Response to selection for rapid chill-coma recovery in *Drosophila melanogaster*: physiology and life-history traits

**Alisha R. Anderson**, Ary A. Hoffmann and Stephen W. McKechnie

*Centre for Environmental Stress and Adaptation Research (CESAR), School of Biological Sciences, Monash University, Victoria 3800, Australia

*Centre for Environmental Stress and Adaptation Research (CESAR), La Trobe University, Bundoora 3083, Australia

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**Summary**

Resistance to low temperatures can vary markedly among invertebrate species and is directly related to their distribution. Despite the ecological importance of cold resistance this trait has rarely been studied genetically, mainly because low and variable fitness of offspring from cold-stressed mothers makes it difficult to undertake selection experiments and compare cold resistance of parents and offspring. One measure of cold resistance that varies geographically in *Drosophila melanogaster* and that is amenable to genetic analysis is chill-coma recovery. Three replicate lines of *D. melanogaster* were selected every second generation, for over 30 generations, for decreased recovery time following exposure to 0 °C. Correlated responses were scored to characterize underlying physiological traits and to investigate interactions with other traits. Lines responded rapidly to the intermittent selection regime with realizedheritabilitiesvarying from 33% to 46%. Selected lines showed decreased recovery time after exposure to a broad range of low temperatures and also had a lower mortality following a more severe cold shock, indicating that a general mechanism underlying cold resistance had been selected. The selection response was independent of plastic changes in cold resistance because the selected lines maintained their ability to harden (i.e. a short-term exposure to cool temperature resulted in decreased recovery time in subsequent chill-coma assays). Changes in cold resistance were not associated with changes in resistance to high temperature exposure, and selected lines showed no changes in wing size, development time or viability. However, there was a decrease in longevity in the selected lines due to an earlier onset of ageing. These results indicate that chill-coma recovery can be rapidly altered by selection, as long as selection is undertaken every second generation to avoid carry-over effects, and suggest that lower thermal limits can be shifted towards increased cold resistance independently of upper thermal limits and without tradeoffs in many life-history traits.

1. **Introduction**

Distribution limits of many insects and other ectotherms have been linked to their physiological and fitness responses to low temperatures (e.g. Parsons, 1982; Chown, 2001; Voituron et al., 2002). In general, insects show more variation for lower thermal limits than their upper limits along altitudinal and latitudinal gradients, and some progress has been made towards understanding the physiological basis of species differences underlying these limits (e.g. Kimura, 1988; Addo-Bediako et al., 2000; Hoffmann et al., 2003). However, there have been few microevolutionary studies testing how insects have adapted to low temperatures. Exceptions include research on overwintering responses such as diapause in pitcher plant mosquitoes (Campbell & Bradshaw, 1992; Bradshaw et al., 2003) and *Drosophila* studies involving strain and population comparisons for cold resistance (Chen & Walker, 1993; Gibert et al., 2001; Bubliy et al., 2002; Hoffmann et al., 2002). As a result there is limited information on the types of trait interactions that can limit selection responses to cold temperatures, as well as limited understanding of the...
life-history traits, physiological processes and genes that underlie microevolutionary adaptive shifts in populations challenged by lower temperatures.

The evolution, genetics and mechanisms of cold resistance can be productively investigated in Drosophila, both because the nature and extent of resistance varies so much between and within species (Stanley et al., 1980; Kimura, 1988; Gibert et al., 2001) and because the organism is particularly amendable to detailed, controlled experimentation. A few experiments involving selection for cold resistance have been undertaken in Drosophila (Tucic, 1979; Chen & Walker, 1993; Watson & Hoffmann, 1996) but this approach has been difficult because of carry-over effects brought on by stressful conditions. Cold-exposed parents, especially mothers, often have offspring with a low fitness. Lack of replication has also been an issue (Hoffmann et al., 2003).

Recently a new assay to facilitate microevolutionary studies of cold resistance has been developed in Drosophila involving recovery from a chill coma. Chill coma is a narcosis-like state induced in many species at cool temperatures (Schenker, 1984; Leather et al., 1993). This state is reversible in Drosophila upon returning to warmer temperatures, and the amount of time taken for flies to recover can be used as a reliable measure of cold tolerance (Gibert et al., 2001). This method is likely to be more amenable to genetic analysis than methods based on mortality, because less severe stress levels are used and because the cold resistance of individuals can be scored prior to these individuals being used for breeding.

Using this assay Gibert et al. (2001) have shown that Drosophila species confined to more tropical climates have a lower resistance to cold temperatures than other species from temperate areas. Moreover, at the intraspecific level, Hoffmann et al. (2002) have described geographical clinal variation in chill-coma recovery for Drosophila melanogaster from the eastern coast of Australia, with the high-latitude populations showing an increased resistance to cold. Such clinal variation provides indirect evidence for thermal adaptation within a species (Hoffmann et al., 2003), particularly as it also occurs over a similar geographic gradient in a different species, Drosophila seratta (Hallas et al., 2002).

In this study we describe the response to selection for decreased recovery time from chill coma in D. melanogaster. By starting with cold-sensitive individuals from tropical low-latitude populations, we show that strains can be derived that are as resistant as strains from cool temperate high latitudes. Recent studies involving chill coma have characterized recovery times across a range of chilling temperatures and demonstrated a biphasic response (David et al., 2003; McDonald et al., 2004). A biphasic response is where recovery time increases with increasing stress levels then plateaus, and can even decrease, before increasing again as stress levels become even more severe. It is suggested that two different mechanisms are at work, one influencing recovery at lower levels of stress and the second ‘switching on’ only when a threshold stress temperature is experienced, resulting in the plateau in recovery time. We have therefore characterized the recovery response of cold-resistant and control flies across a range of exposure temperatures to test whether there has been a shift in the biphasic response. We have also tested whether the selection response has altered hardening effects, i.e. whether a short-term exposure to cool temperatures still result in a decrease in recovery time in subsequent chill coma assays. The impact of selection on cold-induced mortality was also characterized for comparison with earlier experiments based on mortality-based selection (Chen & Walker, 1993; Watson & Hoffmann, 1996). Finally, the impact of selection on life-history trade-offs was examined along with any effects on heat resistance, which can also influence the ecology and distribution of species.

2. Materials and methods

(i) Drosophila lines and selection

All populations were reared on an instant mashed potato, sugar and yeast medium at 25 °C with a 12:12 L:D cycle. Carbon dioxide anaesthesia was used to count flies for all assays, except heat knockdown, and 24 h recovery was always allowed before any experiments were carried out. Selection and control lines originated from a mass-bred population derived by mixing five isofemale lines from each of five low-latitude populations (Cape Tribulation 16°01’, Innisfail 17°30’, Townsville 19°15’, Sarina 21°25’ and Gladstone 23°50’) in a population cage. The isofemale lines were established in 2002 and the mass-bred population was established after lines had been reared for three to five generations. The mass-bred population was maintained in the laboratory for three generations before selection. Replicate selection and control lines were set up by placing 25 females and 25 males in each of five bottles for each line. Flies were selected in the parental generation, then F1s were unselected, and F2s became the next selected parental population. For each selected generation 1000 flies (500 males and 500 females) were placed at 0 °C for 4 h. For each selected line the 50 fastest males and 50 fastest females to recover from chill coma were kept as parents of the next generation, 10 males and 10 females being placed in each of five bottles. Eggs laid 3–5 days after selection were used as the next generation, such that selected females were likely to have remated with selected males. Control lines were not exposed to any treatment, but
10 males and 10 females were placed in each of five bottles for each line. The subsequent generation was not selected because carry-over effects inhibit the selection response for cold stress (Watson & Hoffmann, 1996). Instead for each of five bottles for each control or selected line 25 males and 25 females were used as the parents of the next generation.

(ii) Thermal resistance assays

Chill-coma recovery selection: For each selected line and for both sexes, 10 glass vials each containing 50 flies (4–7 days old) were placed for 4 h at 0 °C in a cooled bath containing ethylene glycol antifreeze, and then returned to 25 °C for recovery. Flies were considered recovered when able to stand up.

Analysis of selection: The selection response was measured at each selected generation. For both sexes and each line, five glass vials containing 10 flies (5–8 days old) were placed at 0 °C for 4 h and returned to 25 °C for recovery. At 2 min intervals the number of flies recovered in each vial was recorded and used to determine the recovery time of individual flies. The mean recovery time for each line was used to evaluate the selection response.

Recovery over temperature range: Vials of 4-day-old flies were set up as above and were placed at temperatures ranging from 4 °C to −1 °C for 4 h and returned to 25 °C for recovery. Recovery time was scored as above.

Mortality experiments: Vials of flies were set up as above and were placed at −2 °C for 4 h and survivors were scored after 24 h recovery on fresh medium at 25 °C.

Hardening experiments: For both sexes and each line, three glass vials with medium each containing 10 flies were placed at 13 °C for 48 h. Control vials with medium were left at 25 °C. All flies were transferred to empty vials immediately before being placed at 0 °C for 8 h. We used an 8 h exposure to ensure the hardening effect could be scored accurately. If a 4 h exposure was used the selected lines that were subjected to hardening recovered too quickly and the recovery time estimate was not accurate. Recovery time was scored as above.

Heat knockdown assay: Knockdown time for individual females was scored as described by Anderson et al. (2003). Briefly, individual flies were placed in small glass vials and immersed in a circulating water bath at 39 °C. Knockdown time was scored in four batches, with five hardened and five unhardened flies of each line run in each batch. To harden flies, they were held in batches of 10 in vials with food in a 37 °C incubator for 1 h and then held with food for 6 h at 25 °C prior to testing. Treatment at 37 °C has been shown to provide a strong hardening response in D. melanogaster and the effect is maximized 6 h after the treatment (Krebs & Loeschcke, 1994).

(iii) Life-history measurements

Development time and viability: Egg-to-adult development time was scored by placing 10 eggs into a vial (15 replicate vials per line) and scoring the number of flies eclosing every 6 h. Egg-to-adult viability was measured as the percentage of eggs eclosed per vial.

Wing area: Flies were cultured at 25 °C at low density prior to wing area measurements. This was scored by removing the left wing of 10 females per line and mounting them on microscope slides on double-sided tape. Wing images were captured via video camera, and all wings were randomized before landmarks (described in Gilchrist & Partridge, 2001) were marked using tpsDig version 1.2 written by F. James Rohlf. The x- and y-coordinates of all landmarks were used to measure an overall wing area (centroid size – the square root of the sum of squared coordinates of the landmarks).

Fecundity: This was scored with and without a 4 h 0 °C cold stress. Fecundity without a stress was scored over the first 10 days after eclosion. Fecundity after cold stress was scored 10 days after females were exposed to the cold stress when 4 days old. Each female was placed with a male in a 30 ml vial. For oviposition, a spoon with 2 ml of medium and coated with live yeast paste was placed in each vial. Spoons were replaced every 24 h for 10 days and numbers of eggs counted. Twenty replicate pairs were set up for each line.

Ageing: Flies were reared at 25 °C at controlled densities (300–400 eggs per 50 ml of medium). For each line two replicates of 100 1-day-old males were kept in 2000 ml population cages at 25 °C. Each cage had two vials of food medium replaced every 2–3 days. The number of dead flies was scored each day until all flies were deceased.

(iv) Statistical analysis

The response to selection was analysed by computing realized heritabilities over the first 22 generations of culture (10 generations of selection). To compare selected and control chill-coma recovery times two generations after each episode of selection, nested analysis of variance (ANOVA) was used, with replicate line tested within the selection regime. Nested ANOVA was also used to compare control and selected lines for mortality, heat knockdown, fecundity, development time, wing length and viability. For the experiments with hardening effects, a hardening term was included in the ANOVA. For ageing, the survival module of the JMP statistical package (SAS Institute, Cary, NC) was used.
3. Results

(i) Selection response

For both sexes, lines responded quickly to selection (Fig. 1). The chill-coma recovery time of the selected lines was already significantly decreased compared with that of the control lines after three generations of selection (ANOVA; females: $F_{1,4} = 18.729$, $P < 0.01$; males: $F_{1,4} = 33.59$, $P < 0.01$) as well as in subsequent generations (ANOVAs not presented). Realized heritabilities at $F_{22}$ after 10 generations of selection were $0.38\pm0.04$, $0.46\pm0.09$ and $0.33\pm0.13$, for selected lines 1, 2 and 3 respectively.

(ii) Recovery at other temperatures

For both the control and selected lines and for both sexes, recovery time decreased between 0 and $-1^\circ C$ (Fig. 2). All three selected lines showed decreased recovery times compared with controls at 2, 0 and $-1^\circ C$ but not 4 $^\circ C$, when recovery was rapid for all lines (Fig. 2). Nested ANOVA (Table 1) shows the recovery times for selected lines were significantly decreased compared with those for control lines at all temperatures except 4 $^\circ C$. There were also significant differences among the replicate lines for both sexes (Table 1).

(iii) Cold mortality and cold hardening

For both sexes, selected lines showed significantly increased survival after exposure to $-2^\circ C$ compared with the control lines (Fig. 3a, Table 1).

In the cold hardening experiments there was a significant effect of hardening treatment and a

Table 1. $ANOVAs$ for recovery and cold mortality assays

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Selection (df = 1)</th>
<th>Line (df = 4)</th>
<th>Vial (df = 18)</th>
<th>Error (df)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Females</strong> Recovery assay</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4 $^\circ C$</td>
<td>19.65</td>
<td>5.11</td>
<td>18.10</td>
<td>12.86 (212)</td>
</tr>
<tr>
<td>2 $^\circ C$</td>
<td>1031.57**</td>
<td>34.34*</td>
<td>9.83</td>
<td>8.97 (206)</td>
</tr>
<tr>
<td>0 $^\circ C$</td>
<td>5373.09***</td>
<td>43.19</td>
<td>66.79</td>
<td>25.26 (198)</td>
</tr>
<tr>
<td>$-1^\circ C$</td>
<td>3862.58**</td>
<td>78.93**</td>
<td>18.12</td>
<td>22.98 (206)</td>
</tr>
<tr>
<td>Mortality assay</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$-2^\circ C$</td>
<td>7012.67**</td>
<td>623.04</td>
<td>N/A</td>
<td>503.04 (12)</td>
</tr>
<tr>
<td><strong>Males</strong> Recovery assay</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4 $^\circ C$</td>
<td>67.09</td>
<td>28.75*</td>
<td>8.36*</td>
<td>4.52 (183)</td>
</tr>
<tr>
<td>2 $^\circ C$</td>
<td>651.95*</td>
<td>50.93**</td>
<td>7.45</td>
<td>7.13 (200)</td>
</tr>
<tr>
<td>0 $^\circ C$</td>
<td>2863.97***</td>
<td>69.32**</td>
<td>21.29</td>
<td>18.88 (188)</td>
</tr>
<tr>
<td>$-1^\circ C$</td>
<td>2415.89***</td>
<td>17.66</td>
<td>18.83</td>
<td>12.32 (209)</td>
</tr>
<tr>
<td>Mortality assay</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$-2^\circ C$</td>
<td>10005.35**</td>
<td>143.90</td>
<td>N/A</td>
<td>19.97 (12)</td>
</tr>
</tbody>
</table>

Mean squares for the different exposure temperatures are given and significance of $F$ ratios indicated. Line is nested in Selection and Vial in Line and Selection. *$P<0.05$; **$P<0.01$; ***$P<0.001$.
Heat resistance

Mean heat knockdown times did not differ significantly between control and selected lines (Table 2). There was a significant effect of heat hardening across all lines but we did not detect a selection by hardening interaction.

(iv) Life-history traits

Comparisons between selected and control lines for wing size, development time and viability indicated no difference between selected and control lines (Table 3). For fecundity, there was a significant nested line effect (Table 3), due to the low fecundity of two of the selected lines compared with a relatively high fecundity for the third line (Fig. 4). Fecundity after a cold shock was significantly higher in selected lines than the control lines (Fig. 4, Table 3).

Selected lines had reduced longevity when compared with control lines (Fig. 5). Likelihood ratios indicate a significant difference between control and selected ageing curves ($G = 17.54$, df = 1, $P < 0.001$) and no nested line differences ($G = 6.95$, df = 4, $P > 0.05$). The mean longevity was 32.86, 32.88 and 34.06 days for the Selected 1, Selected 2 and Selected 3 lines respectively, compared with means of 34.57, 38.21 and 34.61 days for Control 1, Control 2 and Control 3 lines. The ageing curve (Fig. 5) suggests...
that there is a shift in the onset of ageing because the rate of death increased earlier in the selected lines compared with the control lines.

4. Discussion

(i) Selection response

The experiments show that Drosophila melanogaster can respond quickly to chill-coma selection, indicating that chill coma is a heritable trait. The mean estimate of heritability was 38.3%, which is similar to an estimate of 30% for selection on cold shock survival (Watson & Hoffmann, 1996).

Physiological mechanisms underlying chill-coma recovery are not understood. Recent experiments on the relationship between recovery time and temperature in D. subobscura (David et al., 2003) and D. melanogaster (McDonald et al., 2004) suggest that there may be two mechanisms involved in recovery, one specific to cooler temperatures and leading to a plateau in recovery time as stress is increased. This plateau was evident in the current experiment (Fig. 2), where for both control and selected lines recovery time decreases between 0 and $x_1^\circ \text{C}$, counter to expectations based on a linear association between temperature and stress levels. The plateau suggests that a mechanism countering the effect of cold stress was ‘switched on’ by the $x_1^\circ \text{C}$ treatment. This ‘switch’ temperature appears to be similar to one observed by McDonald et al. (2004), who also found a plateau between 0 and $-1^\circ \text{C}$. In D. subobscura, a plateau in recovery time from cold exposure occurred between $-4^\circ \text{C}$ and $-6^\circ \text{C}$ (David et al., 2003). D. subobscura is a temperate zone species more resistant to cold than D. melanogaster. Because selected lines showed relatively decreased recovery times across a range of temperatures, selection has altered traits related to overall cold resistance rather than specific cold tolerance traits.
than one of the temperature-specific mechanisms underlying the plateau in recovery times.

The results for the mortality assays show the lines selected for chill-coma recovery also had increased survival after a severe, $-2^\circ$ C, cold exposure. The relationship between chill-coma recovery and mortality assays has not been extensively studied; however, both traits vary clinally along the Australian east coast over a climatic gradient and may be under similar selective forces (Hoffmann et al., 2002).

Selection for increased resistance can influence hardening or the hardening response if mechanisms that underlie the hardening response become constitutively expressed in the selected lines. For instance, lines selected for desiccation resistance showed a reduced response to hardening relative to unselected lines, suggesting that the mechanism responsible for hardening also partly formed the basis of the increased unhardened resistance in the selected lines (Hoffmann, 1990). In the current study the cold-selected females and males retained the ability to harden, although there was a reduced response in the males. The hardening mechanism is still ‘available’ to increase resistance following an acclimating cold stress in the selected lines. Perhaps sex differences in the metabolic responses involved in hardening (Duman et al., 1982; Thomashow, 1999; Ramlov, 2000; Misener et al., 2001) contribute to the different response to hardening in males compared with females.

(ii) Other correlated traits

Previous studies in D. melanogaster have suggested possible relationships between cold and heat resistance. At a geographical level, the two traits have opposing latitudinal clines along the east coast of Australia (Hoffmann et al., 2002). At a genetic level, variation attributable to genes on the right arm of chromosome 3 that conferred cold tolerance has also been associated with heat susceptibility (Anderson et al., 2003). However, the selected lines in this study were tested for both hardened and non-hardened heat knockdown time and showed no difference in control and selected lines, suggesting no genetic trade-off between resistance levels to opposite extremes of thermal stress.

In previous studies fecundity was the only life-history trait affected by selection to increased survival after cold shock (Watson & Hoffmann, 1996). In the present study fecundity was reduced in two of the three selected lines and may not constitute a robust correlated response (Harshman & Hoffmann, 2000). When fecundity was measured directly after a cold exposure, the selected lines produced more eggs after the exposure than the controls. This is most probably a result of indirect selection as genes conferring an increased fecundity after a cold exposure would result in an increased number of offspring in the next generation, independent of recovery time. The other life-history traits measured (body size, development time and viability) did not differ significantly between control and selected lines, consistent with previous results (Watson & Hoffmann, 1996).

In past selection experiments, the correlation between ageing and cold resistance has been inconsistent. In one study, lines selected for delayed senescence resulted in both greater longevity and greater cold resistance in selected lines compared with control lines (Luckinbill, 1998). However, in another study, cold selected lines lived longer than controls at 14$^\circ$C but lived shorter than controls at 25$^\circ$C, although neither of these differences was significant (Norry & Loschke, 2002). In the current study selected lines had a reduced longevity compared with control lines, suggesting the mechanism that increases cold resistance resulted in decreased longevity in these lines. The relationship between longevity and cold resistance remains unclear.

We have found that Drosophila melanogaster can respond rapidly to chill-coma recovery selection as long as selection is only carried out every second generation. This process appears to select a general mechanism of cold resistance affecting cold resistance across a range of low temperatures while being independent of high-temperature resistance and most life-history traits.

The next step in understanding adaptation to extreme cold temperatures will involve linking natural and laboratory-selected variation in cold resistance with underlying physiological, molecular and genetic mechanisms. Molecular analysis in these selected lines should help elucidate important genes and processes involved in cold resistance.

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