Stress specific correlated responses in fat content, Hsp70 and dopamine levels in Drosophila melanogaster selected for resistance to environmental stress

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1. Introduction

Most organisms will at some point be exposed to potentially harmful environmental conditions, and can therefore be said to experience environmental stress (Hoffmann and Parsons, 1991). To counter any adverse consequences, adaptations have evolved that help to cope with stress and to minimize the fitness consequences of stressful episodes. Since stress tolerance from an evolutionary point-of-view is considered to be costly (Hoffmann et al., 2003; Kristensen et al., 2008), the mechanism of adaptation will depend on the specific conditions experienced. Short bouts of adverse conditions are expected to favour selection for phenotypic adjustments (i.e. reversible hardening responses) while genetic modifications in basal traits will evolve to withstand enduring adverse conditions (Bubliy and Loeschcke, 2005; Witcombe et al., 2008). Adaptation to environmental conditions in insects, primarily temperature, has been the focus of a large number of studies (reviewed in Hoffmann et al., 2003; Chown and Terblanche, 2006). Recent advancement in modern technologies (including micro-arrays and SNP scans) has made it possible to search entire genomes for important genes and variation behind adaptation (Sørensen et al., 2007b; Swindell et al., 2007; Hoffmann and Willi, 2008), however, progress in identifying and investigating the cellular, physiological and behavioural mechanisms of stress resistance associated with these genes is still limited.

On the molecular level, there is evidence for the existence of signalling networks, which can transcriptionally activate a wide range of stress resistance mechanisms (Wang et al., 2005; Roelofs et al., 2008). It is unknown how genetic changes in signalling networks affect the physiology of the organism, although several general stress responses have been suggested, e.g. lower metabolic rate, accumulation of fats/carbohydrates, oxidative stress response and expression of molecular chaperones (Vermeulen and Loeschcke, 2007). Physiology provides the mechanistic link between resistance phenotype on one side and genetic or genomic variation on the other side. On the physiological level, some traits have been identified that correlate well with one or more measures of stress resistance. For example, a classical and well documented physiological adaptation to starvation resistance...
is increased fat stores (Service, 1987; Baldal et al., 2006; Vermeulen et al., 2006b). In addition, increased fat stores are sometimes observed with genetically increased longevity (Service, 1987) and increased resistance to some types of cold stress (Chen and Walker, 1994). Since increased starvation resistance occurs as a correlated response to selection for resistance to different types of stress, this makes fat content an interesting character to further explore (Bubliy and Loschke, 2005). Similarly, increased heat resistance has often been shown to correlate well with increased expression of heat shock proteins (Hoffmann et al., 2003 and references therein; Garbuz et al., 2008). With the above mentioned examples as obvious exceptions, the involvement of many physiological factors in adaptation, and its possible trade offs, are yet little investigated. As a consequence, a large suite of physiological traits has been more loosely associated with stress resistance. For example, dopamine levels are known to respond to heat stress (Rauschenbach et al., 1993) and show a correlated response with genetically increased longevity (Service, 1987; Baldal et al., 2006; Vermeulen et al., 2006b). In addition, increased fat stores are sometimes associated with general stress resistance, we would detect a common physiological fingerprint of selection across selection regimes.

2. Materials and methods

2.1. Stocks and selection procedure

The flies were derived from a mass laboratory population of D. melanogaster established in September 2002. The population was established from a mix of four pre-existing laboratory stocks. Selection and control lines were established by flies from the fourth generation of the mass population (for details see Bubliy and Loschke, 2005). Eight experimental regimes were established with five biological independent replicate lines each. These were unselected control (UC), cold shock resistance selection (CS), heat shock resistance selection (HS), heat knockdown resistance selection (KS), desiccation resistance selection (DS), starvation resistance selection (SS), longevity selection (LS) and “laboratory natural selection” at constant 30 °C (C30). The selection procedures are described in Bubliy and Loschke (2005) and Sørensen et al. (2007b). All selection and control lines were established in parallel and had similar general maintenance conditions. For all stress regimes, selection was implemented every second generation to allow the population to recover and avoid any cross generational effects (see, e.g. Watson and Hoffmann, 1996; Hercus and Hoffmann, 2000). Selection lines were maintained similar to the control lines (UC) during the relaxed generations. All flies were reared at 25 ± 1 °C, at a 12:12 h light-dark cycle in 100 ml bottles containing 35 ml of standard oatmeal–sugar–yeast–agar medium (60 g dead yeast, 40 g sugar, 16 g agar, 30 g oatmeal, 16 ml nipagine solution and 1.2 ml acetic acid per litre). Each replicate line was kept in five culture bottles using ca. 60 pairs of parents per bottle.

2.2. Rearing of experimental flies

Experimental flies from all selection regimes had been subjected to selection every other generation, resulting in 34 generations of actual selection, except for the life span (LS) regime (16 selected generations) and the starvation selection (SS) regime (42 selected generations). For determination of Hsp70 and fat content flies from each replicate selection line were allowed to lay eggs on spoons. Eggs were collected into nine vials with 40 eggs from each of the five replicate lines per selection regime, resulting in 45 vials (1800 eggs) per selection regime. Eggs were allowed to develop, and females were collected into vials containing 4 ml of standard medium (10 females per vial) for Hsp70 assay or frozen within 8 h after emergence for fat content assay. For determination of locomotor activity and dopamine levels experimental flies were collected using the protocol described above, with the exception that we used a density of 100 eggs per vial and flies were collected as virgins. Female flies were collected into vials as above, and stored in an incubator at 25 °C until the tests were performed. In these assays, ampicillin was added to the medium (100 mg/l) to avoid bacterial infection.

2.3. Hsp70 expression levels

Two sets of 4–5-day-old female flies were prepared for analysis of Hsp70 expression levels. The flies were exposed to a 1 h heat shock of 35 °C or 37 °C, respectively, followed by recovery for 1 h at 25 °C. Both sets were snap frozen in liquid nitrogen and stored at −80 °C in centrifuge tubes until analysis. For the measurement of inducible Hsp70, flies were homogenized (10 flies per replicate vial) and the Hsp70 level was determined by the ELISA technique utilizing the antibody 7.FB, which is specific for the inducible Hsp70 in D. melanogaster (Welte et al., 1993). The assays were run on 96 micro-well ELISA plates with one replicate of all selection regimes measured in quadruplicate on each plate. The grand mean of each replicate (plate) was normalized to the grand mean of replicate (plate) 1 in each set. The ELISA protocol used is described in detail in Sørensen et al. (1999).

2.4. Dry weight and relative fat content

We determined dry weight and ether-extractable fat content as described in Fairbanks and Burch (1970). We used nine samples per replicate line, consisting of 5 < 8 h old females per sample. Flies were collected into open glass 6 ml scintillation vials and dried at 80 °C for 48 h. Thereafter, samples were weighted to the nearest 0.01 mg. To each scintillation vial, 2 ml of diethyl ether was added and the samples were gently agitated for 24 h at room temperature. After the ether was discarded, the samples were dried again at 80 °C for 48 h and subsequently reweighed. Relative fat content (RFC) was calculated using

\[
\text{RFC} = \frac{\text{DW} - \text{FDW}}{\text{DW}}
\]

with DW = dry weight and FDW = fat free dry weight.

2.5. Locomotor activity

Activity data were collected with the Drosophila Activity Monitoring System (TriKinetics Inc., Waltham, MA, USA), which is routinely used in mutant screens, studies of environmental and chemical sensitivities and social interaction (e.g. Dimitrijevic et al., 2004; Hirsch et al., 2009). We used 3 units each with 32 positions, so 96 5–6–day-old flies could be monitored simultaneously. The capillary glass vials were sealed with a drop of sugar–agar solution (40 g sugar and 16 g agar per litre) at the one end to provide food and moisture. This end was further sealed on the outside with
parafilm to avoid desiccation of the medium. The other end of the vial was closed with a small cotton wool stopper to allow gas exchange. Single female flies were loaded into the capillary glass vials without anaesthesia and inserted into the monitoring units. The data from the first hour after handling the flies were discarded. After 1 h the variances of the average activity were stabilized. The activity data were registered for 24 h, in which the photocells in the plates registered the number of times that the flies pass through the beam. The counts were binned every 15 min.

2.6. Dopamine levels

For every replicate line four samples of 10 4-day-old female flies were assessed. Flies were killed by snap freezing them in liquid nitrogen and were stored at −80 °C. The samples were weighed to the nearest 0.01 mg for correction of body weight, homogenized in 0.5 ml 0.1 M perchloric acid and centrifuged for 5 min at 14,000 rpm at 4 °C. The HPLC procedure and the description of the chromatographic system can be found in Vermeulen et al. (2006a).

2.7. Statistics

To analyse the results we performed analysis of variance (ANOVA) on single traits, supplemented with principle component analysis (PCA) of all traits. ANOVA was performed by analysing each trait as the dependent variable, and replicate line nested within regime. Whenever the replicate line factor became significant, this was used as the error term for regime. In the analysis of Hsp70 expression levels, we only had a single measurement for each replicate line, but we had replication at two temperatures (35 °C and 37 °C). Therefore, we dropped the replicate line factor, and added temperature treatment and the interaction between treatment and regime. To test for a general effect of selection for increased stress resistance, we performed an a priori orthogonal comparison of the unselected control against the selection regimes. Post hoc multiple comparisons of means were performed with the Scheffé test. We performed principal component (PC) analysis (PCA) on all traits. The signals were scaled to obtain unit variance. The explained variation was used to identify the traits having a large contribution from PC1 and PC2. The PCA was performed using Simca-P (Umetrics, Umeå, Sweden).

3. Results

3.1. Hsp70 expression levels

Hsp70 expression levels are shown in Fig. 1. ANOVA showed significant effects of temperature treatment ($F_{1,64} = 76.7$, $P < 0.001$), selection regime ($F_{7,64} = 10.8$, $P < 0.001$) and the interaction between the two ($F_{7,64} = 3.4$, $P = 0.004$). Flies from all regimes expressed more Hsp70 after exposure to 37 °C compared to 35 °C, although not significantly so in three regimes (DS, KS and CS; $t_6 = 0.6$, $t_6 = 1.4$ and $t_6 = 2.0$, n.s. respectively). The remaining five regimes showed a significant increase in expression at higher induction temperature. HS and LS showed the largest increases in induction between 35 °C and 37 °C ($t_6 = 6.1$, $P < 0.001$ and $t_6 = 4.1$, $P = 0.004$). Assuming that increased expression of Hsp70 is part of a general mechanism of stress resistance, an a priori prediction is that the UC control should show a general difference in Hsp70 expression levels compared to all selection regimes. However, this was not the case ($F_{1,64} = 0.03$, n.s.), indicating that increased stress resistance is associated both with up- and downregulation of Hsp70 expression among selection regimes. Post hoc tests of differences among selection regimes within temperature treat-
ments were reasonably consistent between the two test temperatures (Fig. 1). Overall, KS had significantly higher expression than CS, HS and C30 at 35 °C, and LS had higher levels of expression than DS, C30 and CS at 37 °C. The UC control regime and the remaining regimes displayed intermediate levels.

3.2. Dry weight and relative fat content

Results of dry weight and relative fat content, respectively, can be seen in Figs. 2 and 3. For dry weight, nested ANOVA showed significant effects of line within selection regime ($F_{2,32} = 3.6$, $P < 0.001$) and selection regime ($F_{7,32} = 9.5$, $P < 0.001$). The a priori contrast of UC control regime vs. selection regimes was non-significant ($F_{1,32} = 0.02$, n.s.). Among selection regimes, the post hoc Scheffé test showed significant differences. SS had significantly higher dry weight than all other regimes, whereas HS had significantly lower dry weight than all but LS. The UC control and remaining selection regimes had intermediate dry weights (Fig. 2).

For relative fat content nested ANOVA also showed significant effects of line within selection regime ($F_{2,32} = 2.0$, $P = 0.001$) and
selection regime \((F_{7,32} = 6.5, P < 0.001)\). The \textit{a priori} contrast of UC control vs. all selection regimes was non-significant \((F_{1,32} = 2.0, \text{ n.s.})\), whereas the post hoc Scheffé test showed significant differences among selection regimes. The starvation selected regime SS had significantly higher fat content than all other regimes. Furthermore, CS and DS regimes had higher fat content than HS, which had the lowest. The UC control and remaining selection regimes had intermediate relative fat contents (Fig. 3).

3.3. Locomotor activity

Mean spontaneous locomotor activity is shown in Fig. 4. Nested ANOVA showed significant effects of line within selection regime \((F_{32,152} = 1.9, P < 0.01)\), but no significant effect of selection regime \((F_{7,32} = 1.2, \text{ n.s.})\).

3.4. Dopamine levels

Fig. 5 shows the dopamine levels for all selection regimes. Analysis by nested ANOVA showed no significant effects of line within selection regime \((F_{32,152} = 1.5, \text{ n.s.})\) and a significant effect of selection regime \((F_{7,152} = 3.6, P < 0.01)\). The \textit{a priori} contrast of UC control regime vs. all selection regimes was non-significant \((F_{1,115} = 1.2, \text{ n.s.})\), whereas the post hoc Scheffé test indicated that LS had significantly higher dopamine levels than HS (Fig. 5).

3.5. Joint PCA analysis

We performed principal component analysis (PCA) incorporating Hsp70 expression at 35 °C and 37 °C, fat content, locomotor activity, dopamine levels and dry weight. The first two principle components (PC1 and PC2) were significant, explaining 34% and 24% of the total variation respectively. PC1 and PC2 readily separate most of the selection regimes (Fig. 6a). PC1 is mainly loaded by fat content and dry weight, whereas PC2 is loaded mainly by Hsp70 expression at both temperatures (data not shown).

4. Discussion

4.1. General and trait specific stress mechanisms

The UC (unselected control) regime had intermediate phenotypes in all traits measured, and did not significantly differ from the other selection regimes as a group. Thus, in the traits measured in this study there is no strong signal of a general response to selection for increased stress resistance at the physiological level. This is also shown by the central position of the UC regime in the PCA graph (Fig. 6a). However, we found significant differentiation among the selection regimes, except for locomotor activity, as evidence of strong responses to selection in all traits. This finding and the low level of correlation among Hsp70 expression levels, fat content and dopamine levels suggest that these physiological correlates do not belong to the general mechanism of adaptation to stress (Bubliy and Loeschcke, 2005; Sørensen et al., 2007b), but rather are associated with stress specific mechanisms and pathways. The same results can be deducted from the PCA analysis, where the two first PCs were loaded by different traits (not shown). This reflects that these traits are poorly correlated, and represent different stress adaptations. The significance of the “line” factor for dry weight, relative fat content and locomotor activity potentially indicates an additional influence of genetic drift on these traits. Alternatively, this may indicate the existence of several different ways to achieve a given resistance phenotype.

As selection regime was also significant in most cases, there is no indication on the phenotypic level that genetic drift obscured the effect of selection.
Hierarchical clustering showed the HS regime to be very different from all other regimes, and showed DS, SS and LS to be a tightly clustered group. This cluster is separated from, but closer to, UC control than to HS. These data on gene expression are reflected only partly in the physiological traits. The HS regime indeed seems to be distinct from all other regimes, but the DS, SS and LS regimes do not group together in our study.

Stress tolerance phenotype measures are also available and we expect the physiological measures to be correlated with the stress tolerance phenotype. Bubliy and Loeschcke (2005) have reported measures of stress resistance, body size, early fecundity and developmental time for this set of lines. Although expression of Hsp70 at 35 °C and 37 °C are correlated with each other, they do not correlate with the same phenotypic traits. Hsp70 expression at 35 °C is positively correlated with knockdown resistance \( r = 0.55, P < 0.001 \) and developmental time \( r = 0.46, P = 0.006 \). However, Hsp70 expression at 37 °C is not significantly correlated with either of these traits, but rather with cold shock resistance \( r = -0.45, P = 0.006 \). Earlier studies have suggested Hsp70 expression level to be negatively related with heat shock resistance (Sørensen et al., 1999, 2001), due to the lower need for Hsp70 in heat tolerant lines/populations. This was confirmed in larvae of D. buzzatii from Eastern Australia but not adult males nor females (Sarup et al., 2006). Nielsen et al. (2005) investigated the effect of a mutation which abolished the inducible heat shock protein response on heat shock, knockdown and cold tolerance and found no effects on knockdown and cold tolerance. These results seem not to be confirmed here and the understanding of the complex role of Hsp70 and other chaperones for stress adaptation and responses in an ecological context is still elusive.

Surprisingly, body size and body weight were not correlated \( r = 0.09, \text{n.s.} \). This is due to the fact that variation in body weight was mainly caused by variation in fat content \( r = 0.82, P < 0.001 \). As expected, fat content correlates significantly with starvation resistance \( r = 0.65, P < 0.001 \), but also with developmental time \( r = 0.48, P = 0.004 \), early fecundity \( r = -0.40, P = 0.017 \) and activity \( r = -0.42, P = 0.012 \). This suggests fat levels mediate a trade off between starvation resistance on the one hand and fecundity and developmental rate on the other. Bubliy and Loeschcke (2005) found that increased starvation resistance was a correlated response to all selection regimes, except selection for heat resistance. They hypothesised that all increases in starvation resistance were mediated by increased fat and carbohydrate stores, and considered this to be especially likely for the CS and LS lines, because of a suspected mechanistic link. We confirm that for resistance to cold this link is supported, but the other selection regimes do not have significantly higher fat content than the UC control. Apparently, the correlated response in starvation resistance is established by mechanisms unrelated to fat accumulation. Notably, the HS line, which was the only selection line without a correlated response to all selection regimes, except selection for heat resistance were mediated by increased fat and carbohydrate stores, and considered this to be especially likely for the CS and LS lines, because of a suspected mechanistic link. We confirm that for resistance to cold this link is supported, but the other selection regimes do not have significantly higher fat content than the UC control. Apparently, the correlated response in starvation resistance is established by mechanisms unrelated to fat accumulation. Notably, the HS line, which was the only selection line without a correlated response to starvation resistance, had the lowest fat content. Finally, dopamine levels appear not to be correlated to any other phenotypic traits. On the basis of its role as neurotransmitter, one might have expected a correlation with locomotor activity, but this was not found.

It will be instructive to include more biological levels into the comparison among these selection regimes. In particular, metabolomic and proteomic studies will provide a broader perspective, by showing the relative importance of the traits investigated in this study with respect to whole organismal changes in physiology.

**4.3. Comparison with other studies**

Despite the significant differentiation in Hsp70 expression among lines, none were significantly different from the UC control line in post hoc comparisons. High inducible levels of Hsp70 are known to be phenotypically correlated with increased heat resistance measured as survival (Dahlgaard et al., 1998), but populations selected for high temperature tolerance and populations naturally adapted to frequent occurring high temperatures show decreased expression (Bettencourt et al., 1999; Sørensen...
et al., 2001). The low level of inducible Hsp70 in C30 is concordant with this, although it is not statistically different from UC. The third heat resistance measure (KS) shows relatively high expression at 35 °C, but nearly no further induction at 37 °C. Thus, the three different regimes selected for heat resistance yield clearly different selection responses. This is in concordance with similar data on the role of Hsp70 for heat resistance (Sørensen et al., 2003, 2007b). There was also a tendency (not statistically significant) for a higher Hsp70 level in LS. This was not unexpected altogether, as a relationship between Hsp70 expression and life span earlier has been shown in transgenic lines of Drosophila overexpressing Hsp70 (Tatar et al., 1997) and lines lacking a heat stress response (Sørensen et al., 2007a).

The positive relationship between fat content and starvation resistance is well established (Djawdan et al., 1998; Hoffmann and Harshman, 1999; Baldal et al., 2006), so it was not surprising that starvation resistant SS flies had significantly increased fat content. None of the other regimes were statistically different from the UC regime, but the heat resistant HS flies tended to be leaner.

There was no clear pattern in locomotor activity in this set of lines. There seems to be a negative correlation with fat content and starvation resistance, but that may be an artefact of the assay. We tested the activity of flies with only sugar as a food source. It has been shown that starved flies have higher activity levels (Knoppien et al., 2000). Starvation resistant flies may remain inactive, because they are not so easily starved as flies from the other selection regimes. Alternatively, the selection for increased starvation resistance has affected activity directly, possibly through effects on metabolism.

With respect to dopamine levels, none of the lines were statistically different from the UC control regime. The fact that LS has the highest dopamine levels corroborates the findings of Vermeulen et al. (2006a) that it is a correlated response to selection on life span or age at reproduction. It should be noted that we assessed virgin females, whereas for the selection procedure and stress assays mated individuals were used. The use of mated females may have given a slightly different outcome, but to the best of our knowledge, no data are available on the effect of mating status on dopamine levels.

5. Conclusion

Overall, our results fit previous findings and demonstrate that variation in fat content and Hsp70 and dopamine levels represent links to stress adaptations. However, changes in these physiological traits seem to be stress specific rather than part of a general stress mechanism. Both up- and downregulation occurred, depending on the selection regimes that were applied. Therefore, our results are concordant with the suggestion, that cross resistance occurs, when different stressors require similar adaptive mechanisms, rather than that it is mediated by a general stress response. More detailed analysis of cross resistance, at the cellular and molecular level, will increase our understanding and show whether this interpretation is correct. In most cases, however, cross resistance is not well understood and can not be easily predicted among traits, e.g. cross resistance among measures of heat resistance traits is not often reported while cross resistance among seemingly unrelated traits are found frequently.

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