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The effect of selection for desiccation resistance on cold tolerance of *Drosophila melanogaster*

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Abstract. Low temperature and desiccation stress are thought to be mechanistically similar in insects, and several studies indicate that there is a degree of cross-tolerance between them, such that increased cold tolerance results in greater desiccation tolerance and *vice versa*. This assertion is tested at an evolutionary scale by examining basal cold tolerance, rapid cold-hardening (RCH) and chill coma recovery in replicate populations of *Drosophila melanogaster* selected for desiccation resistance (with controls for both selection and concomitant starvation) for over 50 generations. All of the populations display a RCH response, and there is no effect of selection regime on RCH or basal cold tolerance, although there are differences in basal cold tolerance between sampling dates, apparently related to inter-individual variation in development time. Flies selected for desiccation tolerance recover from chill coma slightly, but significantly, faster than control and starvation-control flies. These findings provide little support for cross-tolerance between survival of near-lethal cold and desiccation stress in *D. melanogaster*.

Key words. Chill coma, cold tolerance, cross tolerance, desiccation, laboratory selection.

Introduction

There are a remarkable number of overlaps in the tolerances of insects to differing environmental stresses. For example, mild desiccation elicits increased cold tolerance in the springtail *Folsomia candida* (Bayley *et al.*, 2001), and a period of anoxia induces increased cold tolerance in the house fly *Musca domestica* (Coulson & Bale, 1991). Cross-tolerance is thought to be a useful way to approach complex traits, and selection experiments in *Drosophila* have been frequently used to examine the relationships between environmental stressors, including high and low temperatures, desiccation and starvation (Nghiem *et al.*, 2000; Hoffmann *et al.*, 2003; Bubliy & Loeschcke, 2005). Selection experiments attempt to replicate the processes that have produced cross-tolerance in the field, with the intention that responses shared between selected stress tolerance traits will identify candidate mechanisms for the observed tolerances (Gibbs, 1999).

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Of the many environmental stresses that insects face regularly, desiccation and cold are thought to be particularly closely related (Ring & Danks, 1994). At subzero temperatures, the low energy state of ice removes water vapour from the air, placing insects in a desiccating environment. Many overwintering insects consequently show reduced water content, and many of the biochemical adaptations to cold also serve to protect against desiccation (Ring & Danks, 1994). This relationship is well-supported in insects that are highly permeable (Holmstrup *et al.*, 2002), exposed to very cold, desiccating conditions (Ramløv & Lee, 2000) or are freeze tolerant (Sinclair & Wharton, 1997). However, in non-cold-hardy insects, such as *Drosophila melanogaster*, the link between cold and desiccation is less well-explored.

In a selection experiment where lines of *D. melanogaster* are selected for resistance to multiple different stressors and their tolerances to other stressors examined, selection for desiccation resistance results in an increase in cold tolerance (measured at 50 h at 0 °C) above controls (Bubliy & Loeschcke, 2005). Conversely, flies selected for cold resistance (exposed to 50 h at 0 °C) do not show any corresponding increase in desiccation tolerance. However, several studies observe either no relationship, or a complex relationship,

between cold and desiccation resistance in *Drosophila*. Hoffmann *et al.* (2005b) show that although there is a strong latitudinal cline in cold tolerance in *D. melanogaster* (time to recover from chill coma after 3 h at 0 °C), there is no such cline in tolerance to either desiccation or starvation. Telonis-Scott *et al.* (2006) demonstrate a decrease in cold tolerance (measured as time survived at –2 °C) in one line selected for desiccation resistance, and no change in two others from the same selection regime. Finally, Sinclair *et al.* (2007) find few similarities in quantitative expression of five stress-related genes in wild-type *D. melanogaster* during exposure to and recovery from desiccation and cold stress.

Most studies of selection for, or response to, desiccation fail to take the concurrent starvation stress into account in their controls (Gefen *et al.*, 2006). This omission may be a significant confounding factor: Hoffmann *et al.* (2005a) show that flies selected for starvation have reduced cold tolerance (survival at –2 or –4 °C), whereas those selected for reduced chill coma recovery time show a corresponding decrease in starvation tolerance. By contrast, the methods used in examining cold tolerance in *Drosophila* have been highly variable, and probably not comparable (Sinclair & Roberts, 2005). In particular, the mechanisms underlying mortality from acute cold exposure (defined by Sinclair and Roberts as < 6 h) and chronic cold exposure are probably not closely related, and the mechanisms underlying chill coma recovery are likely also unrelated to the mechanisms associated with the other cold tolerance metrics. Cold tolerance is plastic in *D. melanogaster*, responding to long-term acclimation (Goto, 2000) and short-term hardening (Overgaard *et al.*, 2005), and also has a genetic basis in that cold tolerance responds to selection (Bubliy & Loeschcke, 2005). In the latter case, acute cold tolerance can be substantially increased by a pre-exposure to a less severe low temperature in a process called rapid cold-hardening (RCH) (Czajka & Lee, 1990).

In the present study, replicate lines of flies selected for desiccation resistance, with appropriate controls for the concomitant starvation effects, are used to determine the effect of selection for desiccation resistance on cold tolerance. Two commonly used metrics of cold tolerance are used (i.e. tolerance to acute cold shock and recovery from chill coma) and the effects of desiccation selection on the RCH response are also examined.

Materials and methods

Selection experiments

Fly stocks. The *Drosophila* lines used in these experiments were derived from approximately 400 females collected in New Jersey in 1999. They have been maintained at 24 °C as a large outbred population subsequent to collection. To minimize the possibility of artefacts due to adaptation to a new environment, the population was maintained on a standard three-week stock cycle for 12 generations before selection was started. Pre-adult stages were reared at moderate densities (approximately 60 larvae per vial) in vials containing 10 mL of corn meal–sucrose–yeast medium. After 2 weeks, adult flies (approximately 4 days post-eclosion) were dumped into a 5.5-L acrylic population cage containing two Petri dishes of medium. A cloth sleeve covered one end and allowed access to the cage. The medium was changed every 2 days. After 4 days, yeast paste was added to stimulate egg production. Approximately 1200 eggs were collected after 7 days to found the next generation. The selected flies are more thoroughly characterized by Gefen *et al.* (2006).

Desiccation selection. Selection for desiccation resistance was performed by removing food from the cages 1–4 h after the flies were dumped. A cheesecloth-covered dish containing approximately 200 g of silica gel desiccant was placed inside, and the open end of the cage was loosely covered with plastic wrap to allow gas exchange at the same time as reducing the influx of water vapour from the surroundings. Initially, each cage contained approximately 7500 flies. The cages were checked hourly until 80–85% of the flies had died. The desiccant was then removed, and fresh food provided to the survivors. The flies were given several days to recover before egg collection for the next generation.

Because desiccation selection required the removal of both food and water, each selection line (*D*) was matched to a starved control population (*S*), whose cage received two plates of 1% agar instead of desiccant. At every generation, each of these stocks was starved for the same length of time as its corresponding desiccated population. To control for the effects of starvation stress, each pair of stressed populations had a matched, unstressed, fed control population (*F*). The *F* populations were maintained under the original 3-week stock cycle. The selection and control treatments were replicated three times each. Population sizes in all treatments were maintained to provide an estimated 1000–1500 adults after selection.

After 30 generations of selection, mean desiccation resistance in the *D* males (4-day old nonvirgins) had increased from approximately 12 h to approximately 35 h (A. G. Gibbs and C. H. Vanier, unpublished observations). Males from the *S* control populations survived desiccation for an average of approximately 14 h. In subsequent generations, desiccation selection was relaxed. The *D* flies were exposed to desiccating conditions for 24 h each generation, and the *S* controls were starved for 24 h. Because control flies die of desiccation stress in this period, and very few *D* flies do (A. G. Gibbs, unpublished observations), the relaxed selection regime maintained differences in desiccation resistance between selection treatments. Flies used in the experiments described here underwent approximately 50 total generations of selection.

Fly handling and management

Flies used for cold tolerance determination had been in the selection regime for 50 generations, whereas those used for chill coma determination had been in the selection regime for 72 generations. Flies were removed from the selection regime for one generation before use in this experiment and eggs

collected from population cages and reared in vials containing approximately 10 mL of *Drosophila* medium [Tucson stock centre recipe: 0.9% agar, 2.4% cornmeal, 3.9% sugar, 1.4% dried yeast (w/v), 0.3% (v/v) propionic acid] at a uniform density of approximately 50–70 individuals per vial. For the cold tolerance experiment, eggs were collected on two occasions 2 days apart, to ensure a large number of flies emerging for five consecutive days. Upon emergence, flies were sorted into their experimental groups (ten adult males per food vial) under light CO_2 anaesthesia and given 2 days to recover before 3 day-old flies were used in experiments; for details on CO_2 effects on cold tolerance, see Nilson *et al.* (2006).

Cold tolerance and RCH

Groups of flies were assigned randomly to temperature and RCH pre-treatment groups on the day of the experiment. Cold tolerance was measured by exposing three groups of ten adult male flies in 2-mL cryo-vials acutely to each of six temperatures for two hours (–4, –5, –6, –7, –8 and –9 °C; for a full description of the method, see Nilson *et al.* (2006). For the RCH pretreatment flies were transferred to 2-mL cryovials (Nunc, Rochester, New York), placed in sealed bags and immersed in an ice-water slurry (0 °C) for 2 h prior to exposure to lower temperatures. After exposure, flies were transferred to a well of a six-well cell culture plate containing a 0.5-cm^3 piece of *Drosophila* food, and survival (ability to stand and walk in a coordinated fashion) assessed after 24 h. The cold tolerance and RCH experiments were conducted at the same time on five successive days. Low numbers of flies for one of the lines on day 1 and for two of the lines on day 2 meant that those days were not used in statistical analysis.

Chill-coma recovery

Chill coma recovery was assessed using the method of Nilson *et al.* (2006). From each selection group, 10 flies were transferred to each of ten 2-mL cryo-vials. These vials were then placed into sealed bags and immersed in an ice-water slurry (0 °C) for 4 h to induce chill coma. After exposure, flies were transferred to room temperature and into a well of a six-well cell culture plate containing a 0.5-cm^3 piece of *Drosophila* food. Recovery (ability to stand and walk in a coordinated fashion) was assessed every 60 s until all individuals were recovered or when 30 min had lapsed. Exposures were staggered by treating 30 vials every 30 min to allow time for recovery scoring.

Data analysis

The LT_{50} values were derived from cold tolerance and RCH survival data from cold exposure for each line/treatment/day combination using Proc Probit in SAS, version 9.1 (SAS Institute, Inc., Cary, North Carolina) and the respective LT_{50} were used for a nested repeated-measures analysis of variance (with lines as the individual replicates) examining the effects of day, line, selection regime and pretreatment on LT_{50} using Proc GLM with Tukey's post-hoc test in SAS. Days 1 and 2 had missing values for entire lines; thus, to preserve statistical power, the analysis was conducted only on data from days 3–5. The design of the selection experiment did not give enough degrees of freedom to examine the effects of selection on the magnitude of the RCH response. An additional repeated-measures GLM examined the effect of selection regime on RCH magnitude. RCH magnitude was expressed as the difference between the LT_{50} of nontreated and pretreated (rapidly cold-hardened) flies of the same line on the same day.

Time to 80% chill-coma recovery was analysed as by Nilson *et al.* (2006), using a type 3 generalized linear model with a Poisson error distribution, logit-link and scaled deviance (Proc Genmod in SAS). Confidence intervals for least-squares means were computed and used to compare groups at $\alpha = 0.05$. A nested design was used, with line nested within selection regime.

Results

There was no effect of selection regime on cold tolerance ($F_{2,6} = 2.34$, $P = 0.177$). A power analysis for each day individually using Proc GLM POWER in SAS indicates that a sample size in excess of 72 selection lines would have been necessary to detect a significant effect of selection regime on day 3, and in excess of 810 and 1080 selection lines on days 4 and 5. All of the lines displayed a significant RCH response ($F_{3,6} = 43.91$, $P = 0.0002$; Fig. 1), but

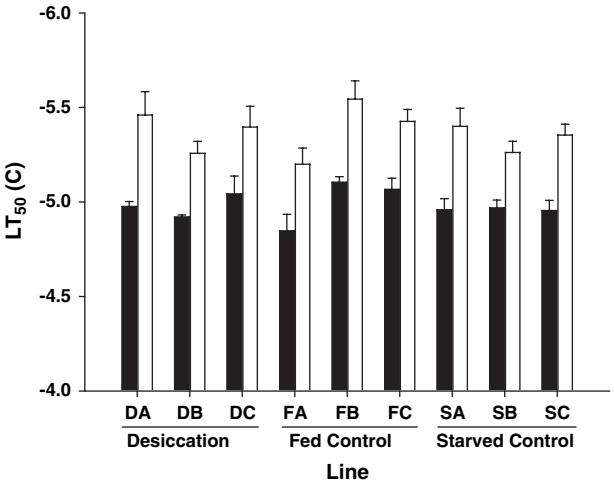

Fig. 1. Temperatures (mean ± SE) at which 50% of adult *Drosophila melanogaster* males from different selection regimes died after a 2-h exposure (LT_{50}). Filled bars indicate flies that had no pretreatment, open bars are for flies that had a 2-h pre-exposure at 0 °C to induce the rapid cold-hardening response. Data are pooled from determinations on five consecutive days.

there was no effect of selection regime or day on the magnitude of the RCH response ($P > 0.5$ in both cases). There was a significant difference in cold tolerance between lines ($F_{4,6} = 7.47$, $P = 0.016$). A post-hoc test shows that this is because of the control line 'FC', which had an unusually low basal cold tolerance on day 3 and differences between the lowest and highest values on days 4 and 5 (Fig. 2). These significant differences between lines on various days are also reflected in the significant effect of Day in the repeated measures analysis, which suggests a slight decrease in basal cold tolerance as time progressed ($F_{2,12} = 11.23$, $P = 0.0018$). None of the interactions between Day and the other terms in the model were significant ($P > 0.32$ in all cases).

Flies selected for desiccation survival showed a significant (approximately 2 min) decrease in chill coma recovery time compared with starvation controls, or flies that were not selected at all (Wald $\chi^2 = 18.84$, d.f. $= 2$, $P < 0.0001$; Fig. 3). There was significant interline variation (Wald $\chi^2 = 23.35$, d.f. $= 6$, $P = 0.0007$), most notably within the starved control (Fig. 3).

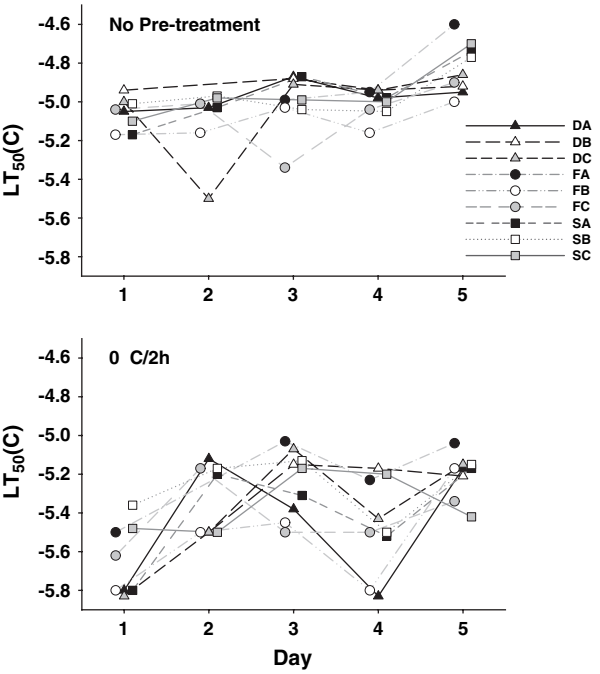

Fig. 2. The LT_{50} values of adult male *Drosophila melanogaster* after a 2-h exposure to various subzero temperatures on five consecutive days. The LT_{50} for each line is displayed separately for each day to display the interday and interline variation. Lines DA, DB and DC were selected for desiccation resistance; FA, FB and FC are fed controls; and SA, SB and SC are starved controls. The top graph shows flies that were exposed without a pretreatment; the bottom shows flies that had a 2 h pre-exposure to 0 °C prior to exposure. Because of missing values on days 1 and 2, only days 3–5 were used in statistical analyses.

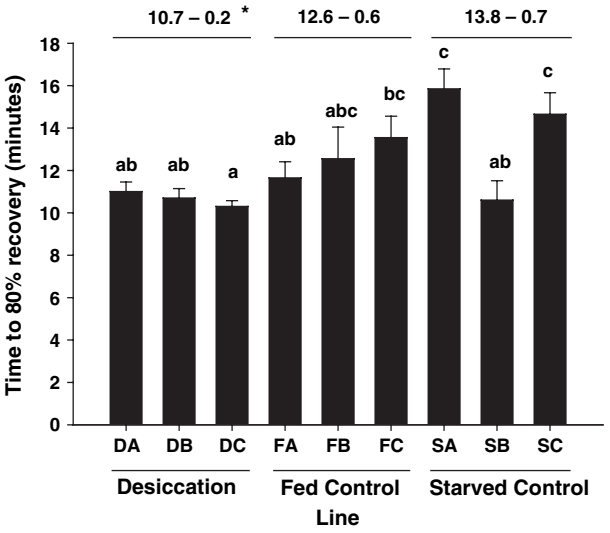

Fig. 3. Time taken until 80% of *Drosophila melanogaster* males selected for desiccation (plus starved and fed controls) recovered from chill coma after a 4-h exposure to 0 °C. Data are shown for ten replicates (mean ± SE) from each line, and an overall least-squares mean for each treatment is shown above the bars. Bars with differing letters are significantly different ($P < 0.05$), in that the overall mean of the desiccation-selected flies differed from the other two groups.

Discussion

Effects of desiccation selection on basal cold tolerance and RCH

Selection for desiccation (and the starvation control) does not affect the basal tolerance of adult *D. melanogaster* to acute cold exposure, nor does selection affect the ability of *D. melanogaster* to exhibit the RCH response. The present study appears to be the first to examine the effects of desiccation and starvation selection on the plasticity of another stress resistance trait (in this case, the RCH response). The lack of change in basal cold tolerance observed here contrasts with the results of a study by Bubliy & Loeschcke (2005), who show that desiccation selection confers increased tolerance to a long exposure at 0 °C; with Telonis-Scott *et al.* (2006), who show a decrease in cold tolerance after selection for desiccation resistance in one of their two selected lines; and with Hoffmann *et al.* (2005a), who show that *D. melanogaster* selected for starvation tolerance have decreased survival of an acute cold shock. Telonis-Scott *et al.* (2006) employ an acute cold exposure (2.5 h at –2 °C) as their assay, so their metric is likely directly comparable with that used in the present study. Moreover, these three studies combined with the present one offer three different relationships between laboratory-evolved desiccation resistance and basal cold tolerance. It is unclear whether this divergence in relationship is due to chance variation in trajectories of selection lines, or due to genetic differences between the founding populations. Another possible source of discrepancy may be the sex of the flies: the present study uses males, whereas

other studies perform cold tolerance experiments on mated females (Bubliy & Loeschcke, 2005; Hoffmann *et al.*, 2005a; Telonis-Scott *et al.*, 2006). However, Telonis-Scott *et al.* (2006) perform many of their stress assays (but not cold tolerance) on both males and females, and demonstrate that the direction of response is consistent between genders for other traits.

The expected relationship between cold and desiccation tolerance in a chill-susceptible species like *D. melanogaster* is not as clear-cut as in species that tolerate extreme subzero temperatures because the causes of injury under the two stresses are still unclear. Bubliy & Loeschcke (2005) link the likely adaptations associated with starvation resistance and cold tolerance, in that carbohydrate and energy stores have been linked to survival of each stress. However, given the broad array of both potential carbohydrate responses and patterns of cold tolerance (Sinclair & Roberts, 2005), such a connection may be premature, particularly in light of the lack of cross-tolerance revealed in the present experiment.

There are significant differences in basal cold tolerance between lines of flies (although not localized to any particular selection regime), and on different days on which the experiment was run. Although the interday differences could reflect subtle differences in the efficiency of the cooling systems or handling, it is noted that that although flies are the same age at the time of each experiment, the experimental populations of flies derive from two egg collection events (the first provides the bulk of the flies on days 1 and 2, the second provides the bulk of the flies on days 4 and 5, and day 3 is an approximately even mix of the two cohorts). The flies that eclose from the same cohort but on different days thus represent differences in development time. Thus, 'Day' may indicate a more complex relationship associated with the effects of development time on cold tolerance. Indeed, this effect is also reported by Nilson *et al.* (2006). *Drosophila melanogaster* selected for fast development are significantly smaller (Nunney, 1996; Prasad *et al.*, 2000; Chippindale *et al.*, 2003) and, although the relationship has been poorly explored (Chown *et al.*, 2002), body size is correlated with chilling tolerance in some insects (Renault *et al.*, 2003). The body size of the flies is not measured on the different days. Gefen *et al.* (2006) report increased body size in desiccation-selected flies, which would predict increased cold tolerance if body size were the sole determinant of cold tolerance. Thus, the influence of development time on cold tolerance appears to be complex, but perhaps worthy of exploration because the many factors affecting cold tolerance in *D. melanogaster* are unravelled. Nilson *et al.* (2006) demonstrtate an effect of development time on the RCH response, although an effect of 'Day' on RCH is not observed in the present experiments.

Effects of desiccation selection on chill coma recovery

Chill coma recovery is a commonly measured trait in *Drosophila* that responds to selection both in the laboratory (Anderson *et al.*, 2005) and in the field (Gibert *et al.*, 2001). Selection for desiccation resistance results in a decrease of approximately 2 min in chill coma recovery time compared with starved or fed control flies, equating to a 22 and 15% decrease from starved and fed control lines, respectively. Anderson *et al.* (2005) employ a 4-h exposure at 0 °C, and show that selection for rapid chill coma recovery results in a decrease in recovery time in male flies from approximately 25 to 12 min. This indicates that the effect of desiccation selection on chill coma reported in the present study is not as great as when selection is specifically directed at chill coma recovery. In *D. melanogaster* in the field, Hoffmann *et al.* (2005b) show that strong clinal patterns in chill coma recovery (and basal cold tolerance) are not mirrored in tolerances to desiccation or starvation, suggesting that selection for the stresses may not be closely linked in the field. The observed differences between the desiccation-selected and starved-control lines would be even greater, if one of the lines (SB) did not show a considerably lower chill coma recovery time than the other two (Fig. 3). Previous analyses, including biochemical, physiological and whole-genome expression microarrays, have not detected any unusual properties of the SB line (A.G. Gibbs and C.H. Vanier, unpublished observations), although these assays have not been conducted with respect to low temperature exposures.

The physiological mechanisms occurring during recovery from chill coma have not been investigated. If it can be assumed that the processes during recovery mimic those during onset of chill coma, then recovery is likely contingent upon the re-establishment of ion gradients, allowing nervous and muscle activity (Goller & Esch, 1990; Kostal *et al.*, 2006). In a different desiccation selection experiment, Folk & Bradley (2003) show that improved regulation of ions at the whole body level during recovery is an important aspect of the response to desiccation selection. However, they find no substantial differences between desiccation-selected and control lines prior to desiccation treatments, and responses of cellular-level ion are not investigated. Nevertheless, the possibility that changes in cellular-level ion regulation are common to plasticity of recovery from both chill coma and desiccation merits further work, and emphasizes the importance of investigating the mechanisms of recovery from environmental stresses.

Conclusions

The present study finds no effect of desiccation selection on basal cold tolerance or RCH response, which differs from the results gathered from other lines and using different methods. Together with gene expression data (Sinclair *et al.*, 2007) and equivocal evidence from multiple selection studies, this implies that the evidence for cross-tolerance between cold and desiccation tolerance in *D. melanogaster* is limited. An apparent development time effect on basal cold tolerance is observed, which is consistent with other work (Nilson *et al.*, 2006), and merits further investigation. These results do indicate a significant effect of desiccation selection on chill coma recovery although, given the lack of understanding of the

mechanisms underlying the latter, this result does not shed light on the mechanism explaining that cross-tolerance.

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