Chill-Coma Temperature in *Drosophila*: Effects of Developmental Temperature, Latitude, and Phylogeny

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ABSTRACT

We modify and apply a nonlethal technique for rapidly quantifying the cold tolerance of large numbers of *Drosophila* and other small insects. Flies are transferred to individual vials, cooled in groups in progressive 0.5°C steps, and checked for loss of righting response (chill-coma temperature [T_{cc}]). Flies recover quickly when transferred to ambient temperature, and thus this technique potentially can be used in selection experiments. We applied this technique in several experiments. First, we examined the sensitivity of T_{cc} to developmental temperature. *Drosophila melanogaster* (Congo, France), *Drosophila subobscura* (Spain, Denmark), and *Drosophila ananassae* (India) were reared from egg to adult at 15°, 18°, 25°, or 29°C, transferred to 15°C for several days, and then progressively chilled: T_{cc} was positively related to developmental temperature, inversely related to latitude of the population, but independent of sex. The sensitivity of T_{cc} to developmental temperature (acclimation flexibility) was marked: T_{cc} shifted on average 1° for each 4°C shift in developmental temperature. Among 15 species of the *obscura* group of *Drosophila*, T_{cc} varied from −0.1° to 4.5°C; T_{cc} was inversely related to latitude in both nonphylogenetic and phylogenetically based ANCOVA (standardized independent contrasts) and was unrelated to body size.

Introduction

Estimates of cold and heat tolerance have long played a central role in evolutionary physiology (Cowles and Bogert 1944; Brett 1956; Lee and Denlinger 1991; Leather et al. 1993). An ideal measure of cold tolerance would meet two criteria: it should facilitate rapid and repeatable measurements of many individuals and be nonlethal, thus conserving animals and facilitating opportunities for artificial selection experiments (Huey et al. 1992). A variety of measures of cold tolerance are in use, but perhaps the most commonly used categories of measures are the critical thermal minima ([CTMin] temperature at which the righting response is lost [Cowles and Bogert 1944]; or equivalently the chill-coma temperature [T_{cc}; Lee and Denlinger 1991; Fitzgerald and Underwood 2000]), percentage surviving exposure to a cold temperature (Parsons 1977; Tucić 1979), and either freezing or supercooling points (Salt 1961; Lee and Denlinger 1991). Of these, only the CTMin potentially meets both criteria (above).

Here, we describe a method of assaying the cold tolerance of small insects. Our protocol is based on that used to measure CTMin or T_{cc}, but our implementation enables simultaneous measurements of large numbers of individuals, whereas traditional protocols for CTMin and T_{cc} are typically measured on only one or a few individuals at a time. We apply this technique to determine the impact of development temperature and of geographic origin on cold tolerance in several species of *Drosophila*. At least some of these species inhabit regions where winter temperatures are cold enough to reduce survival (Izquierdo 1991), so measures of cold tolerance are thus ecologically relevant. In addition, we present a comparative analysis of interspecific variation in flies of the *obscura* group of *Drosophila*. These species are very suitable for comparative analyses because they are widely distributed, occur in diverse latitudes and habitats, vary in body size (Ashburner 1989), and have been the subject of recent phylogenetic analyses (O'Grady 1999).

Material and Methods

Large samples of *Drosophila melanogaster* were collected from Bordeaux (France, 45°N) in December 1997 and in Pointe Noire (Congo, 4.5°S) in September 1998. *Drosophila ananassae*, a cold-sensitive species (Morin et al. 1997), was collected in Rohtak (India, 28.9°N) in 1997. Flies were kept in bottles as laboratory mass cultures (~200 pairs per generation; cornmeal, sugar, yeast, agar, Nipagine) at 20°C and 16L : 8D photoperiod. Samples of *Drosophila subobscura*, a cold-tolerant species (Moreteau et al. 1997), were collected from Malaga (Spain, 37°N) and from Aarhus (Denmark, 56°N) in April 1998 (founded

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from ~25 isofemale lines) and maintained in population cages at 20°C and 12L : 12D photoperiod.

The geographical origin of the *obscura*-group species are given in Table 1. Most of these flies were obtained from the Bloomington Drosophila Stock Center (Indiana) shortly before these experiments were conducted. The length of time each stock had been in captivity is unknown, but they certainly had experienced some evolution to a common laboratory environment. Flies were maintained at low density in vials in Seattle for one generation before testing (~100 pairs per generation; cornmeal, sugar, yeast, agar, Nipagine; at 20°C and 16L : 8D photoperiod). Flies were tested in spring and summer 1999.

Shortly after emergence, adult flies were briefly anesthetized (CO_2), sorted by sex, and transferred to fresh vials at 15°C. Two to four days later, individual adult flies were quickly transferred (without anesthesia) into empty Eppendorf vials (1.5 mL) at room temperature (about 22°C). As soon as 60 vials had been prepared, we submerged them all in a computer-controlled cooling bath (Neslab) at 10°C. After 15 min at that temperature, we began lowering the temperature in 0.5°C steps (~10 min/°C). At each temperature step, we quickly removed each vial (<2 s/vial) one at a time and then checked for a fly's righting response. We could check all 60 flies in less than 3 min.

Flies used in the development temperature tests were reared at controlled densities from egg to adult at 15° (all populations except *D. ananassae*), 18°, 21°, 25°, or 29°C (except *D. subobscura*) and then transferred into fresh vials at 15°C for 2–4 d before testing (as above). Flies of the *obscura*-group species ($n = 14$) were all reared at 21°C and then treated as above.

Statistics

For analyses of development temperature, we used a two-way ANOVA with latitude and with development temperature as ordered factors. Preliminary analysis showed that the effects of development time and latitude on T_{cc} were mainly linear, and so we did not include quadratic contrasts for these factors (see Huey et al. 1999). Statistical results presented below are for the full data set, which is unbalanced (not all species were measured at all developmental temperatures). However, to ensure that the results were robust, we eliminated developmental temperature treatments of 15° and 29°C, which were conducted only on a subset of populations (see above) and thereby achieved a balanced design for analysis.

For the comparative studies of *obscura*-group flies, we first conducted standard nonphylogenetic ANCOVA, with T_{cc} as the dependent variable and with latitude and body size as covariates. We used wing length of females (left wing measured from the thoracic articulation to the distal tip) as the measure of body size. This nonphylogenetic analysis assumes (unrealistically) that the true phylogeny is a "star" (i.e., that all populations diverged at the same time; Felsenstein 1985). We next conducted phylogenetic analyses based on a recent phylogeny

Table 1: Origin, latitude (lat), chill-coma temperature (T_{cc}; mean ± SE), and female wing length (wing) of flies of the *obscura* group used in the comparative analyses

Species	Origin	Lat	*n*	T_{cc} ± SE	Wing	Source
Drosophila affinis	Athens, Ga.	33.10	60	.45 ± .147	259.68	J. R. David[a]
D. ambigua	Moscow, Russia	55.75	60	.87 ± .144	322.40	J. R. David[a]
D. azteca	Chilpancingo, Mexico	17.53	50	3.15 ± .151	293.44	Stock 14012-0171.0[b]
D. bifasciata	Kuusamo, Finland	65.98	60	1.91 ± .181	328.16	D. Sperlich[c]
D. guanche	Canary Islands, Spain	29.25	59	2.64 ± .149	286.24	A. M. Gonzalez[d]
D. imaii	Sapporo, Japan	43.00	58	3.53 ± .211	273.76	Y. Tobari[e]
D. madeirensis	Madeira, Portugal	32.68	37	2.26 ± .178	317.92	A. Bremh[f]
D. microlabis	Mt. Elgon, Kenya	1.00	59	3.04 ± .119	273.17	M. L. Cariou[a]
D. miranda	Mather, Calif.	37.88	58	2.62 ± .291	343.36	Stock 14011-0101.8[b]
D. obscura	Utrecht, Holland	52.02	60	1.40 ± .160	304.32	D. Sperlich[c]
D. persimilis	Mt. San Jacinto, Calif.	33.78	59	1.31 ± .164	289.12	Stock 14011-0111.27[b]
D. pseudoobscura	Flagstaff, Ariz.	35.20	58	−.10 ± .090	296.96	R. Huey
D. subobscura	Aarhus, Denmark	56.15	60	−.08 ± .112	290.24	R. Huey
D. tolteca	Coroico, Bolivia	16.52	59	4.51 ± .094	274.08	Stock 14012-0201.0[b]
D. tristis	Gif-sur-Yvette, France	48.85	51	1.50 ± .199	319.52	J. R. David[a]

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for these flies (O'Grady 1999), with branch lengths measured from Figure 6*a* in O'Grady (1999). We then used Felsenstein's standardized independent contrasts (Felsenstein 1985), as implemented with PDAP (Phenotypic Diversity Analysis Program [Garland et al. 1999; Garland and Ives 2000]).

Preliminary analyses of residuals showed that the absolute value of the standardized contrast in T_{cc} was weakly but significantly correlated with the square root of the sum of the corrected branch lengths. To attempt to correct this, we followed techniques detailed in Garland et al. (1997) and tried several branch-length transformations ($\log_{10}$, setting branch lengths equal to 1; Nee's or Grafen's arbitrary methods). None of these transformations eliminated the problem, yet all yielded similar conclusions in analyses of the contrasts themselves. Consequently, we present an analysis based only on untransformed data.

Results

Effects of Development Temperature and Latitude

Our first analysis examines the effect of developmental temperature on three species (two latitudinally separated populations of *Drosophila melanogaster*, two of *Drosophila subobscura*, and one of *Drosophila ananassae*). T_{cc} is inversely related to development temperature ($P \ll 0.001$), directly related ($P \ll 0.001$) to latitude (Fig. 1; Table 2), but independent of sex ($P = 0.807$). All populations showed similar quantitative responses to developmental temperature, as evidenced by the nonsignificant interaction between developmental temperature and latitude ($P = 0.066$). All these patterns held in an analysis of the reduced data set, which is fully balanced (see "Material and Methods").

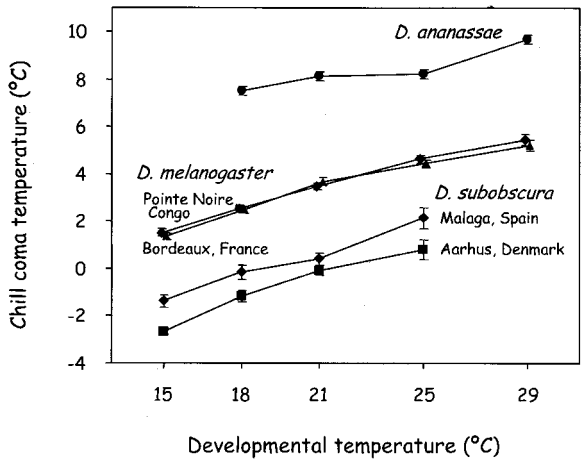

Figure 1. Phenotypic plasticity of chill-coma temperature in relation to developmental temperature in several *Drosophila* species. We used two populations of *Drosophila melanogaster* (a cosmopolitan species), two populations of *Drosophila subobscura* (a relatively cold-tolerant species), and one population of *Drosophila ananassae* (a relatively cold-intolerant species; see "Material and Methods"). Data from males and females are pooled, and each point is the mean (±95% confidence interval) of 60 values.

Table 2: ANOVA for chill-coma temperature (T_{cc}) for *Drosophila* spp.

Factor	df	MS	*F*	*P*
Sex	1	.3	.1	.8070
T_{dev}	1	5,330.7	1,054.3	<.0001
Latitude	1	3,505.1	693.2	<.0001
Sex × T_{dev}	1	1.2	.20	.6174
Sex × latitude	1	1.0	.20	.6547
T_{dev} × latitude	1	17.1	3.3	.0658
Sex × T_{dev} × latitude	1	.1	.00	.8722
Residuals	1,303	5.1		

Note. Developmental temperature (T_{dev}) and latitude are treated as ordered factors (see "Material and Methods") and thus have associated single degree of freedom contrasts.

The impact of developmental temperature is large. To quantify this, we calculated the flexibility of developmental acclimation as the slope of a regression of T_{cc} on developmental temperature (see Levins 1969; Kingsolver and Huey 1998): flexibility quantifies by how much T_{cc} shifts for each 1°C shift in developmental temperature. Flexibilities for these species are 0.285° for *D. melanogaster*, 0.332° for *D. subobscura*, and 0.180° for *D. ananassae*. On average, therefore, T_{cc} shifts by 1°C for each 4°C shift in developmental temperature.

Two species (*D. melanogaster*, *D. subobscura*) were represented by two geographic samples (Fig. 1), and we analyzed each independently (summary statistics shown only). (Note: To correct for multiple comparisons here, we used the Dunn-Šidák method to set the critical *P* value for $P = 0.05$ equal to 0.0253 [Sokal and Rohlf 1995].) Patterns for *D. subobscura* match the overall results (above): no effect of sex ($P = 0.42$), but highly significant effects of developmental temperature and latitude (both $P \ll 0.001$). However, patterns for *D. melanogaster* differ somewhat. Developmental temperature again shows a big effect ($P \ll 0.001$), but females have marginally higher T_{cc} than do males ($P = 0.025$) and latitude is surprisingly not significant ($P = 0.073$).

Comparative Study of obscura*-Group Flies*

We first conducted nonphylogenetic ANCOVA, with T_{cc} as the dependent variable and with latitude and body size as covariates (see "Material and Methods"). T_{cc} decreased significantly with latitude (Fig. 2*a*; $P = 0.042$) but was unrelated to body size ($P = 0.484$).

Next, we conducted a phylogenetic analysis that was based on a recent summary phylogeny (Fig. 6*A* in O'Grady 1999). Because the above nonphylogenetic analysis showed no influ-

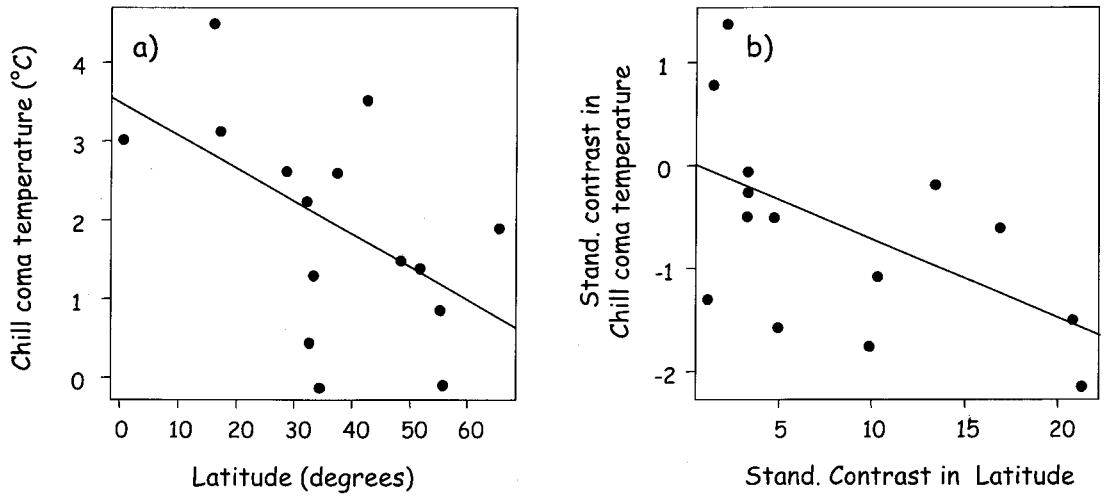

Figure 2. Correlations between chill-coma temperature and latitude for 15 species of *obscura*-group flies. *a*, Nonphylogenetic analysis of chill-coma temperature versus latitude, with a conventional regression line. *b*, Phylogenetic analysis showing standardized independent contrasts for chill-coma temperature and for latitude. Note that this regression equation is forced through the origin (see Felsenstein 1985).

ence of size (Garland and Janis 1993), we analyzed only contrasts involving latitude and T_{cc}. The standardized contrast for T_{cc} was negatively correlated with that for latitude (Fig. 2*b*; least squares linear slope $= -0.076^{**}$; $r^2 = 53\%$). Thus, both nonphylogenetic and phylogenetic approaches yield congruent interpretations.

Discussion

We have developed an efficient technique for assaying cold tolerance of large numbers of small insects. The technique is based on that used to measure CTMin (Cowles and Bogert 1944), which is widely used in ectotherms. However, rather than measuring one individual at a time, we measure many individuals simultaneously. Our analyses below show that T_{cc} varies with developmental temperature and with source population and thus is suitable for studies of plasticity and of geographic variation, as well as potentially the mechanistic bases of T_{cc}. We also compare our findings involving T_{cc} with those from previous studies of cold tolerance (% survival); patterns are generally congruent.

The first experiment examined whether T_{cc} was influenced by sex, developmental temperature, and latitude of origin in three species of flies. Sex was unimportant, either as a main effect or in any interactions. In the within-species analyses, sex was marginally significant only for *Drosophila melanogaster* (females had slightly higher T_{cc}), but sexes in this species differed only trivially in mean T_{cc} (0.1°C). In prior studies of cold tolerance (% survival of a cold stress), males and females had similar cold tolerance in one study of *D. melanogaster* from Australia (Davidson 1990*b*), but females had slightly greater cold tolerance in two other studies (Davidson 1988, 1990*a*). Sex differences in various indices of cold tolerance thus appear somewhat idiosyncratic, at least in *D. melanogaster*.

T_{cc} is phenotypically plastic and increased with developmental temperature, and the effect was quite marked both statistically ($P \ll 0.001$; Table 2) and quantitatively (Fig. 1). Indeed, acclimation flexibilities (Levins 1969; Kingsolver and Huey 1998) for these species show that on average, T_{cc} shifts about 1°C for each 4°C shift in developmental temperature (Fig. 1). Flexibilities for lower lethal temperatures of insects also show an average shift of 1°C for each 4°C shift in developmental temperature (Table 1 in Kingsolver and Huey 1998). Cold tolerance (% survival) is also inversely related to developmental temperature in many studies of *Drosophila* (e.g., Parsons 1977; Yamamoto and Ohba 1984; Hoffmann and Watson 1993). Thus, T_{cc} and cold tolerance seem to show parallel responses to developmental temperature.

T_{cc} was inversely related to latitude among species (Table 2) and also within *Drosophila subobscura* but surprisingly not within *D. melanogaster*. Previous studies consistently show that cold tolerance (% survival) is inversely related to latitude in Australian *D. melanogaster* (Parsons 1977; Davidson 1990*a*; Hoffmann and Watson 1993), but little geographic variation within species of the *melanogaster* group in Japan (Kimura 1988) or within *D. melanogaster* from latitudinally very disjunct populations in the Old World (Guerra et al. 1997). The two populations of *D. melanogaster* used in the present experiments had been in captivity for 4–13 mo before testing, and perhaps their cold resistance had converged as a result laboratory evolution.

Overall, T_{cc} and cold tolerance (% survival) seem to yield generally consistent patterns. However, we believe that T_{cc} will often be a more useful measure of cold tolerance than is percentage survival of a cold shock, which is probably the most commonly used index of cold tolerance of insects. T_{cc} has several logistic advantages. First, T_{cc} can be measured within hours; percentage survival experiments typically last 24 or more hours. Second, T_{cc} can be measured on individual insects, whereas percentage survival experiments typically involve sets of individuals; thus, T_{cc} greatly enhances statistical power. Third, all insects survive T_{cc} and can potentially be used in selection experiments. Although artificial selection can also be done on percentage survival (Tucić 1979), such selection is very labor intensive and may require family selection approaches (Chen and Walker 1993), which are even more labor intensive. Fourth, with T_{cc} one always obtains useful data. In contrast, survival experiments are frustratingly hit or miss. If one guesses the wrong temperature or duration of exposure, all the subjects can survive (or die).

Hori and Kimura (1998) recently described a new technique ("cold stupor temperature") that is somewhat similar to that described here and that correlates strongly with lower lethal temperature. They transfer flies to prechilled vials at a series of temperatures (between 5° and 13°C) and then check the flies every 5 min, recording at each census the percentage of individuals that are motionless. They then estimate the maximum percentage of individuals that were in stupor at each temperature and then visually estimate the temperatures at which 25%, 50%, and 75% of flies were in stupor. This technique is effective but laborious, does not yield data on individual flies, and is not directly usable in selection experiments.

In summary, we describe an efficient way of assaying the cold tolerance of small insects. Our analyses show that T_{cc} is very sensitive to developmental temperature and often to latitude but is generally independent of body size and sex. Moreover, the technique has clear logistic advantages over traditional measures of insect cold tolerance (e.g., percentage survival of a cold shock).

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