

Cold tolerance of the pupae in relation to the distribution of swallowtail butterflies

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A steep decline in the diversity of swallowtail butterfly species at high latitudes could be due to limited cold tolerance of overwintering pupae. If this is so, species with unusually northerly distributions should be unusually cold tolerant. We compared the northerly distributed *Papilio canadensis* with its southern relative, *P. glaucus*. Pupae were exposed for 2–5 months to four acclimatization treatments: outdoors in Alaska, outdoors in Michigan, constant 5°C, and constant –25°C. Field temperatures encountered by pupae in Alaska were lower than in Michigan. The supercooling point of *P. glaucus* pupae was unaffected by acclimatization (mean \pm SE = $-23.5 \pm 0.52^\circ\text{C}$). The supercooling point of *P. canadensis* pupae did not differ from that of *P. glaucus* pupae, except following acclimatization in Alaska, when it dropped to $-27.0 \pm 0.55^\circ\text{C}$. Survival of pupae in Michigan was high for all populations (70–90%); in Alaska, survival of *P. canadensis* was just as high, but survival of *P. glaucus* dropped to 14%. Freezing was usually fatal in both species, but death was not immediate. No pupae survived 6 weeks at –25°C. Trehalose was the most conspicuous metabolite revealed by nuclear magnetic resonance spectroscopy of live pupae and hemolymph. Labelled glucose was metabolized differently by the two species, which may underly the difference in acclimation potential and cold tolerance. The results support the hypothesis that winter temperatures limit swallowtail distributions.

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La brusque chute de la diversité des queues d'hirondelle aux latitudes élevées s'explique peut-être par la faible tolérance des chrysalides au froid durant l'hiver. Dans ce cas, on peut penser que les espèces de queues d'hirondelle qui ont des répartitions particulièrement nordiques sont plus tolérantes au froid. Nous avons comparé *Papilio canadensis*, à répartition plutôt nordique, à son cousin plus austral, *P. glaucus*. Des chrysalides ont été exposées pendant 2–5 mois à quatre conditions d'acclimatation : dehors en Alaska, dehors au Michigan, température constante de 5°C, température constante de –25°C. Les chrysalides gardées en Alaska étaient soumises à des températures plus froides que les chrysalides gardées au Michigan. Le point de surfusion des chrysalides de *P. glaucus* restait inchangé après acclimatation (moyenne \pm écart type = $-23,5 \pm 0,52^\circ\text{C}$). Le point de surfusion des chrysalides de *P. canadensis* ne différait pas de celui de *P. glaucus*, sauf après acclimatation en Alaska où il a baissé jusqu'à $-27,0 \pm 0,55^\circ\text{C}$. La survie des chrysalides au Michigan a été élevée chez toutes les populations (70–90%); en Alaska, la survie de *P. canadensis* était tout aussi élevée, mais la survie de *P. glaucus* a baissé jusqu'à 14%. Le gel a été fatal aux deux espèces, mais la mort n'était pas immédiate. Aucune chrysalide n'a survécu 6 semaines à –25°C. La spectroscopie de résonance magnétique nucléaire a révélé que le tréhalose était le métabolite le plus évident chez les chrysalides vivantes et dans l'hémolymphe. Du glucose marqué s'est avéré métabolisé différemment chez les deux espèces, ce qui explique peut-être la différence entre le potentiel d'acclimatation et la tolérance au froid chez les deux espèces. Les résultats supportent l'hypothèse selon laquelle les températures d'hiver régissent la répartition des queues d'hirondelle.

[Traduit par la rédaction]

Introduction

The family Papilionidae (Lepidoptera) is diverse in the tropics but poorly represented at high latitudes. The northern tiger swallowtail, *Papilio canadensis* R. & J., is extraordinary in maintaining a distribution that extends as far north as the arctic circle in interior Alaska. Of the 508 described species of Papilioninae, only *P. canadensis* and the distantly related *Papilio machaon* L. are reported to occur north of latitude 60° (Scriber 1973, 1984). The ability of *Papilio* pupae to tolerate winter temperature extremes may play a role in defining distribution limits. This hypothesis represents an alternative to explanations based on host-plant distributions (Scriber 1982, 1984; Scriber et al. 1991; Ayres et al. 1991) or summer temperature regimes (Ritland and Scriber 1985). If distributions are determined by

cold tolerance, papilionid taxa with unusually northerly distributions should exhibit an unusual capability to tolerate cold. In fact, *P. machaon*, unlike its more southerly distributed relative *P. xuthus*, is known to be freezing tolerant (Shimado 1988). *P. machaon* pupae can survive freezing at temperatures below –35°C, whereas *P. xuthus* pupae are invariably killed by temperatures below their supercooling point (–20 to –25°C). This pattern supports the ecological importance of cold tolerance. Here we examine the relationship between cold tolerance and distribution in two species within the *Papilio glaucus* species-group: *P. glaucus* L. and *P. canadensis* (formerly *P. glaucus canadensis*; Hagen et al. 1991).

Many aspects of the ecology and evolutionary biology of *P. canadensis* and *P. glaucus* have been investigated, including host

relations (Scriber 1986; Scriber et al. 1991; Ayres et al. 1991), larval temperature responses (Scriber and Lederhouse 1983; Ritland and Scriber 1985), reproductive biology (Lederhouse and Scriber 1987; Lederhouse et al. 1989, 1990), Batesian mimicry (Brower 1958), and sexual selection (Burns 1966; Makielski 1972; Levin 1973). The genetics of some key ecological attributes have been elucidated through studies of a hybrid zone that runs through the Great Lakes region at about 45°N, separating *P. glaucus* to the south from *P. canadensis* to the north (Scriber 1987, 1988; Hagen and Scriber 1989; Hagen 1990). The multivoltine *P. glaucus* enters pupal diapause when cued by short-day photoperiods (Rockey et al. 1987a), but the univoltine *P. canadensis* carries an "obligate diapause" allele on the X chromosome (Rockey et al. 1987b; Hagen and Scriber 1989) which induces pupal diapause regardless of photoperiod. Other differences between the species include the ability to feed on quaking aspen (lacking in *P. glaucus*; Lindroth et al. 1988), the ability to feed on tulip tree (lacking in *P. canadensis*; Lindroth et al. 1986), and the expression of a mimetic dark morph in the adult males (suppressed by an X-linked allele in *P. canadensis*; Clark and Sheppard 1962; Hagen and Scriber 1989). We know of no studies investigating the cold hardiness of *P. glaucus* or *P. canadensis*.

Our study compared pupae from two *P. canadensis* populations (Alaska and Michigan) and one *P. glaucus* population (Georgia), experimentally exposed to Alaska field conditions, Michigan field conditions, and two laboratory acclimation treatments. We had three objectives: (i) to provide a description of low-temperature pupal physiology in *P. glaucus* and *P. canadensis*, including a characterization of supercooling points, lower lethal temperatures, cryoprotectants, and capacity for acclimatization; (ii) to assess genetic variability and phenotypic plasticity in the cold hardiness of tiger swallowtail pupae; and (iii) to test the importance of winter temperatures in defining their distribution limits. No differences between the species would suggest that the cold hardiness of pupae is of little importance in determining their distribution. Alternatively, enhanced cold hardiness in *P. canadensis* pupae may have permitted the northern radiation of this species. Patterns at the population level allow additional inferences. The absence of any differences between *P. canadensis* populations separated by 4000 km and encountering quite different thermal regimes would suggest that cold hardiness is an evolutionarily conservative trait.

Materials and methods

Insect collection and rearing

Adult *P. canadensis* females were collected from the Upper Peninsula of Michigan (MI, Gogebic and Iron counties) and from the vicinity of Fairbanks, Alaska (AK), during June 1988. Their progeny were reared simultaneously under identical conditions in our Alaska laboratory (24°C, photoperiod of 18 h L : 6 h D). Similarly, *P. glaucus* females were collected from southern Georgia (GA) in July 1988, and their progeny were reared under the same conditions in our Michigan laboratory, except for a diapause-inducing short-day photoperiod (10 h L : 14 h D) (Rockey et al. 1987a). Pupae from all three populations were sexed, weighed, and held in darkness at 5°C from the time of pupation (August and September 1988) until early January 1989, when they were allocated to one of four treatments: Alaska field site, Michigan field site, 5°C dark, and -25°C dark (35 *P. canadensis* AK, 35 *P. canadensis* MI, and 22 *P. glaucus* GA to both field sites; 15 *P. canadensis* AK, 15 *P. canadensis* MI, and 10 *P. glaucus* GA to both laboratory acclimations; 264 pupae in total). *Papilio canadensis* treatment groups contained equal numbers of male and female pupae drawn from 8–10 families per population; *P. glaucus* treatment groups contained male pupae only, drawn from 29 families.

Field sites, climatic monitoring, and laboratory acclimations

The field sites for overwintering pupae were in the arboretum of the University of Alaska Fairbanks (AK) and on the campus of Michigan State University (MI). Pupae were individually enclosed in mesh bags (5 × 5 cm) sewn from "no-see-um" netting (0.8 mm mesh). At each site, pupae were placed on the ground in a screen box (75 × 75 × 5 cm wooden frame, sealed on the top and bottom with 5 mm mesh wire screen). The mesh bags and screened box minimized interference with natural light and temperature regimes, afforded protection from predators, and allowed us to label the pupae individually. Pupae were under natural snow cover (30–60 cm until late April in Alaska, 2–3 cm for 3 d in Michigan).

Temperature and snow cover were monitored at each field site from the time pupae were placed there (7–13 January 1989) until the last adult eclosion (21 May in Michigan, 30 June in Alaska). In Michigan, daily maximum and minimum temperatures were recorded at ground level and 1 m above ground. In Alaska, thermocouples were fixed at ground level and 1 m, and temperatures were recorded each morning. (We were unable to record daily maxima and minima at this site, but diurnal temperature fluctuations tend to be modest during January and February in Fairbanks, especially beneath the snow.) Air temperatures and snow cover at the Alaska field site approximated those recorded by the National Weather Service at a nearby station (2 km distant). Temperatures at the Michigan field site tended to be slightly higher than at local National Weather Service stations, probably due to heat escape and reflective insolation from adjacent greenhouses.

At the same time as field experiments were initiated (13 January 1989), pupae from each population were allocated to constant-temperature laboratory treatments of 5°C and -25°C in darkness.

Supercooling points

On 22 February 1989, 42 of the Alaska pupae were excavated from the snow and shipped overnight to East Lansing, Michigan (20 h in transit). At the same time, 42 of the Michigan field pupae, 17 of the 5°C pupae, and 17 of the -25°C pupae were removed from their treatments, and, like the travelling pupae, were exposed to 20 h at room-temperature. Upon receipt of the pupae from Alaska, pupae from both sites were stored together at 5°C in darkness until after the supercooling point measurements. Supercooling points of 73 of these pupae (3 populations × 4 treatments × 4–8 pupae) were measured on 24–25 February 1989 (40–65 h after their removal from the acclimation treatments), using a Sensortek BAT-12 telethermometer wired to a chart recorder through a four-line channelyler. The temperature of the pupae was monitored constantly (MT-29/1B probes taped to the pupal exoskeleton) while their temperature was lowered at the rate of 1°C · min⁻¹ (Cole–Parmer digital refrigerated bath). The supercooling point (SCP) (= crystallization temperature, T_c) was detected as the heat of fusion released due to the freezing of the pupal body.

Following the supercooling point measurements, 61 pupae (3 populations × field treatments × 7–10 pupae, plus 10 pupae from the -25°C treatment) were transported on ice to Columbus, Ohio, where they became available for nuclear magnetic resonance (NMR) studies as described below; 23 of these pupae were not included in the supercooling point measurements (i.e., were not frozen in our laboratory). The remaining pupae that had been at the Alaska field site (14), along with 19 of the pupae from the 5°C treatment and 14 from the -25°C treatment, were shipped to Alaska and reunited with the pupae beneath the snow in Alaska. Similarly, all pupae that had been at the Michigan field site (14), along with 19 pupae from the 5°C treatment and 14 from the -25°C treatment, were reunited with the pupae overwintering outdoors in Michigan. (Nine pupae were misplaced and therefore lost from the experiment.) These transfers were completed by 9 March 1989. On the various occasions when pupae were moved from laboratory to field and vice versa, they were never exposed to air temperatures of less than -10°C, and therefore should not have experienced undue cold stress during transfer. When it was necessary to disturb pupae beneath the snow, the snow was carefully replaced to approximate the depth and compaction of surrounding undisturbed snow.

Pupal respiration

Pupal respiration rates were measured in the autumn (19 October 1988), spring (18 April 1989), and midwinter (15 December 1989). After 1 h of equilibration at 24°C, individual pupae were enclosed in airtight 236-mL flasks (8-oz jelly jars) and held at 20–30 h at 24°C. The elevation in CO₂ concentration above ambient was measured using a gas chromatograph (Analytical Instrument Development Inc. model 512, Avondale, Pa.). Ambient CO₂ (estimated from control flasks without pupae) remained constant within and across experiments at 0.03%. Final CO₂ concentrations typically ranged from 0.30 to 1.00%. Respiration rates measured in this way were very similar to those collected from a subset of animals by means of a Scholander microrespirometer (Mark Co., Brockton, Mass.) that measured the rate of O₂ uptake. Measurement of autumn respiration included *P. canadensis* pupae from Alaska and Michigan that were at 5°C in darkness immediately prior to the measurements. Spring respiration measurements were made on all pupae overwintering in Alaska (*N* = 97), which were outdoors beneath 30 cm of snow just prior to the measurements. A subset of pupae was weighed in August, October, and again in April to quantify mass loss over the winter. Midwinter respiration measurements were made on pupae reared the following summer (using same rearing protocol as in 1988) and held at 5°C in darkness prior to the measurements.

Cold tolerance, postdiapause development, and eclosion success

The relative cold tolerance of the three populations exposed to the four treatments was assessed in terms of their ability to supercool and their survival to eclosion. We were able to categorize pre-eclosion mortalities as occurring during the winter (low or unmeasurable respiration on 18 April 1989), early in postdiapause development (normal respiration on 18 April, but no recognizable development of imaginal structures), or late in postdiapause development (normal respiration on 18 April, development of imaginal form, and a weakening of the pupal exoskeleton along the lines of ecdysial separation). The extent of imaginal development was determined by dissection about 2 weeks after the last butterfly emergence; most determinations were clear, indicating that death had occurred either early in postdiapause development or just prior to eclosion. A two-way fixed-factor ANOVA was used to test for effects of population and acclimatization treatment on supercooling point (General Linear Model, SAS Institute Inc. 1985). Differences in the eclosion success of three populations overwintering at two field sites were tested using a multiway contingency analysis (CATMOD procedure, linear model, weighted least squares estimator, SAS Institute Inc. 1985).

Metabolic studies using NMR spectroscopy Instrumentation and materials

¹H-decoupled ¹³C NMR spectra of pupae and hemolymph were obtained on a Bruker AM-500 and an MSL-300 Fourier-transform spectrometer, each equipped with a pulse programmer and quadrature phase detection. Spectra were obtained with ~70° pulse widths and a recycle time (acquisition + relaxation delay) of 1 s; 16–32K real points were obtained over a 0–190 ppm spectral width. Chemical shifts are reported in parts per million relative to the C1 signal of deuteriochloroform. Labelled glucose, D-[1-¹³C]glucose (99 at. % ¹³C), was obtained from Omicron Inc. (Chemistry Department, University of Notre Dame, Notre Dame, Ind.). All spectra were collected at a probe temperature of 25°C.

Natural abundance ¹³C NMR of live pupae and hemolymph

¹³C NMR spectra were obtained on live unfrozen pupae within 10-mm NMR tubes inserted within the 300-MHz spectrometer. Hemolymph was bled from each pupa by puncturing its abdomen with an insect pin. ¹³C NMR spectra of the hemolymph were obtained with the 500-MHz spectrometer, using a 5-mm NMR tube. Spectra were compared with the standard chemical shifts obtained by Kukal et al. (1988).

The major comparison was between *P. canadensis* AK and *P. glaucus* GA. A further experiment tested for the effect of short-term acclimation: spectra from all three populations were compared after 10–14 days at 5 and 25°C. Spectra were collected from two to five

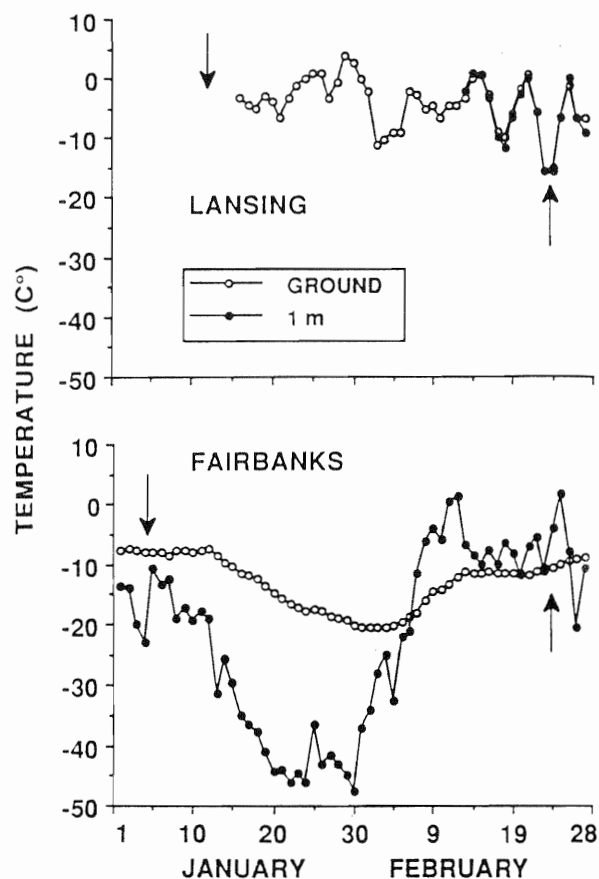


FIG. 1. Ambient temperatures at ground level and 1 m above ground recorded in East Lansing, Michigan, and Fairbanks, Alaska, during January and February 1989. Temperatures measured at ground level correspond to the body temperatures of overwintering pupae. Arrows in January indicate when the acclimatization treatments were begun, and arrows in February indicate when supercooling points were measured. For Lansing, temperatures are daily minima; for Fairbanks, temperatures represent midmorning observations.

individuals *in vivo* prior to the collection of corresponding spectra of their hemolymph.

¹³C NMR of pupae injected with labelled glucose

The lifetimes of metabolic intermediates were compared in pupae of *P. canadensis* AK and *P. glaucus* GA. Two pupae of each species were injected (50-μL Hamilton syringe) with 10 μL of 1 M labelled glucose (D-[1-¹³C]glucose) into their abdomen. After the wound annealed, pupae were inserted into a 10-mm NMR tube, and ¹³C spectra were obtained as a function of time, using an automated NMR pulse program on the 300-MHz spectrometer. The spectra were collected for 2 h each over a 16- to 24-h period.

Results

Microclimate

Fairbanks, Alaska, encountered an unusually sustained bout of cold weather during the period of pupal acclimatization in January and February 1989 (Fig. 1). Air temperatures remained at -35°C or less for 16 consecutive days, reaching an extreme of -48°C on 30 January. The maximum air temperature during the 2-month period was 1°C. The temperatures actually encountered by the pupae, insulated by ~50 cm of snow, ranged from -21 to -7°C, with an average of -13°C (Fig. 1). Pupae encountered 19 consecutive days at -15°C or lower, and 7 consecutive days at -20°C.

Pupae overwintering at the same time in Michigan were

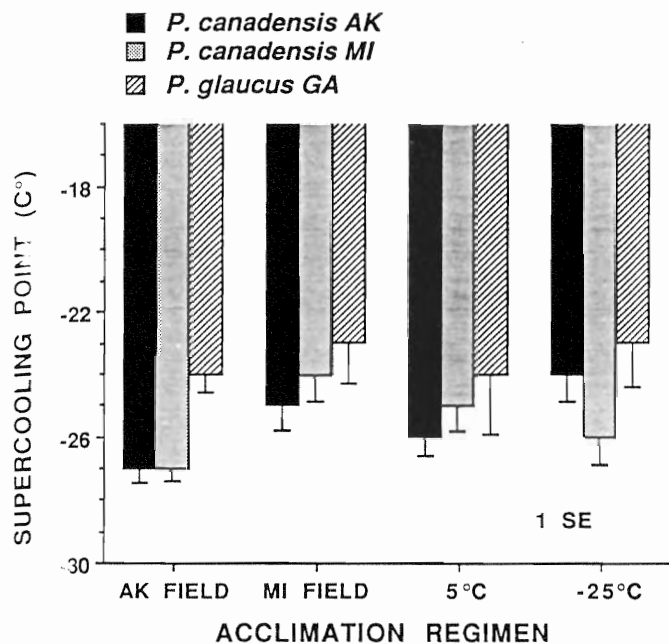


FIG. 2. Supercooling points (= crystallization temperatures) of pupae from three populations following 6 weeks of exposure to Alaska field conditions, Michigan field conditions, constant 5°C, or constant -25°C. From left to right, $N = 8, 8, 5; 7, 9, 5; 5, 6, 4$; and $6, 6, 4$.

exposed to a very different thermal regime (Fig. 1). There was no snow cover and the litter-layer temperatures encountered by the pupae were identical with the aboveground air temperatures. This greatly increased the variability of temperatures experienced by the pupae. The average pupal temperature was much higher in Michigan than Alaska (8°C compared with -13°C), but the minimum temperatures were not so disparate (-16°C in Michigan compared with -21°C in Alaska; see Fig. 1). The highest daily maximum temperature in Michigan was 15°C (Fig. 1 shows daily minima). The average difference between the daily maximum and minimum temperature was 8°C.

Supercooling capacity

Mean supercooling points ranged from -22.9°C for *P. glaucus* exposed to Michigan field conditions to -27.1°C for *P. canadensis* AK exposed to Alaska field conditions (Fig. 2). *Papilio canadensis* supercooling points tended to be significantly lower overall: least squares mean \pm SE = -25.4 ± 0.44 , -25.2 ± 0.42 , and -23.5 ± 0.52 °C for *P. canadensis* AK, *P. canadensis* MI, and *P. glaucus* GA, respectively; $F_{[2,61]} = 4.92$, $P = 0.010$, ANOVA population effect; the population \times treatment interaction was nonsignificant. Supercooling points tended to be lowest following acclimatization in Alaska (least squares mean \pm SE = -25.9 ± 0.49 vs. -23.9 ± 0.50 °C for Alaska and Michigan field treatments, respectively; $F_{[3,61]} = 2.9$, $P = 0.040$, ANOVA treatment effect). Laboratory acclimations were intermediate (least squares mean \pm SE = -24.8 ± 0.58 and -24.3 ± 0.56 °C for the 5 and -25°C treatments, respectively). In response to the Alaska field treatment, both *P. canadensis* populations lowered their supercooling point by 2–3°: -27.1 vs. -24.9°C for *P. canadensis* AK ($P = 0.068$) and -26.9 vs. -23.9°C for *P. canadensis* MI ($P = 0.0057$). However, the *P. glaucus* population exhibited no such acclimatization response: -23.6 vs. -22.9°C ($P = 0.58$).

The variability in supercooling points was generally low. The standard deviation within treatments averaged 2.20°C (= ANOVA

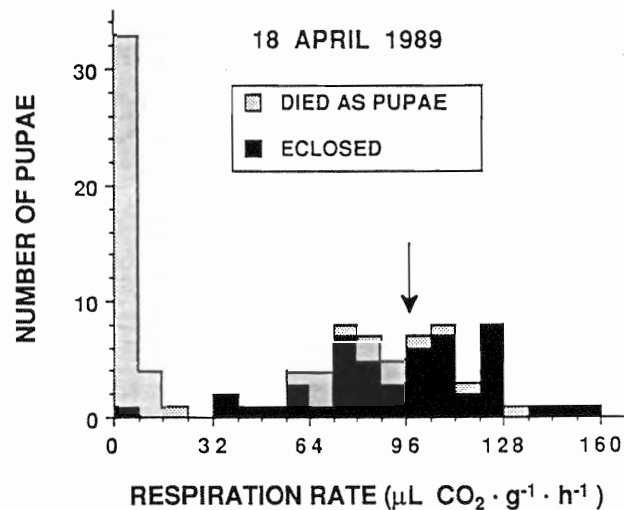


FIG. 3. Spring respiration rates of pupae at the Alaska field site ($N = 97$). The frequency distribution of animals that died as pupae was stacked onto the distribution of animals that survived to produce adults (eclosed). The arrow indicates the mean respiration rate of pupae that produced adults (mean \pm SD = $98 \pm 31 \mu\text{L CO}_2 \cdot \text{g}^{-1} \cdot \text{h}^{-1}$, $N = 48$).

TABLE 1. Pupal respiration rates (at 24°C) of *P. canadensis* populations from Alaska and Michigan in the autumn, spring, and midwinter

	Pupal respiration rate ($\mu\text{L CO}_2 \cdot \text{g}^{-1} \cdot \text{h}^{-1}$)		
	Mean	SD	N
19 October 1988			
Alaska	36*	17	56
Michigan	28*	19	32
18 April 1989 ^a			
Alaska	111**	33	20
Michigan	87**	27	24
15 December 1989			
Alaska	67***	34	56
Michigan	45***	22	42

NOTE: *, $t = 2.01$, $df = 86$, $P < 0.05$; **, $t = 2.71$, $df = 42$, $P < 0.01$; ***, $t = 3.66$, $df = 96$, $P < 0.001$.

^aIncludes only pupae that subsequently emerged (see Fig. 3).

MSE^{0.5}; data in Fig. 2). The variance in *P. canadensis* supercooling points appeared to decline following acclimatization in Alaska: SD in Alaska = 1.26°C (range -23.7 to -28.2°C; $N = 16$); SD in Michigan = 2.49°C (range -19.4 to -27.7°C; $N = 16$); $F_{[15,5]} = 3.09$, $P = 0.02$. The standard deviation of *P. glaucus* supercooling points was comparable to that of *P. canadensis* MI pupae: SD = 2.62°C (range -18.3 to -27.9°C; $N = 14$; AK, MI, and 5°C treatments combined). There was no evidence for a reduction in the variance of *P. glaucus* supercooling points following acclimatization in Alaska, but sample sizes were too small for meaningful comparisons to be made of the variance within treatments.

Papilio canadensis sexes did not differ in their supercooling points ($F_{[1,54]} = 0.37$, $P = 0.54$, ANOVA sex effect in a three-way

TABLE 2. Survival in Alaska of pupae from Alaska (AK), Michigan (MI), and Georgia (GA) subjected to three acclimatization treatments

Treatment	Total no. of pupae	Respiring on 18 April		Postdiapause development		Successful emergence	
		<i>N</i>	%	<i>N</i>	%	<i>N</i>	%
<i>P. canadensis</i> AK							
AK field	24	21	88	18	75	16	67
AK field SC	4	4	100	3	75	1	25
5°C	3	3	100	3	100	3	100
5°C SC	3	0	0	0	0	0	0
-25°C	4	0	0	0	0	0	0
-25°C SC	2	0	0	0	0	0	0
<i>P. canadensis</i> MI							
AK field	21	19	90	20	95	19	90
AK field SC	2	2	100	2	100	1	50
5°C	4	4	100	4	100	4	100
5°C SC	3	1	33	0	0	0	0
-25°C	5	0	0	0	0	0	0
-25°C SC	1	0	0	0	0	0	0
<i>P. glaucus</i> GA							
AK field	13	1	8	2	15	1	8
AK field SC	—	—	—	—	—	—	—
5°C	4	3	75	3	75	3	75
5°C SC	2	1	50	0	0	0	0
-25°C	2	1	50	0	0	0	0
-25°C SC	—	—	—	—	—	—	—

NOTE: N, number of pupae. Emergence occurred from 4 to 30 June 1989. SC, pupae supercooled until they froze on 24 February.

model that also included population effects and acclimatization treatments; *P. glaucus* GA pupae were exclusively male and therefore were omitted from this model; all interactions were nonsignificant).

Pupal respiration, postdiapause development, and eclosion success

Respiration rates of *P. canadensis* pupae were lowest in the autumn (18 October, shortly after the induction of diapause), intermediate during midwinter (15 December), and highest in the spring (18 April; Table 3). The respiration rates of Alaskan pupae were 28–49% higher than those of Michigan pupae (Table 1). Pupae overwintering in Alaska lost 5–6% of their fresh mass between October 1988 and April 1989: mean \pm SD = $5.28 \pm 1.93\%$ ($N=11$) and $5.79 \pm 1.81\%$ ($N=12$) for *P. canadensis* AK and *P. canadensis* MI; pupal mass in October: mean \pm SD = 713 ± 95 and 721 ± 113 mg for *P. canadensis* AK and *P. canadensis* MI, respectively. Mass loss per month was about 3 times greater from October to April than from August to October: $0.94\%/month$ vs. $0.26\%/month$ (*P. canadensis* AK).

The frequency distribution of respiration rates in April was bimodal (Fig. 3), with one peak at $0 \mu\text{L CO}_2 \cdot \text{g}^{-1} \cdot \text{h}^{-1}$ and another near $100 \mu\text{L CO}_2 \cdot \text{g}^{-1} \cdot \text{h}^{-1}$. With the one exception, none of the 37 pupae with respiration rates $<25 \mu\text{L CO}_2 \cdot \text{g}^{-1} \cdot \text{h}^{-1}$ produced adults or gave evidence of any postdiapause development. In contrast, 47 of 60 pupae that were respiring at $>25 \mu\text{L CO}_2 \cdot \text{g}^{-1} \cdot \text{h}^{-1}$ produced eclosing adults, and 7 of the remaining 14 pupae contained dead imagoes within the pupal exoskeleton when they were retrieved from the field in early July (indicating substantial postdiapause development before death). Thus, pupae that failed to produce adults fell into two categories,

those that died during the winter (low or unmeasurable respiration on 18 April) and those that died during postdiapause development in the spring (respiration $>25 \mu\text{L CO}_2 \cdot \text{g}^{-1} \cdot \text{h}^{-1}$ on 18 April).

Contingency analysis indicated that emergence success varied among populations ($\chi^2 = 34.98$, $df = 2$, $P = 0.0001$) and that overwintering sites affected the populations differently (population \times site interaction: $\chi^2 = 15.76$, $df = 2$, $P = 0.0004$; Fig. 4). Most of the *P. canadensis* pupae from Alaska and Michigan that were acclimatized in Alaska and not supercooled in the laboratory were respiring on 18 April, completed postdiapause development, and successfully emerged as adults (16 of 24 and 19 of 21, respectively; Table 2, Fig. 4). In contrast, most of the *P. glaucus* from Georgia exposed to the same conditions appeared to be dead by 18 April, and only 1 of 13 produced an adult. Most pupae treated at 5°C and not supercooled produced adults (3 of 3, 4 of 4, and 3 of 4 for populations from Alaska, Michigan, and Georgia, respectively). Of those pupae treated at -25°C only one was still respiring on 18 April, and none completed any recognizable postdiapause development or produced an adult. Survival of *P. canadensis* in Michigan was similar to that in Alaska (Fig. 4). However, *P. glaucus* had much higher survival in Michigan than Alaska (9 of 12 vs. 1 of 13 pupae produced adults; Tables 2 and 3). *Papilio canadensis* pupae from Michigan were significantly more likely to produce adults than pupae from Alaska, whether exposed to winter conditions in Michigan or Alaska (Fig. 4; comparison of *P. canadensis* AK and *P. canadensis* MI: $\chi^2 = 5.95$, $P = 0.015$).

Pupae that were frozen in the laboratory usually did not survive. If we exclude the -25°C treatment (which caused 100% mortality), only 4 of 29 frozen pupae produced adults (14%, two

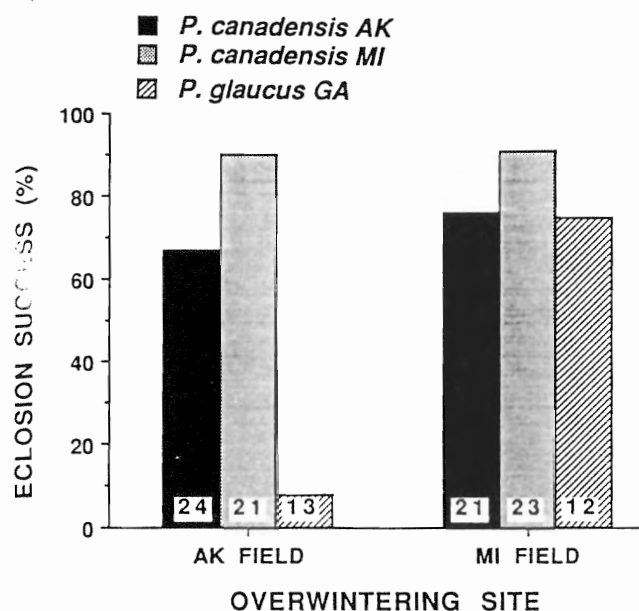


FIG. 4. Eclosion success of *P. glaucus* populations from Alaska, Michigan, and Georgia after overwintering at Alaska and Michigan field sites. See text for corresponding contingency analysis. Values within bars indicate the number of pupae.

TABLE 3. Survival in Michigan of pupae from Alaska (AK), Michigan (MI), and Georgia (GA) subjected to three acclimatization treatments

Treatment	Total no. of pupae	Postdiapause development		Successful emergence	
		N	%	N	%
<i>Papilio canadensis</i> AK					
MI field	21	16	76	16	76
MI field SC	3	0	0	0	0
5°C	5	5	100	4	100
5°C SC	3	2	67	1	33
-25°C	5	0	0	0	0
-25°C SC	—	—	—	—	—
<i>Papilio canadensis</i> MI					
MI field	23	22	96	21	91
MI field SC	5	2	40	1	20
5°C	5	5	100	5	100
5°C SC	2	0	0	0	0
-25°C	4	0	0	0	0
-25°C SC	2	0	0	0	0
<i>Papilio glaucus</i> GA					
MI field	12	10	83	9	75
MI field SC	—	—	—	—	—
5°C	2	2	100	1	50
5°C SC	2	1	50	0	0
-25°C	3	0	0	0	0
-25°C SC	—	—	—	—	—

NOTE: N, number of pupae. Emergence occurred from 3 to 21 May 1989. SC, pupae supercooled until they froze on 24 February.

from each *P. canadensis* population, no *P. glaucus*; Tables 2 and 3). However, death due to freezing was not necessarily immediate. Eight of 14 frozen pupae had normal respiration rates on 18 April (7 weeks after freezing), but only 2 of them successfully eclosed (Table 2). Ten of 29 frozen pupae completed much of their postdiapause development as an imago before dying.

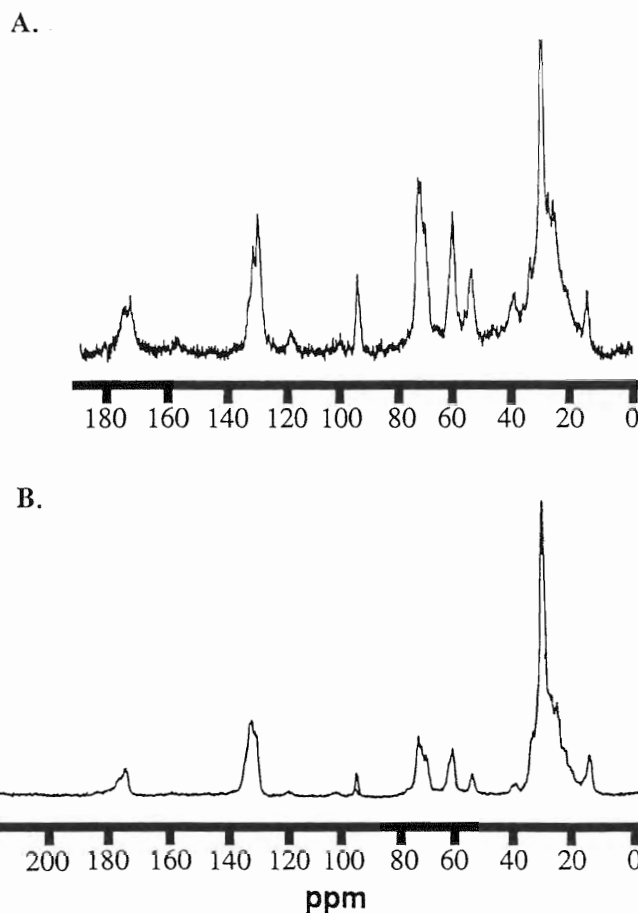


FIG. 5. Representative *in vivo* ^{13}C NMR spectra of diapausing pupae acclimatized in Alaska: *P. canadensis* from Alaska (A) and *P. glaucus* from Georgia (B). Both spectra showed prominent resonances near 30, 130, and 175 ppm which correspond to saturated (C—C) and unsaturated (C=C and C=O) lipids. Trehalose gave rise to the six carbon resonances between 62 and 95 ppm. Glycogen is indicated by a small broad peak at 100 ppm. The peak at 55 ppm may correspond to glutamine.

Natural abundance of metabolites in pupae

In vivo ^{13}C NMR of pupae of different species indicated the presence of a similar set of metabolites (Fig. 5), primarily lipids (resonances near 30, 130, and 175 ppm) and trehalose (95, 74, 73, 71, and 62 ppm). Some glycogen was probably present (101 ppm), and the resonance at 55 ppm may correspond to glutamine. ^{13}C hemolymph spectra also indicated large quantities of trehalose; both *P. canadensis* and *P. glaucus* showed prominent resonances from trehalose between 62 and 95 ppm (Fig. 6; the peaks were more sharply defined with the compounds in solution than *in vivo*). The minor peaks between 20 and 40 ppm arose from contaminant fatty acids suspended in the hemolymph of *P. glaucus* (Fig. 6B).

Influence of acclimation on steady-state metabolites

There were no apparent differences in the steady-state metabolites of warm-acclimated pupae (10–14 days at 25°C) compared with cold-acclimated pupae (10–14 days at 5°C). Major peaks were attributed to lipids and trehalose.

Metabolism of labelled glucose

The two *Papilio* species differed in their metabolism of the glucose precursor. *Papilio canadensis* quickly incorporated

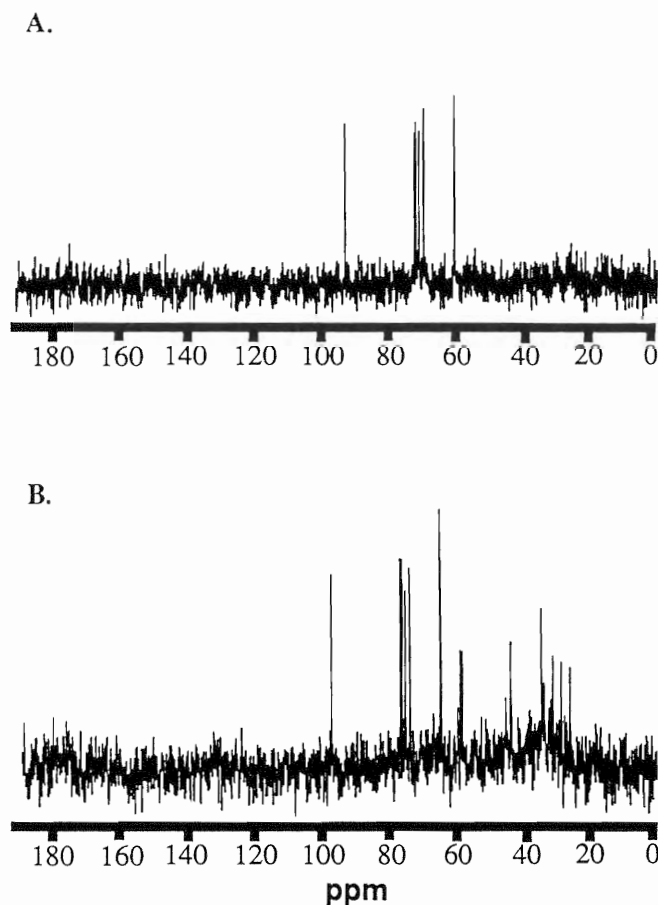


FIG. 6. Representative ^{13}C NMR spectra of pupal hemolymph from *P. canadensis* from Alaska (A) and *P. glaucus* from Georgia (B) after acclimatization in Alaska. Both spectra showed resonances corresponding to trehalose. The *P. glaucus* hemolymph sample also reveals signals from saturated fatty acids resulting from contamination by disaggregated fat body.

much of the label into trehalose (after 16 h at 25°C) and eventually accumulated it into an unidentified compound with a resonance near 63 ppm (Fig. 7A); during the same time *P. glaucus* incorporated much of the C1 label into a different unidentified compound with a resonance near 18 ppm (Fig. 7B). These differences were not absolute, but they were conspicuous. In *P. canadensis*, the signal intensity of the compound at 63 ppm was 97% of that of unsaturated fatty acids (at 30 ppm), compared with only 34% in *P. glaucus*. In *P. glaucus*, the signal intensity of the compound at 18 ppm was 78% of that of unsaturated fatty acids, compared with only 28% in *P. canadensis*. The resonance of the *P. canadensis* unknown corresponds to ethylene glycol, and the resonance of the *P. glaucus* unknown corresponds to alanine.

Because these measurements indicated a surprising difference in glucose metabolism between the species, we repeated the experiments in the fall of 1989 (using pupae from similar stocks, collected and reared as in 1988) and obtained the same results.

Discussion

Physiology of cold tolerance

Papilio canadensis pupae were more cold tolerant than their southern relative, *P. glaucus*. Survival of *P. canadensis* pupae was as high in Alaska as in Michigan (78% vs. 84%). However, survival of *P. glaucus* pupae was only 8% in Alaska compared

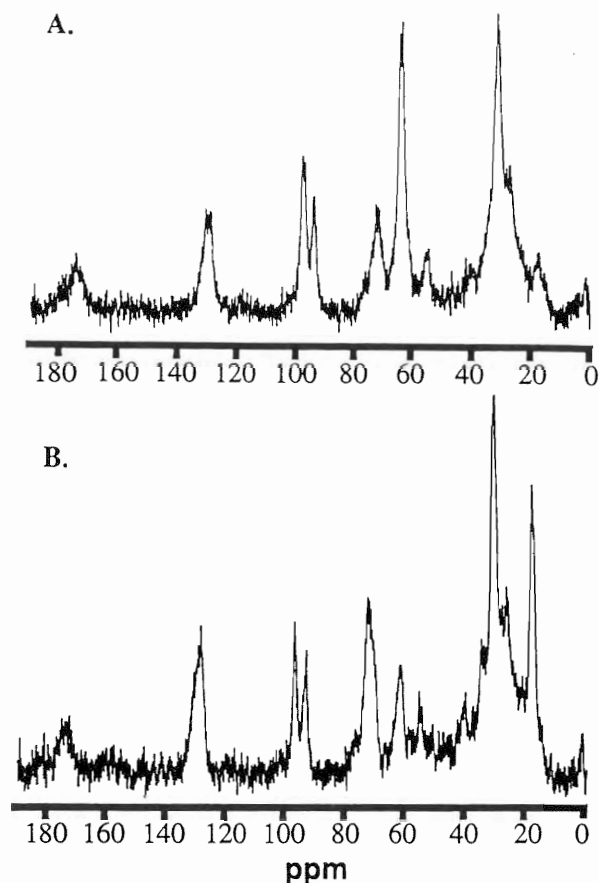


FIG. 7. Representative *in vivo* ^{13}C NMR time-lapse spectra from pupae of *P. canadensis* from Alaska (A) and *P. glaucus* from Georgia (B) injected with $10\ \mu\text{L}$ of labelled glucose. The spectra were collected at 2-h intervals over 24 h. Only the final spectra are shown. The remainder of injected C1-labelled glucose appears at 97 ppm in both spectra, but its metabolic products differed between the species: *P. canadensis* incorporated more of the label into an unidentified compound with a resonance near 63 ppm, whereas *P. glaucus* incorporated more label into a different unidentified compound with a resonance near 18 ppm.

with 75% in Michigan. The high mortality of *P. glaucus* pupae in Alaska may have been due to freezing. We never recorded temperatures beneath the snow as low as the average measured supercooling point of *P. glaucus* pupae (-23.5°C), but temperatures remained at -20°C for 7 consecutive days, and the probability of nucleation (freezing) increases with prolonged exposure (Salt 1958, 1966; Miller 1978, 1984). Like *P. glaucus*, *Cephus cinctus* (Cepidae) had a mean supercooling point of about -24°C when exposed to a high laboratory cooling rate ($\sim 5\%$ of the larvae froze at -20°C or higher); however, 50–70% of the same *Cephus* population were frozen after 7 days at -20°C (Salt 1950, 1958). *Papilio canadensis* pupae acclimatized in Alaska had a mean supercooling point of -27°C , 3.5°C lower than *P. canadensis* acclimatized in Michigan or *P. glaucus* at either site. This physiological adjustment, though seemingly modest, may have allowed *P. canadensis* to avoid freezing and survive in an environment that was too severe for *P. glaucus*.

Differential susceptibility to nonfreezing cryoinjury is an alternative explanation for the differences in survival between *P. canadensis* and *P. glaucus*. In some insect species, substantial mortality may result from prolonged exposure to low but nonfreezing temperatures (Pullin and Bale 1988; Turnock et al.

1983, 1990; Turnock and Bracken 1989). We cannot judge the importance of nonfreezing cryoinjury in *P. glaucus*, but our data suggest that freezing per se is a critical determinant of winter survival in *P. canadensis*. *Papilio canadensis* pupae in Alaska sustained 7 consecutive days at -20°C and 19 consecutive days at $\leq -15^{\circ}\text{C}$, yet 35 of 45 produced healthy adults. A matched set of pupae experienced the same or higher temperatures except for a 5-min excursion to subfreezing temperatures while we measured their supercooling point; only 16% (4/25) of these frozen pupae produced adults (Tables 2 and 3). This difference is highly significant ($\chi^2 = 24.86$, $P < 0.001$). There was a suggestion of nonfreezing cryoinjury in that the eclosion success of *P. canadensis* kept at 5°C tended to be higher than that of *P. canadensis* beneath the snow in Alaska (16/17 (94%) vs. 35/45 (78%), Tables 2 and 3; $P = 0.13$, Fisher's exact test).

Both *P. canadensis* and *P. glaucus* appeared to be intolerant of freezing, and, at least in *P. canadensis*, the supercooling point approximated the lower lethal temperature. Pupae frozen briefly did not usually die immediately, but the tissue damage was sufficient to prevent successful completion of imaginal development. Whether this syndrome of delayed developmental disruption is common in other taxa is unclear, although it has been argued that the distinction between freezing tolerance and freezing sensitivity has been overemphasized (Miller 1978; Baust and Rojas 1985; Bale 1987; Kukal and Duman 1989). Similar delayed effects from nonfreezing cryoinjury have been reported (Turnock et al. 1983, 1990). A few *P. canadensis* pupae (4/25) were able to survive freezing. We would expect occasional extreme winters (low temperatures with shallow snow cover) to exert strong selection of traits that confer freezing tolerance, but the very low frequency of freezing tolerance, even within the Alaska population, argues against the efficacy of such selection. This could be a consequence of stochastic nonheritable variation in the site of nucleation, which affects the pattern of subsequent freezing and tissue damage. Infrared thermography studies of *Manduca sexta* indicate that the site of ice nucleation and the pattern of tissue crystallization can vary among individuals (Block 1990).

Neither *P. canadensis* nor *P. glaucus* exhibited any of the traits typically associated with adaptations to extreme cold, i.e., freezing tolerance (Storey and Storey 1988) or high concentrations of glycerol (Salt 1966; Miller and Werner 1987a, 1987b). *In vivo* NMR identified trehalose as a possible cryoprotectant in *P. canadensis* and *P. glaucus*. Although a cryoprotective function has sometimes been ascribed to trehalose (Morrissey and Baust 1976; Ring and Tesar 1980; Baust and Lee 1981; Crowe et al. 1987), it is an important constituent of pupal hemolymph even in tropical Lepidoptera (Wyatt and Calf 1957), suggesting that its primary role is that of a storage carbohydrate and metabolic intermediate (Kramer et al. 1978). *Papilio canadensis* and *P. glaucus* differed from *P. machaon* and *P. erichsonii* in lacking any measurable glycerol (Shimado 1988).

Papilio canadensis and *P. glaucus* differed in their glucose metabolism, which could underlie the differences in their acclimatization capacity and cold tolerance. From labelled glucose, *P. canadensis* synthesized a compound with a resonance corresponding to that of ethylene glycol, and *P. glaucus* synthesized a compound with a resonance corresponding to that of alanine. Ethylene glycol is thought to function as an effective antifreeze in a beetle (*Ips acuminatus*) inhabiting alpine areas of Norway (Gehrken 1984). Alanine, a byproduct of anaerobiosis, is thought to enhance the freezing tolerance of intertidal bivalves (Loomis et al. 1989). The presumably derived

ability of *P. canadensis* to synthesize ethylene glycol may have permitted its radiation into the boreal forests of North America.

Ecological implications

The differential mortality of *P. canadensis* and *P. glaucus* when exposed to a subarctic winter (Fig. 4) supports the hypothesis that the northern distribution of *P. glaucus* is limited by the cold tolerance of the pupae. This result does not by itself reduce the importance of other factors such as natural enemies, host distribution, and summer temperature regime. The hypothesis could be further tested through a careful analysis of winter weather records at sites on either side of the present distribution (e.g., southern versus central Michigan). Close concordance between the northern limits of *P. glaucus* and the probability of encountering lethal winter temperatures would provide additional support for the hypothesis. Snow cover is a critical determinant of litter-layer temperatures (Whitney 1970; Werner 1978; Turnock et al. 1983) and therefore a necessary parameter. Ideally, such analyses should also incorporate the effects of duration of exposure on the probability of freezing (Salt 1958) and on nonfreezing mortality (Turnock et al. 1983).

By extension, this result supports the more general hypothesis that the distribution of papilionids is commonly constrained by cold tolerance, perhaps resulting in the pattern of reduced diversity at high latitudes. Further tests could be generated through studies of temperate and subtropical relatives of *P. canadensis* and *P. machaon*. If the hypothesis is true, the two northern species should retain their status as species uniquely able to tolerate low winter temperatures, and the northern limits of many southern taxa should coincide with the occurrence of lethal winter temperatures. Similarly, Copeland and Craig (1990), working with mosquitos, Kimura (1988), working with fruit flies, and McClure (1989), working with adelgids and scale insects, argued that the cold tolerance of overwintering life stages was a key determinant of northern distribution limits. Arris and Eagleson (1989) proposed that the boreal-deciduous forest ecotone is maintained in its present location by the limited supercooling ability of many deciduous tree species.

There was only limited evidence for local adaptation of *P. canadensis* populations. Michigan pupae were more likely to survive to eclosion at both sites (Fig. 4). However, Alaskan pupae had substantially higher respiration rates than Michigan pupae (Table 1). Metabolic compensation for cold (a general elevation of the metabolic rate at all temperatures, presumably allowing the necessary metabolic processes to occur at lower environmental temperatures) is thought to be a common attribute of poikilothermic organisms inhabiting polar areas (Scholander et al. 1953; Block and Young 1978; Block 1990).

Both genetic divergence and phenotypic plasticity (acclimatization) contribute to matching the cold tolerance of swallowtail pupae with the winter environment that they encounter. A key adaptation in *P. canadensis* may be the capacity for lowering the supercooling point upon exposure to low temperature (Fig. 2).

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