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The fly that came in from the cold: geographic variation of recovery time from low-temperature exposure in *Drosophila subobscura*

J. R. DAVID*, P. GIBERT†, B. MORETEAU*, G. W. GILCHRIST‡ and R. B. HUEY§¶

**Laboratoire Populations, Genetique, et Evolution, Centre National de la Recherche Scientifique, 91198 Gif-sur-Yvette, Cedex, France, †Laboratoire de Biometrie, Biologie Evolutive, Universite Lyon, 43 Boulevard du 11 Novembre, 69622 Villeurbanne Cedex, France, ‡Department of Biology, Box 8795, College of William & Mary, Williamsburg, Virginia 23187–8795, USA, and §Department of Biology, Box 351800, University of Washington, Seattle, Washington 98195–1800, USA*

Summary

1. The time required for an ectotherm to recover from cold exposure is a useful, non-lethal index of cold tolerance. We explore how recovery times are affected by exposure to low temperatures, develop statistical methodologies, and study geographic variation in recovery time in four populations of *Drosophila subobscura*, a cold-tolerant species.
2. We exposed flies to a low temperature (–7 °C to 1 °C) for 16 h, returned them to ambient temperature, and recorded the elapsed time ('recovery time') until they stood. Other flies were exposed to even colder temperatures (–11 °C to –7 °C), but for shorter times.
3. Recovery times were inversely related to exposure temperature, but had a plateau between −6 °C and −4 °C.
4. Populations had similar recovery times at 'warm' temperatures, but two subtropical populations had relatively long recovery times at colder temperatures.
5. Inter-population differences were also evident in a regression analysis, and recovery times were inversely related to latitude (ordered-factor analysis). Populations differed slightly in the slopes of regressions but differed strongly in their intercepts.
6. The physiological mechanisms underlying the non-linear responses are unknown, but the plateau region suggests that recovery time is governed by the interplay of two temperature-dependent processes. Two models are proposed for the interaction of these processes.

Key-words: Chill coma, climatic adaptation, cold tolerance, geographic variation, thermal physiology

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Introduction

For most terrestrial ectotherms, temperature is a key environmental factor influencing all aspects of their ecology and evolution (Precht *et al.* 1973; Cossins & Bowler 1987). In recent years, the tolerance of insects to low temperature has been explored extensively (Addo-Bediako, Chown & Gaston 2000; Hoffmann, Sorensen & Loeschcke 2003); and the physiological mechanisms involved in cold tolerance, especially of freeze tolerance and resistance, are increasingly well understood (Lee & Denlinger 1991; Leather, Walters & Bale 1993). However, the study of the evolutionary diversification of cold tolerance itself is less comprehensive, in part because traditional methods of characterizing cold tolerance (e.g. percentage survival of a cold shock, LD50s) are labour intensive, kill some animals, and discourage large-scale comparative analyses and selection experiments.

David *et al.* (1998) recently developed a simple and efficient index of cold tolerance that can be used even in remote situations, as long as ice is available. They exposed *Drosophila melanogaster* to 0 °C for varying lengths of time, took the flies back to ambient temperature, and measured the length of time before the flies had recovered (that is, until they could stand). Recovery time increased with the length of exposure to 0 °C and decreased with ambient temperature experienced during recovery.

This 'chill-coma recovery time' has proved to be a rapid and sensitive index of climatic adaptation:

¶Author to whom correspondence should be addressed. Fax +1 206 5433041. E-mail hueyrb@u.washington.edu.

recovery times are much faster for temperate species than for tropical ones (Gibert *et al*. 2001). However, several temperate zone species such as *D. subobscura* are relatively cold tolerant and recover almost instantaneously after exposure to 0 °C. To explore whether geographic populations of such species might differ in cold tolerance, one must obviously expose them to below-zero temperatures, which should induce a chill coma and lengthen recovery times, and thereby provide the opportunity for detecting differences among populations.

Here we explore how recovery time is influenced by test temperature (1 °C to −11 °C). We detected a significant negative (but decidedly non-linear) relationship between recovery time and test temperature, and we therefore developed companion statistical methods to characterize these non-linear relationships. We apply these methods to evaluate geographic variation in cold tolerance of four Old World populations (31·6 °N to 55·8 °N) of *D. subobscura.* Between-population differences were evident, but mainly at very low temperatures (less than −5 °C). In all populations, recovery times showed a distinct insensitivity to temperatures between −6 °C to −4 °C, but the physiological reasons underlying this interesting 'plateau' is unknown.

Materials and methods

We used *D. subobscura* from four populations spanning almost 24° of latitude (two subtropical: Marrakech, Morocco, and Madeira Island, Portugal; and two cool-temperate populations: Moscow, Russia, and Mt Genèvre, France; latitudes are listed in Table 1). Flies were maintained in mass cultures at 21 °C under a photoperiod L : D 16 : 8 for one year (Marrakech and Mt Genèvre) or two years (Madeira and Moscow) before testing. Each population was founded by at least 10 pairs of adults.

EXPERIMENTAL PROCEDURE

Flies were reared at 21 °C in half pint bottles on a cornmeal medium seeded with live yeast and enriched with a piece of killed yeast medium (David & Clavel 1965). Rearing densities were kept low by transferring parents (about 50 pairs) each day into a fresh bottle. Freshly emerged flies were transferred to fresh food and aged for 2 to 4 days before testing, thereby avoiding a possible ageing effect (David *et al*. 1998).

In a previous investigation in *D. melanogaster*, males had slightly longer recovery times than did females (David *et al*. 1998). Such a physiological dimorphism was not evident in preliminary studies on *D. subobscura*: consequently, we did not distinguish sexes (see also Gibert *et al*. 2001).

For experiments we tossed over flies (without anaesthesia) to empty glass vials (50 ± 10 flies per vial) and transferred them acutely to a refrigerated bath set to a predetermined temperature. Regulation was ± 0·1 °C, and bath temperature was checked (with a precision thermometer) during each experiment. For temperatures between −7 °C and +2 °C, flies were held at the test temperature for 16 h and then were taken acutely back to room temperature (22 °C to 24 °C), poured onto a Petri dish, and then monitored. When a fly was able to stand on its legs, we recorded its recovery time and then removed it from the Petri dish (David *et al*. 1998). For colder temperatures, a 16-h treatment induced significant mortality and was thus too long. Consequently, we shortened exposure times for very cold temperatures (3 h for −8 °C; 2 h for −9 and −10 °C; 1 h for −11 °C). Note that recovery times in Table 1 for these low temperatures are standardized to 16-h exposures. For example, the actual recovery time following a 1-h exposure at −11 °C was multiplied by 16: here we assume a linear relationship between treatment duration and recovery, as occurs in *D. melanogaster* (David *et al*. 1998) and *D. ananassae* (Gibert *et al*. 2001).

We measured multiple replicates ('*n*' in Table 1) per population per temperature. All analyses are based on the mean recovery time (based on about 50 flies, see above) for a given replicate. The total number of replicates was 353 replicates (about 18 000 individual flies).

The number of replicate samples that could be handled in a day was either two or four, and so the full experiment took more than a year to complete. However, we always investigated all four populations in the same week, and replicates were randomized over time. Thus, no temporal bias (or laboratory adaptation) should be significant.

STATISTICAL ANALYSIS

For an initial exploration of population differences, we used separate one-way analyses of variance (ANOVA) to compare recovery times of the four populations at each temperature (Table 1), but used a sequential Bonferroni procedure to correct critical *P*-values for multiple comparisons (Benjamini & Hochberg 1995). Because these tests were always made within a temperature, they are unaffected by the reduced exposure times for the lowest temperatures (above).

Next we wanted to compare the differences across temperature in the sensitivity of recovery time among populations. For these analyses, we used only data for temperatures of −7 °C and warmer (temperatures at which all flies had a 16-h exposure). As shown below, recovery time is non-linearly related to exposure temperature, and variances are inversely related to temperature (Fig. 1). A natural-log transformation of recovery time homogenized the variances: however, the relationship remained non-linear (Fig. 2). Accordingly, we modelled temperature as a fourth-order, orthogonal polynomial to compare populations. Also, we used join-point linear regressions (Crawley 2002) for the range −7 °C to 0 °C (one regression for the

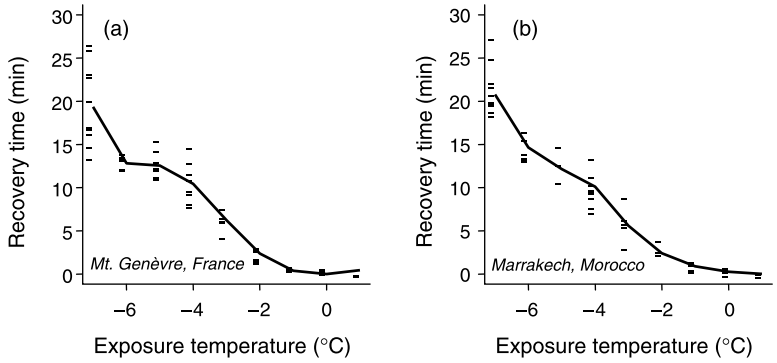

Fig. 1. Raw recovery times of *Drosophila subobscura* from (a) a temperate zone (Mt Genèvre, France) and (b) a subtropical (Marrakech, Morocco) population following exposure to low temperature for 16 h (see Materials and methods). The solid line is from a non-parametric curve fit (supersmoother). Note the non-linear responses as well as the marked increase in variance at low temperatures.

plateau region, another for warmer temperatures), with confidence intervals obtained by bootstrap methods.

Results

Recovery times were inversely and non-linearly related to exposure temperature (Table 1, Figs 1 and 2). To determine whether populations differed in recovery time, we ran one-way ANOVAS at each temperature (Table 1, with adjusted *P*-values, see Materials and methods). Populations did not differ significantly at the 'warm' test temperatures (> –6 °C). At cold temperatures (< –6 °C), however, the two subtropical populations generally had significantly longer recovery times than the cool-temperate species (Table 1). At –11 °C, for example, the two subtropical populations had recovery times that averaged 1·6 times longer than those of the cool-temperate populations.

RECOVERY TIMES BETWEEN –7 °C AND + 1 °C

Next we looked at the thermal dependence of recovery time. Initially, we restricted the analyses to temperatures of –7 °C and warmer (temperatures with the same exposure time). Representative data for two populations (Mt Genèvre, Marrakech) are shown in Fig. 1. Recovery times were inversely and non-linearly related to exposure temperature. A plateau in recovery times is evident between –6 °C and –4 °C, and recovery time increased markedly at the lowest temperature (–7 °C). The plateau remains conspicuous when recovery times are log-transformed (Fig. 2) and appeared relatively narrow (–6 °C to –5 °C) for the two subtropical populations. The plateau is also evident when the first derivative of the response curve is plotted (dashed lines in Fig. 2).

To examine inter-populational variation in the relationship between recovery time and exposure temperature, we used a fourth-order orthogonal polynomial regression (Fig. 2) of temperature nested within

Table 1. Mean recovery time (min) of flies from four populations at temperatures ranging from +1 to –11 °C. Exposure times were reduced at the lowest temperatures (–11 °C to –8 °C), but reported recovery times are standardized here to a 16-h exposure (see Materials and methods). Latitude (Lat.) of origin is listed for each population. Significance levels of one-way ANOVAS (corrected for multiple comparisons by sequential Bonferroni test) are indicated below each temperature (*$P < 0{\cdot}05$, ***$P < 0{\cdot}001$). *n* is the number of replicates (groups of about 50 flies) measured at each temperature

Temperature of treatment (°C)		–11		–10		–9		–8		–7		–6		–5	
Exposure (h)		1		2		2		3		16		16		16	
Population	Lat.	Mean	(*n*)	Mean	(*n*)	Mean	(*n*)	Mean	(*n*)	Mean	(*n*)	Mean	(*n*)	Mean	(*n*)
Moscow	55·8	169	(11)	84	(6)	53	(7)	31·3	(7)	16·6	(11)	11·6	(6)	11·0	(6)
Mt Genèvre	45·0	160	(8)	107	(7)	72	(6)	43·9	(8)	19·3	(11)	12·9	(6)	12·4	(9)
Madeira	32·7	264	(11)	160	(8)	97	(8)	41·7	(8)	21·8	(10)	15·9	(6)	13·4	(7)
Marrakech	31·6	256	(10)	177	(8)	97	(5)	50·9	(7)	20·9	(11)	14·1	(4)	13·1	(5)
ANOVA		*		*		*		***		NS		*		NS	

Temperature of treatment (°C)		–4		–3		–2		–1		0		1	
Exposure (h)		16		16		16		16		16		16	
Population	Lat.	Mean	(*n*)	Mean	(*n*)	Mean	(*n*)	Mean	(*n*)	Mean	(*n*)	Mean	(*n*)
Moscow	55·8	8·7	(10)	4·9	(7)	1·57	(6)	0·50	(6)	0·11	(5)	0·05	(3)
Mt Genèvre	45·0	10·1	(7)	6·2	(7)	2·14	(7)	0·51	(5)	0·20	(5)	0·10	(2)
Madeira	32·7	10·3	(6)	6·4	(7)	2·32	(7)	1·06	(5)	0·15	(4)	0·17	(4)
Marrakech	31·6	9·8	(9)	5·6	(7)	2·70	(4)	0·81	(5)	0·16	(5)	0·14	(3)
ANOVA		NS		NS		NS		NS		NS		NS	

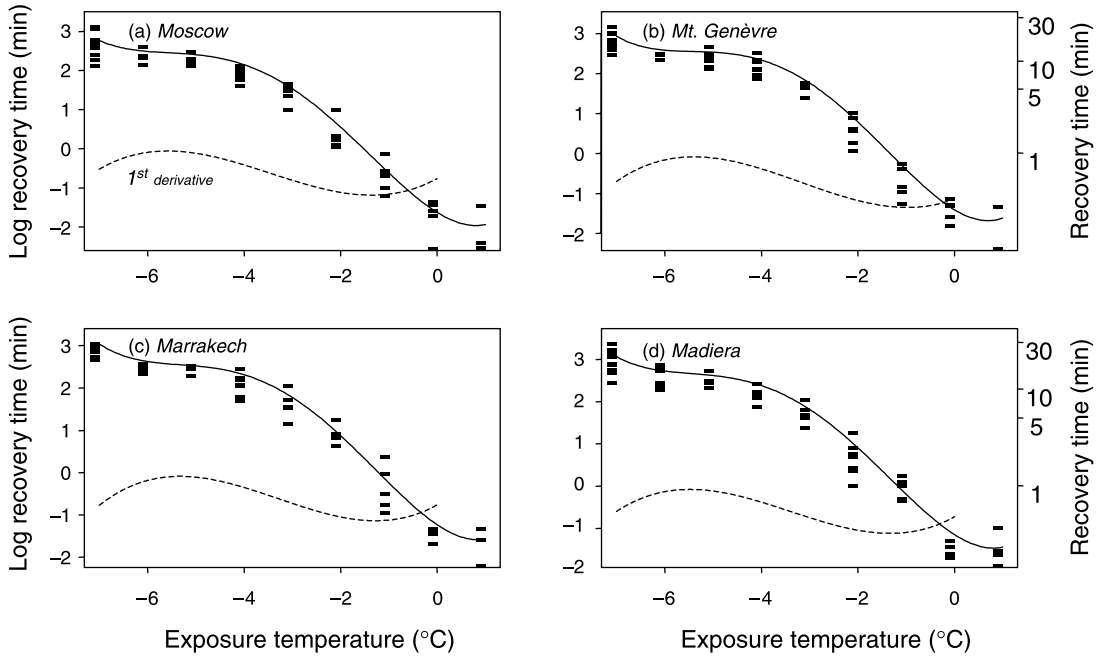

Fig. 2. Natural log-transformed recovery times of *D. subobscura* from four populations. The solid line is a fitted, fourth-order polynomial; and the dashed line plots the derivative of that polynomial. Log transformation homogenizes the variances across temperature (contrast with Fig. 1), but does not eliminate the non-linearity.

population. This regression provided a good fit (multiple $R^2 = 0{\cdot}96$) and was a significant improvement (P values < 0·02) over a second- or third-order polynomial. The population by temperature interaction was not significant (comparison of slopes: $P = 0{\cdot}096$), so we could not reject the null hypothesis of parallel slopes. However, the intercepts differed significantly among populations (ANCOVA: $P << 0{\cdot}001$, assuming a common slope). Moscow had significantly shorter recovery times than did the remaining populations (*a posteriori* Tukey test), but the remaining populations were not significantly different from each other.

We repeated the polynomial analysis using latitude as an ordered factor. Recovery time decreased significantly with latitude (linear and quadratic contrasts P values << 0·01, cubic contrast was non-significant).

We also used a join-point linear regression for the temperature interval –6 °C to 0 °C, with confidence intervals estimated by bootstrap techniques (data not shown). This analysis separated the data into two groups (above –3 °C vs. below –4 °C). For all populations, the slope of the lower regression (–6 °C to –4 °C, the plateau region) was significantly less steep than that of the upper regression (–3 °C to 0 °C: confidence intervals were non-overlapping). However, no significant difference was found between any pair of populations in the slopes of the lower or upper regressions, in the join-points, or in the intercepts.

RECOVERY TIMES BETWEEN –11 °C AND +8 °C

Recovery times for the four coldest temperatures (following standardization, see Materials and methods) were log-transformed and analyzed as above, except that the four temperature intervals allowed only a third-order polynomial fit. The regression provided a good fit (multiple $R^2 = 0{\cdot}90$). The population by temperature interaction was not significant ($P = 0{\cdot}15$), indicating parallel slopes for the populations. However, the intercepts differed significantly among populations ($P << 0{\cdot}001$, assuming a common slope), with the subtropical populations having relatively long recovery times (Table 1). All populations were significantly different (*a posteriori* Tukey test), except Madeira vs. Marrakech.

Discussion

GENERAL PATTERNS

Many studies have examined geographic variation in cold tolerance in *Drosophila* (reviewed in Hoffmann *et al*. 2003). Most such studies searched for variation in percentage survival of adults after exposure to one temperature, and many focus exclusively on *D. melanogaster*. In general, cold tolerance of adults increases with the latitude of the species or source population (Hoffmann *et al*. 2003). For example, chill-coma recovery time (following 8 h at 0 °C) was inversely related to latitude in the Australian flies *D. serrata* (Hallas, Schiffer & Hoffmann 2002) and *D. melanogaster* (Hoffmann, Anderson & Hallas 2002). Nevertheless, not all species show the expected latitudinal pattern: cold tolerance of adult *D. pseudoobscura* from the western USA was independent of latitude, although cold tolerance of pupae did increase with latitude (Coyne, Bundgaard & Prout 1983). The generally greater

cold tolerance of high-latitude populations probably enhances over-winter survival, as has been found in *D. serrata* (Magiafoglou, Carew & Hoffmann 2002).

Only one previous study has examined geographic variation in cold tolerance in *D. subobscura* (Gibert & Huey 2001). The low temperature at which flies became incapacitated (i.e. entered in a chill-coma state) was lower for a high-latitude population (Denmark) than for a low-latitude one (Spain).

In the present study, *D. subobscura* from all sites recovered quickly at relatively 'warm' temperatures (–6 °C and warmer): population differences were small and non-significant at these temperatures (Table 1). Nevertheless, a polynomial regression analysis of recovery time (range –7 °C and warmer) did detect a significant effect of latitude as an ordered factor, mainly because Moscow (high latitude) had a significantly shorter recovery time overall than the other populations (see above). However, differences among populations were conspicuous for cold temperatures (–11 °C to –8 °C; Table 1). The two subtropical populations generally had much longer recovery times than the temperate populations.

Previous studies of chill-coma recovery time of *Drosophila* used an exposure to 0 °C (David *et al*. 1998; Gibert *et al*. 2001; Hallas *et al*. 2002; Hoffmann *et al*. 2002) and demonstrated that this technique can detect geographic variation in cold tolerance, at least for cold-sensitive species (Gibert *et al*. 2001). Recovery times are also technically easy to measure, even in remote sites (only ice is required). Importantly, the flies recover fully, such that artificial selection on recovery time should be feasible.

Cold-tolerant species such as *D. subobscura*, however, recover almost instantly from prolonged exposure to 0 °C (Table 1). Consequently, workers will need to expose such insects to lower temperatures to have any hope of detecting geographic variation in cold tolerance. This requires access to low-temperature baths, and the likely non-linearity of the response will complicate subsequent statistical analyses (above). Of course, logistic and statistical procedures could be simplified by measuring recovery time only at a *single* low temperature (e.g. –10 °C for *D. subobscura*). To select that temperature, however, one would need to conduct pilot studies spanning a range of temperatures (see Fig. 1). In any case, this one-temperature approach would result in the loss of information on the plateau region (Fig. 1).

Latitudinal clines for a diversity of traits (body size and chromosome inversions; Prevosti 1955; Krimbas 1993; Huey *et al*. 2000) have previously been described for *D. subobscura*. Latitudinal shifts in temperature are usually assumed to be the main selective factor generating such clines (but without direct proof). Our data, showing that cold tolerance [either chill-coma temperature (Gibert & Huey 2001) or recovery time (herein), especially from very cold temperatures] varies inversely with latitude is consistent with such an assumption; but direct comparisons of over-winter survival rates in a common garden will be necessary to test that assumption directly (Hoffmann *et al*. 2003).

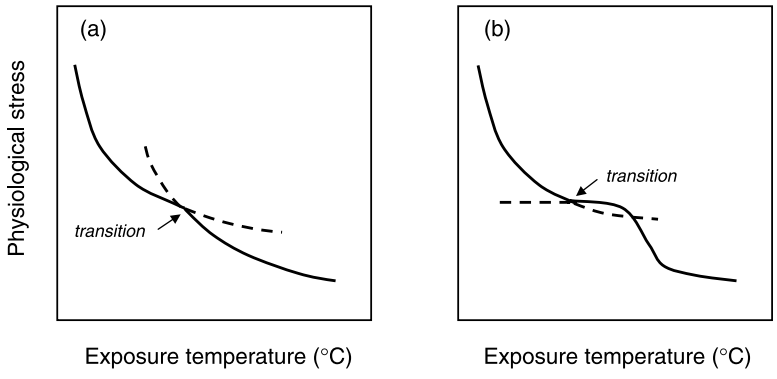

Fig. 3. Conceptual models possibly underlying the bi-phasic, non-linear response curves (see Fig. 1). (a) In this model, physiological stress is governed by two underlying processes that are both exponentially related to temperature, with each process dominating recovery times over adjacent temperature ranges (solid lines). (b) Two processes are also involved over adjacent temperature ranges, but here one is exponential while the other is logistic (see text).

PHYSIOLOGICAL BASES

The observed inverse relationship between recovery time and exposure temperature (Figs 1 and 2) is not surprising. Presumably, cold stress (injury) is magnified at increasingly low temperatures; and so flies take longer to recover. However, the exact mechanisms underlying stress and subsequent recovery here are unclear and will require detailed physiological studies. Potential targets include transient disruption by low temperature of membrane fluidity, of ion gradients, or of microtubule polymerization (Audibert, Debec & Simonelig 1996; Hazel 1995; Rajaram *et al*. 1999).

The conspicuous plateau in recovery times around –6 °C to –4 °C (Figs 1 and 2) is puzzling and indicates that the physiological bases underlying the thermal dependence of recovery time are probably complex. Plateaux of rate vs. temperature relationships are known for a few other processes (e.g. of metabolic rate in some species; see Cossins & Bowler 1987; p. 53). In the specific case of recovery time, at least two processes are likely to be involved in the plateau: one set of physiological processes governs recovery at very cold temperatures, whereas a second set governs recovery at temperatures at or above the plateau. We can envision two conceptual models (Fig. 3). In one case, two underlying processes are both exponentially related to temperature, and the transition from one to the other could occur over a narrow transition zone (Fig. 3a). Alternatively, one process could be exponentially related to temperature, but the other could have a decreasing sigmoid shape (Fig. 3b). A sigmoid shape could occur if some cold-sensitive process was increasingly damaged at progressively colder temperatures, but only up to some maximum (saturation) level.

Whether the bi-phasic pattern in recovery time seen in *D. subobscura* (Fig. 1) is general for other *Drosophila*

and other insects remains to be determined. Tropical species (or cosmopolitan species of tropical origin, e.g. *D. melanogaster*) will be of particular interest. However, because such species are cold sensitive (David *et al*. 1998; Gibert *et al*. 2001), exposure times at temperatures below 0 °C will need to be much shorter than 16 h.

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