphorylase increases below $+5^\circ\text{C}$ (Childress and Sacktor, 1970; Ziegler *et al.*, 1979; Chen and Denlinger, 1990), and that, in some insects, periods ranging from hours to weeks at such temperatures is required to increase chill tolerance (Lee, 1991). The monarch butterfly has previously been shown to increase chill tolerance in response to both brief and prolonged exposure to 4°C (Larsen and Lee, 1994). However, examination of data from the field meteorology station and the original data from the tree data logger showed that neither ambient nor microhabitat temperatures of $\leq 5^\circ\text{C}$ were recorded prior to 29 Sep (our last collection date). It could be argued that the butterflies may have been acclimated due to exposure to colder temperatures farther north. While flight speed is likely to vary greatly during the fall migration, previous analyses indicate an average flight speed of 18 km/h (Gibo and Pallet, 1979) and an average daily time aloft of 5–6 h (Gibo and McCurdy, 1993), suggesting an average daily range of 100 km. This would indicate that butterflies in Oxford, southwest Ohio ($39.5^\circ\text{N } 84.7^\circ\text{W}$), would have been in Ohio 2–3 days and south of 45°N latitude for 5–6 days. Examination of data recorded at 3-h intervals at Cincinnati, Dayton and Toledo indicate no recordings of $\leq 5^\circ\text{C}$ in September 1994, and temperatures below 10°C were only recorded on 7 of the 30 nights. Thirty year normal temperature records indicate that normal lows of 5°C or less aren't found in September in Ohio or Michigan south of 45°N

latitude. While one might object that the meteorology station readings don't properly reflect microhabitat temperature, the unshielded thermistor we placed in an open meadow was more exposed to radiational cooling than typical monarch roosts. This field data logger gave low readings which averaged 5°C less than those recorded by the thermistor placed in a previous monarch roost and by the ERC field meteorological station. Despite these lower readings, the logger placed in the open field recorded few readings $\leq 5^\circ\text{C}$ and none $\leq 0^\circ\text{C}$ during Sep 1994. These observations indicate that the monarchs we observed in September were not exposed to temperatures likely to promote cold acclimation, and would not be exposed to such temperatures in most years.

The other line of evidence that cold acclimation did not occur in the monarchs we observed is that greater supercooling ability was previously observed in overwintering monarchs in Mexico (Anderson and Brower, 1993) and Ohio migrants acclimated in a cold room (Larsen and Lee, 1994), indicating that migrant and overwintering monarchs are capable of greater cold hardening than what we observed from the freshly-caught Ohio migrants. These data suggest that the monarchs of the overwintering cohort from eastern North America rely primarily on avoiding temperatures below 0°C in the northern portion of their range rather than increasing their cold hardiness, and that much cold hardening occurs at the overwintering site.

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