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Inbreeding affects Hsp70 expression in two species of *Drosophila* even at benign temperatures

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ABSTRACT

Heat shock proteins (Hsps) are molecular chaperones that help organisms to cope with environmental stress. Here we report the effect of temperature on Hsp70 expression in inbred and outbred lines of *Drosophila buzzatii* and *D. melanogaster*. For both species, we found significant effects of temperature and inbreeding on Hsp70 expression. In *D. buzzatii*, inbred larvae expressed more Hsp70 at all temperatures except at very high temperatures close to the physiological limit. In *D. melanogaster*, the overall pattern was similar to that of *D. buzzatii*. At benign temperatures, there was a clear trend towards higher Hsp70 expression in inbred than outbred larvae, whereas at higher temperatures, a trend in the opposite direction was observed. The shift from lower to higher expression in outbred larvae with increasing temperatures occurs at a lower temperature in *D. melanogaster* than in *D. buzzatii*. The reason for this difference may be greater sensitivity to high stressful temperatures in *D. melanogaster*. These results provide the first direct experimental evidence that inbreeding influences the expression of Hsp70 even at non-stressful temperatures.

Keywords: *Drosophila buzzatii, Drosophila melanogaster*, Hsp70, inbreeding, protein conformation, temperature stress.

INTRODUCTION

Heat shock proteins (Hsps) are molecular chaperones that act by holding and refolding denatured and misfolded proteins, as well as by turning damaged proteins to the intracellular degradation system (Gething and Sambrook, 1992). They play an important role in the cell in response to potentially deleterious stress conditions (Lindquist, 1986; Hartl, 1996; Feder and Hofmann, 1999; Jolly and Morimoto, 1999). The expression of heat shock proteins is induced by a variety of environmental and physiological conditions, including

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heat shock, parasitism, alcohol, heavy metals, crowding and inflammation (Lindquist, 1986; Gething and Sambrook, 1992; Hartl, 1996; Feder and Hofmann, 1999; Jolly and Morimoto, 1999; Sørensen and Loeschcke, 2001). In *Drosophila*, Hsp70 is thought to be expressed primarily during exogenous stressful conditions, which can be signalled by an accumulation of proteins with non-native conformations (Ananthan *et al.*, 1986; Parsell and Lindquist, 1994; Krebs, 1999).

The expression of heat shock proteins is of major importance for the maintenance of homeostasis in the cell (Rutherford and Lindquist, 1998; Feder and Hofmann, 1999). Homeostasis is disrupted by environmental factors (see above) and by increased homozygosity due to inbreeding (Lerner, 1954), and thus it can be hypothesized that detrimental effects of inbreeding are associated with changes in Hsp70 expression. The aim of this study was to determine whether a relationship exists between inbreeding and Hsp70 expression. Hsp70 expression is commonly interpreted as an emergency response to exogenous stress in *Drosophila*. Inbreeding, on the other hand, is an endogenous stress that is long-lasting. A change in Hsp70 expression with inbreeding will thus emphasize a new role for the Hsp70 protein besides being a part of the emergency response to exogenous stresses. In addition, an association between inbreeding and Hsp70 expression will shed new light on some of the biochemical processes that are ongoing during inbreeding.

The mechanisms for change in Hsp70 expression with inbreeding are two-fold, as are the interpretations. One possibility is that inbreeding has a negative effect on the rate of Hsp70 expression due to reduced enzyme activity, comparable to the negative effect of inbreeding on the rate of growth and metabolism (Lerner, 1954). Greatly reduced expression of Hsp70 may have a severe effect on overall homeostasis, similar to that of low expression of Hsp90 (Rutherford and Lindquist, 1998). Another possibility is that inbreeding may increase cellular stress due to a higher frequency of homozygotes and the expression of recessive deleterious alleles (Charlesworth and Charlesworth, 1987; Lynch and Walsh, 1998). Increased expression of Hsp70 can thus be envisioned to occur in response to increased cellular stress. Changes in Hsp70 expression with inbreeding can, depending on the direction, have the following implications: First, decreased Hsp70 expression may imply that reduced Hsp70 expression *per se* disrupts homeostasis (inbreeding → decreased Hsp70 expression → disrupted homeostasis). Second, increased Hsp70 expression may imply that the cells operate to restore the homeostasis that is disrupted by inbreeding, by increasing the expression of Hsp70 in the cells (inbreeding → disrupted homeostasis → increased Hsp70 expression).

Many studies have investigated inbreeding depression and evolution in small populations. However, direct experimental evidence for the biochemical mechanisms responsible for inbreeding depression is limited. The major stress protein in *Drosophila*, Hsp70, is an interesting starting point for investigations in this field. We show here that inbreeding affects Hsp70 expression in two species of *Drosophila*. Two independent experiments were performed, the first on *D. buzzatii*. To test the generality of the results obtained from this experiment, a similar investigation, but with more lines included, was performed on *D. melanogaster*. Our results clearly show that inbred larvae express Hsp70 at temperatures that are benign to outbred larvae of these species. For the first time, we demonstrate that, in addition to exogenous stress, inbreeding in an otherwise non-stressful environment induces Hsp70 expression. For both species, there is a shift towards higher expression in outbred larvae at highly stressful temperatures.

MATERIALS AND METHODS

Drosophila buzzatii

A laboratory mass-bred population of *D. buzzatii* was founded from flies collected in spring 1998 on Tenerife, Spain. Altogether, 25 males and 25 females collected from each of four localities separated by a few hundred metres founded the population. Three inbred and three outbred lines were created under standard laboratory conditions (25°C and 12/12 h light/dark cycle) by either full sib mating for five successive generations ($F = 0.672$) or by selecting 200 individuals per line at random to create outbred lines ($F = 0$). To keep the inbreeding constant, population sizes in each line were raised to approximately 500 breeding individuals when the predetermined levels of inbreeding were reached. Flies were held at those population sizes until this experiment was performed, 15 generations after the inbred lines reached $F = 0.672$.

Drosophila melanogaster

To test the generality of the results, a second experiment on a different species and with more lines was performed on *D. melanogaster*. A laboratory mass-bred population of *D. melanogaster* was founded by two populations (each population was founded by 30 isofemale lines) collected near Hov on the east coast of Jutland and near Copenhagen on Zealand, Denmark in October 1997. Before crossing, the two populations were maintained as separate laboratory populations each with sample sizes above 1000 breeding individuals. A new population (Hov-Copenhagen) was established by crossing the two populations in February 1998 and was maintained at a sample size above 1000 breeding individuals under standard laboratory conditions until the present experiment was performed in the autumn of 2001. Ten inbred and 10 outbred lines were created from the Hov-Copenhagen mass-bred population under standard laboratory conditions (25°C and 12/12 h light/dark cycle) by the same procedure as described for *D. buzzatii*. As for *D. buzzatii*, the population size for each line was raised to approximately 500 breeding individuals when predetermined inbreeding levels were reached. Flies were held at those population sizes until this experiment was performed, eight generations after the inbred lines reached $F = 0.672$.

The experiment

In both experiments, young adults (12 ± 12 h old) of the two species were fed on standard laboratory medium (sugar, yeast, oatmeal and agar). Flies were transferred to new vials every second day. On day 6, they were allowed to oviposit for 6 h in vials with instant *Drosophila* medium (Carolina Biological Supply). Third-instar larvae from these vials were transferred to fresh vials with agar and then exposed for 1 h to temperatures of 25, 29, 33, 37, 38, 39 or 40°C and transferred to 25°C for 1 h for recovery, before being frozen at −70°C. Hsp70 was quantified on six replicates of approximately 20 larvae (seven and two larvae from each line of *D. buzzatii* and *D. melanogaster*, respectively) per inbreeding level and treatment by the ELISA technique using the monoclonal antibody 7.FB [according to the protocol described by Dahlgaard *et al.* (1998) and Sørensen *et al.* (1999)], which is specific for inducible Hsp70 in *Drosophila* (Velazques and Lindquist, 1984; Welte *et al.*, 1993).

Hsp70 expression was calculated from six replicate ELISA plates per species. One replicate sample of each treatment and inbreeding level was represented on each plate and variation between plates was corrected by adjusting all readings for each plate according to the grand mean of the first plate. Because of the high variation in Hsp70 expression between the low and the high temperatures, a log transformation of the y-axis was used for the graphical presentation of the data. The statistical analyses were performed on untransformed data (performing the same analysis on log-transformed data revealed no changes in significance).

RESULTS

Drosophila buzzatii

Ninety-seven percent of the variation in Hsp70 expression was explained by the factors inbreeding ($F_{1,70} = 32.7$, $P < 0.001$), temperature ($F_{6,70} = 370$, $P < 0.001$) and their interaction ($F_{6,70} = 12.2$, $P < 0.001$). Significant deviations from zero Hsp70 expression were noted at all temperatures for inbred larvae, even though expression at 25 and 29°C was low compared with that at higher temperatures. In contrast, Hsp70 expression in outbred larvae collected after exposure at 25 and 29°C was not significantly different from zero (test results shown only for 25 and 29°C, as the remaining temperatures are known to induce Hsp70) (one-tailed *t*-tests: outbred 25°C, $t = 1.5$, N.S.; inbred 25°C, $t = 3.8$, $P < 0.01$; outbred 29°C, $t = 2.0$, N.S.; inbred 29°C, $t = 3.7$, $P < 0.01$; significance corrected by the sequential Bonferroni technique). Pairwise comparisons of Hsp70 expression in inbred and outbred larvae at each temperature showed that inbred larvae expressed significantly more Hsp70 except at 38 and 40°C (Fig. 1).

Drosophila melanogaster

The factors inbreeding ($F_{1,70} = 4.74$, $P = 0.0329$) and temperature ($F_{6,70} = 123$, $P < 0.001$) had a significant effect on Hsp70 expression. The interaction between inbreeding and temperature was non-significant ($F_{6,70} = 1.57$, $P = 0.1684$). Significant deviations from zero Hsp70 expression were found at all temperatures for inbred larvae (as for *D. buzzatii*, Hsp70 expression was low compared with that at higher temperatures). In contrast, Hsp70 expression in outbred larvae collected after exposure at 25°C was not significantly different from zero (test results shown only for 25 and 29°C, as the remaining temperatures are known to induce Hsp70 in *D. melanogaster* as well) (one-tailed *t*-tests: outbred 25°C, $t = 1.50$, N.S.; inbred 25°C, $t = 4.29$, $P = 0.0039$; outbred 29°C, $t = 3.77$, $P = 0.0065$; inbred 29°C, $t = 2.72$, $P = 0.0209$; significance corrected by the sequential Bonferroni technique). Pairwise comparisons of Hsp70 expression in inbred and outbred larvae at each temperature showed no significant differences in Hsp70 expression. However, we did observe a trend towards higher expression in inbred larvae at 25 and 29°C (Fig. 2). A shift towards higher expression in outbred larvae at the higher temperatures was also observed (Fig. 2).

DISCUSSION

Inbreeding depression, as explained by the widely accepted partial dominance hypothesis, is caused by expression of deleterious recessive alleles. Increased homozygosity increases the expression of deleterious alleles, which may result in an increase in the proportion of

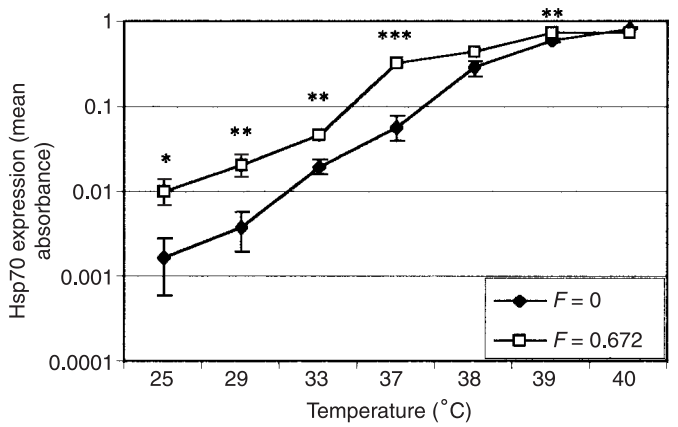

Fig. 1. Hsp70 expression on a log-transformed y-axis (absorbance: mean ± standard error) in third-instar *D. buzzatii* larvae after exposure at 25, 29, 33, 37, 38, 39 or 40°C for 1 h. Significant differences in Hsp70 expression between inbred and outbred larvae assessed by *t*-tests are indicated (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$; significance adjusted according to sequential Bonferroni correction, test results not shown).

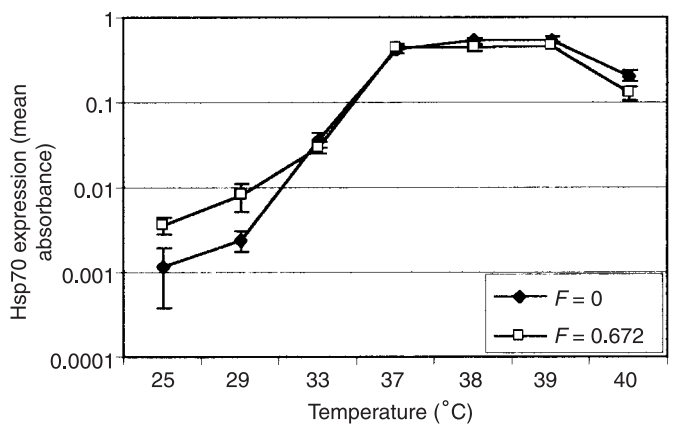

Fig. 2. Hsp70 expression on a log-transformed y-axis (absorbance: mean ± standard error) in third-instar *D. melanogaster* larvae after exposure at 25, 29, 33, 37, 38, 39 or 40°C for 1 h.

proteins with non-native conformation. Proteins with non-native conformation can experience severe problems with misfolding, leaving them in a deleterious state (Bross *et al.*, 1999; Gregersen *et al.*, 2001). The increased expression of Hsp70 observed in this study at benign temperatures may be triggered by greater numbers of misfolded proteins in inbred larvae and may reflect cellular attempts to restore protein homeostasis. Another explanation for the observed pattern could be a change in the epistatic interactions with inbreeding, causing a breakdown of the homeostatic regulation of Hsp70 expression.

The higher Hsp70 expression in inbred than outbred larvae holds for benign to stressful temperatures in *D. buzzatii*. Only at very high temperatures close to the upper physiological

tolerable limit did we observe a tendency towards higher expression in outbred larvae. We suggest that this may be due to a breakdown of the integrated biological system in inbred larvae at such extreme temperatures, whereas in outbred larvae Hsp70 expression is still able to buffer damage in the cell caused by these high temperatures. This shift towards higher expression in outbred larvae is observed at lower temperatures in *D. melanogaster*. *Drosophila melanogaster* is more sensitive to temperature stress (Loeschcke *et al.*, 1997; Krebs and Bettencourt, 1999). It upregulates and exhibits peaks in Hsp70 expression at lower temperatures than *D. mojavensis*, which is closely related to, and has the same temperature niche as, *D. buzzatii* (Krebs and Bettencourt, 1999). This indicates that inbred larvae of *D. melanogaster* reach a physiological limit at lower temperatures than those of *D. buzzatii*. This is to be expected, because the populations used in this study originated from temperate and very warm habitats, respectively. The difference in the pattern of Hsp70 expression observed in this study may, therefore, be explained by differences in temperature sensitivity.

Previous studies have shown that Hsp70 expression in *Drosophila* correlates with inducible tolerance of severe heat shock (Krebs and Feder, 1997; Dahlgaard *et al.*, 1998; Feder and Hofmann, 1999). This benefit may, however, be traded off with deleterious consequences, as Hsp70 expression, even at very low levels, can have severe fitness costs (e.g. reduced reproductive performance and growth rate) (Feder *et al.*, 1992; Krebs and Loeschcke, 1994; Silbermann and Tatar, 2000). The identification of significant effects of inbreeding on Hsp70 expression at benign and stressful temperatures in inbred larvae may, therefore, be an important factor connected to inbreeding depression and evolution in small populations.

Upregulation of Hsp70 expression could be one reason why inbreeding depression in empirical investigations is sometimes observed to be small (Uddin *et al.*, 1994; T.N. Kristensen, J. Dahlgaard and V. Loeschcke, unpublished). Furthermore, it may help explain why inbred lines of *Drosophila* have been observed to have the same or higher thermotolerance than outbred lines (Maynard Smith, 1956). It can be hypothesized, therefore, that upregulation of Hsp70 is an important mechanism (inbreeding $\rightarrow$ disrupted homeostasis $\rightarrow$ increased Hsp70 expression) in coping with the effects of inbreeding that otherwise would be deleterious or lethal. This may have an impact on research on inbreeding and thermotolerance in the future.

Our findings are also of interest in relation to genetic diseases involving abnormal proteins in humans. Recently, it was hypothesized that overexpression of chaperones may modulate the effect of mutant proteins causing conformational diseases (Cummings *et al.*, 1998; Gregersen *et al.*, 2001). Our results, therefore, suggest that upregulation of heat shock proteins is a common cellular response to increased numbers of non-native proteins.

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