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Survival rate and expression of *Heat-shock protein 70* and *Frost* genes after temperature stress in *Drosophila melanogaster* lines that are selected for recovery time from temperature coma

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ABSTRACT

In this study, we investigated the physiological mechanisms underlying temperature tolerance using *Drosophila melanogaster* lines with rapid, intermediate, or slow recovery from heat or chill coma that were established by artificial selection or by free recombination without selection. Specifically, we focused on the relationships among their recovery from heat or chill coma, survival after severe heat or cold, and survival enhanced by rapid cold hardening (RCH) or heat hardening. The recovery time from heat coma was not related to the survival rate after severe heat. The line with rapid recovery from chill coma showed a higher survival rate after severe cold exposure, and therefore the same mechanisms are likely to underlie these phenotypes. The recovery time from chill coma and survival rate after severe cold were unrelated to RCH-enhanced survival. We also examined the expression of two genes, *Heat-shock protein 70* (*Hsp70*) and *Frost*, in these lines to understand the contribution of these stress-inducible genes to intraspecific variation in recovery from temperature coma. The line showing rapid recovery from heat coma did not exhibit higher expression of *Hsp70* and *Frost*. In addition, *Hsp70* and *Frost* transcription levels were not correlated with the recovery time from chill coma. Thus, *Hsp70* and *Frost* transcriptional regulation was not involved in the intraspecific variation in recovery from temperature coma.

1. Introduction

Since temperature affects many physiological processes in ectotherms, such as insects, it is one of the most important factors affecting their distribution, timing of reproduction, and voltinism (Chown and Nicolson, 2004). The two main methods used to investigate temperature tolerance in insects are measurements of characteristics related to temperature coma (stupor or knock-down) and measurements of survival rates (Chown and Nicolson, 2004; Hoffmann et al., 2003). The first method measures the temperature needed to induce coma in insects that are exposed to heat or cold stress, or the time needed to enter to the coma or to recover from the coma (Anderson et al., 2005; David et al., 1998, 2003; Macdonald et al., 2004; Mori and Kimura, 2008). The second method measures the survival rates of insects after severe thermal stress (Goto and Kimura, 1998; Hoffmann and Watson, 1993; Sørensen and Loeschcke, 2002; Worthen and Haney, 1999). Both of these methods have been used to study temperature tolerance in many insects (Chown and Nicolson, 2004) and have provided valuable information about intraspecific and interspecific variations in temperature tolerance. However, it is still unclear whether the characteristics detected by coma-based assays and survival-based assays indicate just different aspects of temperature tolerance developed by the same physiological mechanism or they are operated by different mechanisms (Hoffmann et al., 1997), especially in studies about cold tolerance.

In *Drosophila*, selection experiments have been extensively used to elucidate the mechanism of thermal tolerance (Anderson et al., 2005; Bubliy and Loeschcke, 2005; Folk et al., 2006; Hoffmann et al., 2003; MacMillan et al., 2009; Mori and Kimura, 2008; Norry et al., 2007; Sejerkilde et al., 2003). Recently, Mori and Kimura (2008) used artificial selection and free recombination without selection to establish *Drosophila melanogaster* lines with rapid, intermediate, or slow recovery from heat or chill coma. This led us to use these lines to investigate physiological mechanisms underlying temperature tolerance. First, we explored whether the same physiological mechanisms were responsible for both coma recovery and survival after severe temperature stresses. Second, we investigated the relationship between coma recovery and survival enhanced by rapid cold hardening (RCH) or heat hardening, i.e., enhancement of temperature tolerance by short periods of mild temperature exposures (Lee et al., 1987, 2006; Overgaard et al., 2007; Rajamohan and Sinclair, 2008).

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Third, we studied the expression of two genes encoding for *Heat-shock protein 70* (*Hsp70*) and *Frost* to determine their contribution to intraspecific variation in coma recovery times. In *Drosophila* and other insects, expression of *Hsp70* is induced by heat and cold shock (Burton et al., 1988; Goto and Kimura, 1998; Koštál and Tollarová-Borovanská, 2009; Sinclair et al., 2007). Similarly, *Frost* is a stress-inducible gene that is induced by cold shock as well as septic injury and desiccation (Goto, 2001; De Gregorio et al., 2002; Sinclair et al., 2007).

2. Materials and methods

2.1. Insects and selection of recovery time from heat and chill coma

Mori and Kimura (2008) first established *D. melanogaster* lines with rapid, intermediate, or slow recovery from heat or chill coma by artificial selection and free recombination without selection. Briefly, 15 isofemale lines of *D. melanogaster* from different parts of Japan were mixed and maintained for three generations before selection. The flies were grown under constant light conditions at 25 ± 1 °C on a cornmeal-malt medium. One hundred virgin females and 100 males 5 days after eclosion were exposed to temperature stress to induce coma by either placing them on ice for 4 h or at 38.0 °C for 30 min in a water bath. Flies were considered to have recovered when they stood up on their legs (David et al., 1998). At 2 min intervals, the number of recovered flies was recorded. The 10 fastest or slowest recovering flies of each sex in each temperature stress condition were selected as parents of the next generation. As a result, four lines were produced from these selection regimes: HR (rapid recovery from heat coma), HS (slow recovery from heat coma), CR (rapid recovery from chill coma), and CS (slow recovery from chill coma). Although we produced two replicates for each selection line (HR1, HR2, HS1, HS2, CR1, CR2, CS1, and CS2) and three control lines (CTL1–3) maintained without any treatment, we combined the data for each regime because they were almost identical. Selection was performed every 2–4 generations to avoid any carry-over effect that could inhibit the selection response (Watson and Hoffmann, 1996). Anesthesia was not used at any point in the process.

We transferred the lines at the 25th–27th generations from Dr. Kimura's laboratory (Graduate School of Environmental Earth Science, Hokkaido University, Sapporo, Japan) to our laboratory (Graduate School of Science, Osaka City University, Osaka, Japan). Our selection procedure was slightly modified from that of Mori and Kimura (2008); we used 80–120 virgin flies and selected 10% of the fastest or slowest recovering flies of each sex at each temperature as parents of the next generation. In addition, we employed a slightly higher temperature (38.3 °C, 30 min) for heat selection because HR did not fall into heat coma at 38.0 °C.

We measured the recovery time from heat and chill coma in CTL at the 29th and 31st generations, respectively.

2.2. Survival rates after heat and cold exposure

Prior to experiments, fresh vials with medium were heated or cooled to the experimental temperatures. Only males 5 days after eclosion were used. Survival rates after heat exposure were examined at the 33rd and 35th generations in HR1 and HR2, respectively, at the 33rd generation in HS, and at the 37th generation in CTL. The flies were incubated either at 39.7 °C for 30 min (severe heat stress) or 31.0 °C for 30 min followed by exposure to 39.7 °C for 30 min (heat hardening). Similarly, the survival rates after cold exposure were examined at the 33rd generation in CR, CS, and CTL. The flies were chilled either at −7 °C for 1 h (severe cold stress) or at 0 °C for 2 h followed by exposure to −7 °C for 1 h (RCH). After exposure to temperature stress, the flies were transferred to fresh vials with medium at 25 °C. Finally, survival rates were assessed after 24 h. Measurements were made in triplicate, with each replicate having 28–80 flies.

2.3. Real-time PCR

Accumulation of *Hsp70* and *Frost* mRNAs during and after heat exposure was examined at the 29th generation in HR and HS and at the 33rd generation in CTL. Flies were incubated at 38.3 °C for 0, 10, or 30 min. Some flies also were incubated at 38.3 °C for 30 min, and then returned to 25 °C for 30 min. Likewise, accumulation of *Hsp70* and *Frost* mRNAs before and after cold exposure was examined in CR, CS, and CTL at the 31st generation. Flies were chilled at 0 °C for 4 h, and then returned to 25 °C for 0, 10, or 30 min. After each temperature stress condition, 10 flies were transferred to a fresh tube, and then flash frozen in liquid nitrogen. Only males 5 days after eclosion were used.

Total RNA was extracted with TRIzol reagent (Invitrogen, Carlsbad, CA, USA). RNA pellets were resuspended in water, and then stored at −20 °C. cDNAs were synthesized using a High Capacity cDNA Archive kit (Applied Biosystems, Foster City, CA, USA). Real-time PCR was performed on an Applied Biosystems 7500 Real-Time PCR System with Power SYBR Green (Applied Biosystems). Primers were used at a concentration of 0.05 μM. The primers for *Hsp70* were 5′-GTCAGCGCCAAGGAGATGAG-3′ and 5′-CGTTCAC-CATGCGATCAATC-3′. The primer sequences completely match to *Hsp70Aa*, *Hsp70Ab*, *Hsp70Bb*, *Hsp70Bbb* and *Hsp70Bc* sequences, and therefore the primer set is considered to be able to detect all the *Hsp70* transcripts. The primers for *Frost* were 5′-TGGTCATCATC-CAACAGTCA-3′ and 5′-GGTGGTCAACTCAGGCA-3′. 28S rRNA was used as a control gene for normalization; its primers were 5′-GGAACGTGAGCTGGGTTTAGAC-3′ and 5′-CTGCGGGTTCCTCTC-GTACT-3′. Each set of primers generated amplicons of approximately 100 bp. Quantification of mRNAs was performed using a standard curve. All real-time PCR experiments were performed on 3–6 independent RNA samples. For all reactions, melting analysis confirmed that only a single amplicon was generated.

2.4. Statistical analysis

A natural logarithm transformation was applied to recovery times from heat and chill coma and an arcsine transformation was applied to survival rates. Tukey's test was used to compare the transformed data (Zar, 2009). The relative transcript levels (the ratios to 28S rRNA) were also compared with Tukey's test without transformation.

3. Results

3.1. Selection on recovery time from coma

As shown in Fig. 1, all experimental lines successfully responded to the selection. As a result, the recovery time of the selected lines was significantly different from that of CTL (Fig. 2).

3.2. Survival rates after temperature stress and rapid hardening

After severe heat stress, the survival rates of the HR, CTL, and HS were low, intermediate, and high, respectively, although the differences were not significant ($p > 0.05$) (Fig. 3A). Survival rates after severe heat were higher in flies pretreated at mild heat than in those not pretreated, although the differences were not significant in all lines ($p > 0.05$).

After severe cold stress, the survival rate of CR was significantly higher than those of CS and CTL (Fig. 3B). RCH increased survival rates in all lines; however, the significance of its effects differed among the lines. Specifically, RCH significantly increased the