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Evolutionary adaptation to environmental stressors: a common cellular stress response at the proteomic level

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1 **Abstract**

2 Mechanistic trade-offs between traits under selection can shape and constrain evolutionary
3 adaptation to environmental stressors. Thus, understanding the quantitative and qualitative
4 overlap in the underlying adaptive mechanisms, such as the heat shock protein response, is
5 central for our understanding of evolution. However, our knowledge on this matter is highly
6 restricted by the challenges of comparing and interpreting data between separate studies and
7 laboratories, as well as to extrapolating between different levels of biological organization. We
8 investigated the constitutive proteome (637 proteins) of 35 *Drosophila melanogaster* replicate
9 populations artificially selected for increased resistance to six different environmental stressors.
10 We found strong evidence for a ‘common stress response’, shared among different selection
11 regimes. This common response was especially dominated by processes related to the
12 translational machinery. We found no evidence for negative correlations between environmental
13 stress resistance traits, suggesting that adaptation was not constrained by mechanistic trade-offs
14 among traits. One exception was acute high temperature survival, where the selection response at
15 the proteome level was partly opposite of the response to all other selection regimes. Using
16 global proteomics we show a strong and diverse contribution from the heat shock chaperones to
17 environmental stress resistance. This clear-cut result suggests that the molecular chaperones do
18 not only play a vital role during and after environmental challenge, but that selection adjusts the
19 constitutive expression of individual heat shock proteins in a complex fashion specifically
20 tailored to each environmental stress regime.

Introduction

Natural populations are expected to be under selection for adaptation to several abiotic factors such as temperature and the availability of water and food (Hoffmann and Parsons 1997). Shared genetic and physiological mechanisms between the associated traits can constrain or promote adaptive evolution of individual traits (Hoffmann 2014), making the investigation of such mechanisms important for understanding the evolutionary adaptation of populations. Studies investigating specific candidate mechanisms of individual environmental tolerances (e.g. Sørensen et al. 2009; Udaka et al. 2013; MacMillan et al. 2015; Slotsbo et al. 2016) as well as un-targeted approaches at the genetic or transcriptomic levels have advanced understanding of physiological tolerance mechanisms (Sørensen et al. 2007; Chen et al. 2015; Gerken et al. 2015; Telonis-Scott et al. 2016). However, our ability to quantify the mechanistic overlap across studies investigating different environmental stressors is reduced by several challenges, limiting our understanding of the potential for mechanistic trade-offs constraining adaptive evolution. These challenges include the lack of compliance across studies in both ectotherms and plants, due to huge effects of genetic background and the redundancy of molecular pathways, i.e. the ability to attain a functional change through regulation of several different transcripts (Sarup et al. 2011; Sanchez-Bermajo et al. 2015). Further, transcriptomic studies are likely to only identify a part of the physiological changes due to the potential for substantial post-transcriptional variation (Feder and Walser 2005; Seliger et al. 2009; Colinet et al. 2013). Finally, several studies point to a non-linear and complex relationship between the different levels of biological organizations (Suarez and Moyes 2012; Malmendal et al. 2013; Overgaard et al. 2014). This seems to be especially pronounced for cold resistance, where only relatively few genes have

43 been associated with the physiological changes of the well-established cold acclimation and cold
44 resistance selection responses (Misener et al. 2001; Qin et al. 2005; Sørensen et al. 2007).

45
46 Proteomics directly addresses the functional molecules, downstream from transcription and
47 translation, and thus is likely to reveal relevant information about expression patterns and
48 functional groups of proteins responding to selection. Both a change in the constitutive (non-
49 induced) and the plastic (induced) responses, including those at the proteomic level, play a major
50 role for environmental stress adaptation (Herring and Gawlik 2007; Schimel et al. 2007;
51 Dineshram et al. 2016; Kristensen et al. 2016). However, most studies find a quite limited
52 evolutionary response in the degree of plasticity (e.g. van Heerwaarden et al. 2014; Manenti et
53 al. 2015). For this reason, we argue that the constitutive protein expression may be highly
54 informative of the mechanisms underlying evolutionary responses to different environmental
55 stressors. Such an approach requires separate lineages of a species with evolved differences in a
56 range of different stress resistance traits.

57
58 The aim of this study was to investigate changes in protein expression and deduce functional
59 differences and similarities among *D. melanogaster* lines selected for resistance to a range of
60 environmental stressors. Such lines are useful for disentangling the evolutionary response,
61 including correlated trait-responses, to selection for specific environmental parameters (Bubliy
62 and Loeschcke 2005; Telonis-Scott et al. 2006), as well as for the investigation of the underlying
63 genetic and physiological mechanisms (Travisano et al. 1995; Folk and Bradley 2005).

We investigated the proteomes of replicated control lines and lines selected for desiccation resistance, increased longevity, cold resistance and three types of high temperature resistance. Specifically, we studied the following questions: How much of the selection response to different stressors can be attributed to a ‘common stress response’? How common are negative correlations between responses to stressors at the proteome level? Does the proteome reveal candidate mechanisms for cold resistance previously overlooked by transcriptomic studies? Due to the high amount of proteins detected in this study, our non-targeted approach is supplemented by a targeted investigation of the response of the heat shock protein (Hsp) candidate pathway. Most studies have focused on the induction characteristics of single Hsps (Sørensen et al. 2003), however, the adaptive significance of Hsps might differ among Diptera species and rely more on constitutive expression levels than previously thought (Zatsepina et al. 2016). Thus, we also aimed to investigate: To what extent do the constitutive heat shock protein ‘family’ expression levels show common or stress resistance regime specific selection responses?

Materials and Methods

Origin of experimental flies and selection regimes

The flies used in this study originated from the environmental stress resistance and life history trait selection experiment described in Bublly and Loeschcke (2005) and used by several studies on different levels of biological organization (Sørensen et al. 2007; Sørensen et al. 2009; Malmendal et al. 2013). In short, a diverse mass-bred laboratory population of *D. melanogaster* was established and kept at 25 °C on standard oatmeal-sugar-yeast-agar *Drosophila* medium. Hereafter, five independent replicate lines were established for each of six selection regimes and

one control regime. The experimental regimes were: Unselected control (UC); Desiccation resistance selection (DS); Longevity selection (LS); Cold-shock resistance selection (CS); Heat shock resistance selection (HS); Heat knock down resistance selection (KS); and exposure to constant 30 °C during development (C30). Selection was performed every other generation to allow recovery and avoid trans-generational effects (see e.g. Watson and Hoffmann 1996; Hercus and Hoffmann 2000). The selection procedures are described in detail in Bublly and Loeschcke (2005) and Malmendal et al. (2013).

In this study we report on untreated female flies from all five independent replicate lines from each of the seven regimes (i.e. a total of 35 samples). The flies were collected from the second generation after the most recent selection event, after maintenance at uncrowded common garden conditions (25 °C). For the regimes UC, DS, CS, HS, KS and C30, replicate lines were collected after 51 generations of rearing and 23 selection events (disregarding UC). Replicate lines from the LS regime had been reared for 27 generations and experienced 11 selection events (for a detailed description, see Bublly and Loeschcke 2005). All flies were synchronized for age and collected simultaneously. Newly hatched flies (1-2 days old), from each independent replicate, were transferred to micro-centrifuge tubes and snap frozen in liquid nitrogen. Subsequently the samples were stored at -80 °C until batches of 50 females were separated from males on ice blocks from a -80 °C freezer in a cold room (to avoid thawing the flies) for proteomic analyses.

Proteomic protocol

108 For relative comparison we used five additional replicate populations (selected for starvation
109 resistance) from the original selection experiment described in Bubliy and Loeschcke (2005) as a
110 common reference. The use of this reference allows us to estimate the variance of the different
111 replicate control populations and thereby perform statistical tests of differences between control
112 and the selection regimes of relevance. Proteins were extracted using 1 mL lysis buffer of 100
113 mM Triethylammonium bicarbonate (TEAB) with 0.5% sodium dodecyl sulfate (SDS) and
114 protease inhibitor (cOmplete ULTRA Tablets, Roche Diagnostics) following manufacturer's
115 recommendations. Mechanical homogenization of *D. melanogaster* was performed using a
116 Bio101, Thermo Savant FastPrep FP120 cell disruptor using lysing matrix C by three cycles of
117 40 s at 6.5 rpm. Vials were kept on ice for 1 min in between cycles to avoid heating samples.
118 Homogenate was transferred to Eppendorf tubes and centrifuged at 4 °C and 10,000 rpm for 20
119 min. Protein concentration of samples was determined using a Qubit™ Fluorometer (Invitrogen,
120 Life Technologies) and the Qubit Protein assay kit following manufacturer's protocol.

121 Proteins from each sample (200 µg) were precipitated over-night using 6x sample volume ice-
122 cold acetone (-20 °C), thereafter samples were centrifuged at 14,000 rpm at 4 °C for 10 min.
123 Acetone was removed and pellets were dried shortly before dissolved in 40 µL Dissolution
124 Buffer with 2 µL Denaturant (iTRAQ® Reagents, AB Sciex). Proteins were reduced by
125 incubation at 60 °C for 1 hour with 4 µL 50 mM tris-(2-carboxyethyl)phosphine (TCEP)
126 (Reducing Reagent, iTRAQ® Reagents, AB Sciex) and subsequently alkylated by 10 min
127 incubation at room temperature with 2 µL 200 mM methyl methanethiosulfonate (MMTS) in
128 isopropanol (Cysteine Blocking Reagent, iTRAQ® Reagents, AB Sciex). Proteins were
129 enzymatically digested over-night to peptides using a 1:50 trypsin:protein ratio (Sequencing
130 Grade Modified Trypsin, Promega Biotech AB).

131 Each sample was labeled with isobaric tags for relative and absolute quantitation (iTRAQ)
132 (Pottiez et al. 2012). Peptides were incubated with the specific iTRAQ labeling reagents at room
133 temperature for one hour. Hereafter, all eight samples within one replicate (i.e. the common
134 reference and one replicate of the seven experimental regimes) were pooled for fractionation and
135 purification.

136 Prior to offline strong cation exchange fractionation (SCX) sample volume was reduced to < 50
137 μ L by vacuum centrifugation. Loading buffer (10% formic acid, FA) was added to acidify the
138 samples. SCX fractionation was performed using HyperSep SCX SPE columns (Thermo
139 Scientific) and Visiprep SPE Vacuum manifold (Sigma-Aldrich). Columns were conditioned by
140 1 mL 100% ACN, 500 μ L pH4 SCX elution buffer, 500 μ L pH8.5 SCX elution buffer, 1 mL
141 0.1% Trifluoroacetic acid (TFA) and 40% acetonitrile (ACN). Acidified samples were loaded
142 on columns and fractions were subsequently eluted by increasing pH. SCX elution buffers were
143 20 mM acetic acid, 20 mM boric acid, 20 mM phosphoric acid and 50% ACN, adjusted to 10 pH
144 levels (pH 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.5, 11.0) by 5M NaOH. Fractions 4.0 + 4.5 and
145 8.5 + 11.0, respectively, were pooled to attain eight fractions for further purification and
146 analysis. The volume of SCX fractions of each sample was reduced to < 100 μ L by vacuum
147 centrifugation and fractions were acidified as described above. All sample fractions were
148 purified by Sep-Pak[®] Vac C18 cartridges (Waters) on a Visiprep SPE Vacuum manifold (Sigma-
149 Aldrich) prior to nLC MS/MS analysis. Purification C18 columns were washed by 1 mL 100%
150 ACN, equilibrated with 2 mL buffer A (0.1% FA) prior to loading the acidified sample. After
151 sample loading, the column was washed 2 times with 1 mL buffer A. The purified sample was
152 eluted by 500 μ L buffer B (90% ACN, 0.1% FA). Prior to mass spectrometry analysis the
153 sample volume was reduced by vacuum centrifugation and the sample was acidified by 10% FA.

Purified acidified sample fractions (eight fractions for each replicate) were analyzed by high-resolution electrospray ionization tandem mass spectrometry (ESI-MS/MS) (Köcher et al. 2009). Reverse phase nanoLC separation (Dionex UltiMate RSLCnano System) was performed online coupled to the mass spectrometer (LTQ Orbitrap Velos). Peptides were loaded on the column using 96% buffer A (0.1% Formic Acid) and 4% buffer B (90% acetonitrile, 0.1% Formic Acid). Peptides were eluted by a linear gradient (1.5-2.5 hours gradient) of increasing buffer B. Three technical replicates of each pooled sample replicate were analyzed, each using a slightly different gradient to increase protein coverage. The three gradients used were I) 100 min: 0-3 min at 4% B, 3-5 min increasing to 8% B, 5-103 min increasing to 60% B, 103-108 min at 90% B, II) 120 min: 0-3 min at 4%, 3-5 min increasing to 10% B, 5-123 min increasing to 55% B, 123-128 at 90% B, III) 150 min: 0-3 min at 4%, 3-13 min increasing to 15%, 13-100 min increasing to 35% B, 100-150 min increasing to 60% B, 150-153 min increasing to 70% B, 153-158 min at 90% B. Tandem mass spectrometry parameters were: positive mode, MS scan range 300-1600 with resolution at 30,000, MS/MS fragmentation was performed using HCD (higher-energy collisional dissociation) on the 20 most intense ions with a normalized collision energy of 40, dynamic exclusion of 90 s and a minimum signal threshold of 10,000.

Analyses

All raw data from triplicate injections of the eight fractions were searched together using Proteome Discoverer 1.4, Reporter Ions Quantifier, Percolator validator, Mascot 2.4 Search Engine and Swiss-Prot database restricted to taxonomy '*Drosophila*'. The Percolator algorithm uses semi-supervised machine learning and a target-decoy search strategy with reversed sequences to distinguish correct and incorrect peptide sequence matches (Käll et al. 2007; Spivak

et al. 2009). Search parameters were the following: precursor mass tolerance was 10 ppm and fragment mass tolerance 0.1 Da, maximum two missed cleavages, quantification method iTRAQ 8-plex, dynamic modifications were oxidation (M) and deamidated (NQ) and static modification was methylthio (C), the strict target FDR was 0.01 for high confidence peptide matches and the relaxed target FDR was 0.05 for medium confident peptide matches. Protein and peptide grouping was enabled. Protein quantification was based exclusively on unique peptides and among replicate experimental bias correction was performed (Latosinska et al. 2015). As a quality criterion for the proteins used for analysis we only accepted proteins that were detected reliably in all replicates from all regimes. We found this approach to avoid the potential bias from non-detected proteins, while only a few proteins were removed from the analysis.

Pairwise comparisons between control and selection regimes, respectively, were performed on the resulting dataset with ANOVA. We analysed direction of significant proteins among regimes to determine if the direction of expression change was similar among regimes. All raw P-values were used as input for the PIANO (Platform for Integrative Analysis of Omics data) R-package (vs. 1.8.2)(Varemo et al. 2013) for gene (protein) set analysis (GSA) of pathway enrichment among the proteins. Only GO terms with three or more proteins were considered in the analysis.

A principal component analysis (PCA) was applied to protein expression levels of the proteins fulfilling our quality criteria. This was done to assess overall patterns of differentiation in the global protein expression levels among the seven regimes. A PCA was also applied on the subset of proteins belonging to the heat shock protein (Hsp) family. All data were scaled and centred prior to performing the PCA. We performed an ANOVA on the scores of the first three PCs of

each PCA to ascertain statistical significance of the selection regime. To further evaluate the patterns of heat shock protein expression, we performed Pearson correlations among the individual Hsps, while adjusting the P-values with the Holm-Bonferroni correction method. All analyses were performed using the statistical software R (vs. 3.2.2) (R Development Core Team 2016).

Results

Individual protein responses

The proteomics pipeline identified a total of 1115 unique proteins. By selecting proteins that were detected reliably in all three technical and five biological replicates of all seven selection regimes we retained 637 proteins, of which 506 showed significant differences ($P < 0.05$) in at least one contrast (a selection regime versus the control regime; Suppl. Table 1). The proteins dropped from the final dataset were generally expressed at very low levels. The fold changes of the detected proteins were quite modest, with most proteins showing less than 1.5 fold change in either direction, and only in a few cases exceeding a fold change of 2 (Suppl. Table 1). The number of proteins differentially expressed in the contrasts ranged from 179 proteins in the LS to 290 proteins in the C30 lines (Figure 1A). The significantly differentially expressed proteins represented roughly similar numbers of down and up-regulated proteins. To investigate whether significant proteins shared among regimes (i.e. being significantly differentially expressed from controls in both regimes) represented a common stress response, we calculated to what degree significant proteins shared by two selection regimes were changed in the same direction (Figure 1B). All pairwise comparisons among regimes showed that the majority of significant proteins

shared were changed in the same direction compared to the control, supporting a common response to selection for stress resistance. In most cases the degree of correspondence in direction of expression was $\geq 95\%$ with the notable exception of the HS regime, that shared less correspondence in direction of significant proteins with all other regimes (8-18%) (Figure 1B). We found 58 proteins that were significantly up- or down regulated in all individual contrasts represent a common evolutionary stress response. Excluding HS (which showed the most diverse response among all regimes), the remaining regimes shared a common response of 86 significant proteins. The ten proteins with the highest average fold change among these 86 were: Acyl carrier protein (Q94519), Defense protein I(2)34Fc (Q9V3Y3), LDLR chaperone boc (Q8T9B6), Elongation factor 1-alpha 1 (P08736), 60S acidic ribosomal protein P0 (P19889), GTPase-activating protein (P48423), Glyceraldehyde-3-phosphate dehydrogenase 1 (P07486), Eukaryotic translation initiation factor 4E (P48598), Acyl-CoA-binding protein homolog (P42281), Alcohol dehydrogenase class-3 (P46415) with average fold changes between 12 and 20%. These proteins are associated (among others) with the biological processes 'fatty acid biosynthetic process' (Q94519), 'sensory perception of pain' (Q94519, P48423), 'innate immune response' (Q9V3Y3), 'protein targeting to membrane' (Q8T9B6), 'translation' (P08736, P19889, P48598), 'DNA repair' (P19889), 'glucose' - and 'alcohol metabolic processes' (P07486, P46415), 'transport' (P42281) and 'visual learning' (P46415).

Principal component analysis

To generate an overview of the general pattern of global protein expression levels we performed PCAs of protein expression of the 637 proteins fulfilling our quality criteria. Even though

biological replicate lines showed quite a bit of variability in the response to selection and the amount of variation explained was moderate (Figure 2), the analyses showed a clear and highly significant effect of selection regime along the first three principal component axes ($F_{(6, 28)} = 22.8, 25.9$ and 10.7 for PC1, PC2 and PC3, respectively, $P < 0.001$ for all three PCs). The HS flies showed the most distinct response, primarily along PC2, while all other selection regimes were progressively separated from controls along both PC1 and PC2 in the sequence of least to most different from controls: LS (having fewer selection events than other regimes), DS, KS, C30 & CS. Along PC3 the separation of the three heat resistance regimes (HS, KS and C30) was most noteworthy (Figure 2B).

Pathway enrichment analyses

All raw P-values for the total 637 proteins were used as input for a PIANO analysis. The identified enriched pathways are presented in full in Suppl. Table 2. Distinct and significant enriched proteomic responses were identified for all contrasts. Among the individually significant contrasts were several GO terms with a predicted connection to environmental stress resistance and adaptation. For LS most pronounced were processes related to ‘glycolytic processes’, ‘transmembrane transport’ and ‘protein folding’. For DS we found ‘phototransduction’, ‘RNA binding’ and ‘response to mechanical stimulus’ to be enriched. CS selection yielded processes related to ‘protein folding’, ‘sensory perception of chemical stimulus’, ‘response to pheromone’, ‘adult locomotor behaviour’ and ‘learning or memory’. For KS we found several groups related to ‘translation’, ‘phototransduction’ and ‘adult locomotor behaviour’. HS yielded groups related to ‘translation’, ‘lipid metabolic process’, ‘response to

insecticide', 'sensory perception of chemical stimulus' and 'antimicrobial humoral response'. Finally, C30 yielded groups with relation to 'alcohol dehydrogenase (NAD) activity', 'cold acclimation', 'learning or memory', 'translation', 'lipid particle' and 'response to heat'. While we detected 58 (or 86 excluding HS) proteins contributing to a selection response common among all regimes only 1 (or 2 when excluding HS) GO category was significant for all contrasts (Figure 1C; Suppl. Table 2).

In addition to identifying enriched pathways, we identified all GO categories that we had the power to detect as enriched given our dataset of 637 proteins (i.e. GO categories qualifying would be represented by at least three proteins among our list of 637 proteins) (Suppl. Table 2). These GO terms can be interpreted as not affected significantly by selection.

Overlaps of enriched functional groups

We further investigated the overlap of significantly enriched functional groups among selection regimes to identify shared mechanisms (Figure 1; Suppl. Table 2). Comparing the three heat related regimes (HS, KS and C30), we found little overlap suggesting largely independent functional mechanisms underlying these resistance regimes as illustrated by a Venn diagram (Figure 3A). Further, KS (but not HS or C30) showed high overlap with CS suggesting shared mechanisms. KS shared a similarly large overlap in mechanisms with CS and DS, but CS and DS showed very little overlap suggesting that KS share independent mechanisms with CS and DS, respectively (Figure 3B).

Heat shock proteins

As a prime candidate mechanism for stress resistance we specifically looked at the heat shock proteins (Hsps) (Sørensen et al. 2003). We found six heat shock proteins (Hsp23, Hsp26, Hsp27, Hsp60, Hsp68, Hsp83), a heat shock co-chaperone (Hsp90 co-chaperone), and four heat shock cognates (Hsc70-1, Hsc70-3, Hsc70-4, Hsc70-5) among the 637 proteins in the data set. Neither inducible forms of the major Hsp70 (A and B, respectively) nor the HSF heat-shock transcription factor were consistently identified in all samples and thus not included in the analysed dataset.

A PCA on the 11 identified heat shock related proteins showed a different pattern from the comparison of all selection lines with controls and explained much more variation than the PCA on all proteins (Figure 4). Selection regime was highly significant for the first three principal component axes ($F_{(6, 28)} = 63.0, 12.4$ and 11.8 for PC1, PC2 and PC3, respectively, $P < 0.001$ for all three PCs). The controls were well separated from HS and especially C30 along PC1, and progressively along PC2 for KS, LS and CS, while DS and especially HS showed a marked overlap with controls (Figure 4A). Along the highly significant PC3, especially HS was different from the remaining regimes (Figure 4B). Thus, HS, CS, LS and especially C30 selection regimes seem to produce distinct HSP expression profiles while the remaining selection regimes were little separated from controls (Figure 4).

At the level of individual proteins all selection regimes showed significant differential expression in several Hsps as compared to controls, however, each selection regime seemed to have its own specific profile (Table 1). DS and LS showed the fewest significant Hsps (two and three, respectively), while C30 and CS each had significant expression changes in eight heat shock related proteins. With respect to the degree of regulation (fold change) across all

investigated proteins (significant and non-significant) C30, followed by HS, showed the highest average fold change, while CS only showed a very moderate fold change increase compared to controls and the third heat resistance trait, KS, seems on average even to show a decrease in expression. Hsp68 was only differently expressed from controls in the CS lines, and thus seems specific for this selection regime.

Correlations in expression among heat shocks proteins (Hsps)

The four Hsp70 (supposedly closely related) cognate proteins detected showed a quite diverse response among the selection lines (Figure 5). Hsc70-1 was not correlated to any other Hsc70 but positively correlated to Hsp68, while non-significantly negatively correlated to most other Hsps. Hsc70-3 and Hsc70-4 were strongly correlated to each other, and to Hsp23, Hsp60 and Hsp83. Hsc70-5 was most correlated to Hsp83. Among the three small Hsps, Hsp23 was positively correlated to Hsp60, Hsc70-3 and Hsc70-4, while Hsp26 and 27 showed a high correlation to each other but not to any other heat shock related proteins (Figure 5). The Hsp90 co-chaperone was not significantly correlated in expression to any protein in the analysis (Figure 5).

Discussion

The principal component analysis (Figure 2) showed a clear distinction between selection regimes and the control regime along the first two PCs, suggesting a directional response to selection as the main driver for the evolved proteomic profiles. Furthermore, the evolved

329 direction of expression was highly conserved among selection regimes, with generally > 95% of
330 proteins shared by regimes being regulated in the same direction, suggesting that mechanistic
331 trade-offs during evolutionary adaptation to environmental stressors is unlikely, at least in the
332 majority of the environmental regimes. This result is supported by a less pronounced signal of a
333 shared response among the same selection regimes on the gene expression level (Sørensen et al.
334 2007). A formal comparison of the transcriptomic (Sørensen et al. 2007) and proteomic signals
335 of selection in these lines yielded surprisingly little correspondence. However, due to the fact
336 that the transcriptomic and proteomic investigations were performed on different generations of
337 selection and potentially under slightly different conditions our ability to interpret the limited
338 correspondence among these sensitive methods is restricted. Still, the molecular results on both
339 the transcriptomic and the proteomic level provide evidence for a common cellular stress
340 response and thereby a mechanistic background for the high degree of cross resistance
341 (positively correlated selection responses) in these lines (Bubliy and Loeschke 2005).

342 The functional constituents of the common response to selection for stress tolerance observed
343 here are elusive. Two points are especially noteworthy. First, previous transcriptomic studies of
344 environmental stress tolerance candidate genes have suggested genetic redundancy, meaning that
345 changes to different transcripts might commonly lead to similar functional phenotypes (Sørensen
346 et al. 2007; Telonis-Scott et al. 2016). In accordance with this we would expect the common
347 stress signal to be more pronounced at the functional enriched (GO category) level than at the
348 individual protein level. We did not observe this pattern, as only one or two GO categories were
349 shared among selection regimes, even if quite a number of individual proteins were shared.
350 Shorter lists of genes or proteins suffer from reduced power to detect significant enriched
351 functional groups, however, as we have quite a diverse response among regimes this might not

be the main explanation in this case. Second, we compared the individual proteins shared by all regimes (or all regimes excluding HS) with the ‘minimal stress proteome’ as proposed by Kültz (2005). While several of these proteins were found in our data set, only a single (enolase) was found among our selection of common stress proteins. Instead, the top 10 shared proteins in this study point to a prominent effect of the post-transcriptional machinery (‘translation’) and to the sensing and responses to environmental stimuli (‘sensory perception of pain’, ‘innate immune response’, ‘DNA repair’ and ‘visual learning’) as mechanisms associated with a general stress proteome in addition to a number of metabolic and biosynthetic enzymes not included in the ‘minimal stress proteome’ (Suppl. Table 2) (see also Kültz 2005). This suggests that selection has a major impact on processes downstream from transcription, explaining the discrepancy between results from different levels of biological organisation (Feder and Walser 2005; Malmendal et al. 2013). The consequence on protein turnover of the changes is difficult to predict, however, most of the proteins within this category showed upregulation in the stress selected regimes. This probably does not have a primary stress tolerance effect, but might allow for the activation of downstream processes and more efficient cellular turnover. Thus, this study adds novel candidates to the list for a common stress proteome and helps to explain results on different levels of biological organization.

Several selection regimes shared a particular high number of enriched functional responses, supporting a shared mechanistic background for specific stress resistance traits (Figure 3; Suppl. Table 2). However, while the analyses in this study support the notion of a common stress response, the different regimes vary in the strength of the response along the primary PCs. This suggests a difference in the realized selection coefficients among selection regimes during

375 selection. Furthermore, the results also indicate that while the common stress response
376 constitutes a back-bone of the response, it is further supplemented by selection regime specific
377 responses. In this regard, two sets of comparisons of significantly enriched processes among
378 regimes were especially noteworthy (Figure 3). First, the responses among the three regimes
379 selecting for high temperature resistance (HS, KS & C30) suggest that the three regimes showed
380 differentiated responses, bringing mechanistic support for the discrepancy in results obtained by
381 different heat resistance metrics (e.g. Berrigan and Hoffmann 1998; Sgrò et al. 2010), and might
382 bring light to the ongoing discussion of ecological relevance of different resistance metrics
383 (Sørensen et al. 2005; Rezende et al. 2011; Terblanche et al. 2011; Overgaard et al. 2012). The
384 independence of the heat resistance traits was even clearer when looking at the lack of overlap in
385 enriched functional pathways (Figure 3A), indicating that choice of methodology affects which
386 mechanisms are investigated and thus the results obtained (Sørensen et al. 2005; Terblanche et
387 al. 2011; Castaneda et al. 2015). Second, we compared regimes selected for cold, desiccation and
388 high temperature knock down resistance. The molecular background of desiccation tolerance in
389 *Drosophila* has been investigated thoroughly (see Telonis-Scott et al. 2016), but not compared
390 directly to that of other environmental stressors. While lines selected for heat knock down
391 resistance shared little with the other heat resistance regimes, all pathways (except one) were
392 shared with either DS or CS, but little with both. This pattern is surprising and indicates that heat
393 knock down resistance is constituted partly by aspects of desiccation resistance and partly by
394 aspects of cold resistance. The link to DS is interesting as high temperature and low humidity co-
395 occur under natural conditions and as several studies have linked these traits in *Drosophila*
396 (Hoffmann 1990; Bubliy et al. 2012; Kellermann et al. 2012). The functional overlap between
397 DS and KS seems to be related to environmental sensing (e.g. ‘phototransduction’). The shared

mechanisms between cold resistance (CS) and high temperature knock down seem to be related to maintenance of physiological function ('locomotor activity' and 'protein folding'). Environmental sensing, but not 'locomotor activity' and 'protein folding' were recently identified as associated to *Drosophila* desiccation tolerance strengthening the generality of the this functional group (Telonis-Scott et al. 2016).

Within selection regimes, significant enrichment was found in processes responding to temperature and other types of environmental signals, e.g. immune responses, chemical stimuli and light. Further, different aspects of locomotor behaviour were found significantly enriched in several regimes. Taken together, these results suggest that environmental sensing and downstream responses to these environmental signals constitute an important role of the proteomic selection response, although in a complex way dependent on the exact selection regime. Moreover, the PCA revealed pronounced variation among the biological replicates, which could signify that selection has acted through complementary proteins and mechanisms within selection regimes. This complexity may explain why not all studies find strong support for phenotypic cross-resistance to environmental stress (Sinclair et al. 2007; Sinclair et al. 2009).

A previous transcriptomic analysis of the selection lines used in this study produced no significant signal of selection in the cold resistance lines (Sørensen et al. 2007). In this light it was surprising that the current proteomic analyses revealed several novel candidate mechanisms for cold resistance including 'protein folding', 'sensory perception of chemical stimulus', 'response to pheromone', 'adult locomotor behaviour' and 'learning or memory' (see Suppl. Table 2 for full list). Transcripts related to 'protein folding' and 'sensory perception' were also

420 detected by Telonis-Scott et al. (2009) and Qin et al. (2005) found a response in molecular
421 chaperones and membrane-related transcripts in response to short-term cold acclimation. Our
422 finding that selection for cold resistance was associated with ‘protein folding’, confirms that
423 molecular chaperones are involved in cold resistance in *D. melanogaster* although through
424 pathways not regulated by the heat shock (transcription) factor (Nielsen et al. 2005). The new
425 candidate mechanisms responding to selection for cold tolerance identified by this study might
426 not be activated by other studies addressing induced stress resistance responses (Qin et al. 2005;
427 Overgaard et al. 2014), suggesting that evolutionary cold tolerance is achieved by partly different
428 mechanisms than cold acclimation.

429
430 Several trade-offs between environmental stress resistances have been identified in insects (e.g.
431 Hoffmann et al. 2005; Chidawanyika and Terblanche 2011; Schou et al. 2015) but it remains
432 unclear if adaptation is generally constrained by such negative correlations. The existence of a
433 common stress response, as found in this study, suggests that adaptation by increasing resistance
434 towards multiple stress factors simultaneously should be possible (Sørensen et al. 2015). For HS
435 a specific selection response was evident along the second and third PC which could signify a
436 trade-off with the remaining selection regimes. This finding is in correspondence with earlier
437 studies on physiological and transcriptomic levels where the HS lines also showed differentiation
438 from all other stress regimes (Sørensen et al. 2007; Sørensen et al. 2009). We investigated a
439 potential mechanistic trade-off further, by looking at the discrepancy in the direction of
440 regulation of significantly differently regulated proteins among selection regimes. We found
441 generally very large correspondence (> 95%) in the direction of regulation among regimes,
442 except for the HS regime where a relative large proportion of proteins was regulated in the

opposite direction in this regime compared to all other selection regimes (Figure 1B). Selection for heat shock survival (HS) could induce a mechanistic trade-off with the other environmental regimes, obstructing simultaneous adaptation to multiple traits. However, the more ecological relevant heat resistance trait (KD) (Sørensen et al. 2005) does not show such negative correlations with the other regimes. Thus, most selection regimes seem not to constrain each other, at least based on shared physiological mechanisms.

Heat shock proteins (Hsps) and the heat stress response are prime candidates for environmental stress adaptation, however, the role of Hsps in the evolution of stress resistance is not fully resolved and mainly investigated for Hsp70 (Sørensen et al. 2003; Chown and Nicolson 2004; Sørensen 2010). The fact that the purely inducible forms of Hsp70 were not detected in this study is not surprising as our results are obtained from flies under benign common garden conditions, two generations after selection was performed. Further, earlier studies on the same selection lines suggested a very limited differentiation among lines in expression of inducible Hsp70 after heat exposure (Sørensen et al. 2009). We found a marked response of heat shock and associated proteins to selection for increased stress resistance. The expression of some stress chaperones was highly correlated, which was expected given their relation to the highly conserved stress response (Feder and Hofmann 1999). We found constitutive changes in expression of the small heat shock proteins and constitutive chaperones (heat shock 70 cognates) to be central in the environmental stress adaptation. However, little support was found for a unified positive correlation in expression levels among regimes as a quite large diversity of responses among regimes was found (Figures 4 and 5), consistent with independent regulation across stressors rather than one common heat shock stress response. Such a pattern of regulation

was identified across heat and desiccation resistance in the flesh fly, *Sarcophaga crassipalpis* (Tammariello et al. 1999). Opposed to what Hayward et al. (2004) found in *S. crassipalpis*, we did not find Hsp23 and only one Hsc70 copy to be influenced by selection for desiccation tolerance. Increased longevity has previously been associated with increased expression of both *hsp70* and *hsp22* (Zhao et al. 2005), and was here found positively associated with both Hsp23 and Hsp26 suggesting a particular role of small Hsps for longevity. This testifies diversified functional roles of different stress chaperones, including different copies of Hsc70 and the small Hsps. Selection regimes C30 and CS seemed to depend the most on Hsps, which both significantly increased constitutive expression in eight proteins. With respect to the degree of regulation (fold change) across all Hsps (significant and non-significant) C30, followed by HS, showed the highest average fold change, while CS only showed a very moderate fold change increase compared to controls and the third heat resistance trait, KS, seems on average even to show a slight decrease in expression. Hsp68 was only differentially expressed in CS lines, and thus seems specific for this stress regime. Previous studies have not been able to link cold resistance in *Drosophila* with the major stress protein Hsp70 (Nielsen et al. 2005). Here, CS lines showed a strong response in the small Hsps, and these might therefore be a general component of importance for insect cold resistance (Rinehart et al. 2007).

Conclusion

Using technically and biologically replicated samples and a powerful proteomic approach we consistently detected 637 proteins across all of our *D. melanogaster* samples from six different environmental stress selection regimes. In the response of the proteome to selection for increased

stress resistance we found a ‘common stress response’ shared among selection regimes. However, we also detected a potential trade-off between selection for heat shock survival and each of the remaining environmental stress resistance regimes, whereas the general pattern was a very limited occurrence of negative correlations at the proteome level. Thus, we find little evidence for a mechanistic basis for negative correlations among traits that could inhibit evolutionary adaptation to environmental stress, except for heat shock survival. We found that the strength of the signal from selection for cold resistance was at the same level as the other environmental stress regimes. This was surprising as many other studies have failed to generate strong signals for this trait, leading to the suggestion that few genes and proteins contribute to cold resistance. We here refute this suggestion and provide a set of candidate proteins and pathways, including ‘protein folding’ and ‘locomotor activity’. We found a strong and diverse contribution from the heat shock and related proteins to resistance to environmental stress. Opposed to the current understanding, the data suggests that the molecular chaperones do not only play a vital role during and after environmental challenge, but are molded by evolutionary changes in their basal levels in a complex fashion specifically tailored to each environmental stress regime. Finally, we conclude that the investigation of selection at the proteomic level improves our understanding of the physiological background of adaptation by revealing information complementary to information gained from investigations at the transcriptional level.

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Tables

Table 1. Fold change for all heat shock response related proteins in the proteomic dataset. Significant different expression in individual contrasts is given in bold and asterisks denote P-values (* P < 0.05; ** P < 0.01; *** P < 0.001).

ID	Protein	DS	LS	CS	HS	KS	C30
P02516	Hsp23	0.013	-0.031	-0.035	0.327***	-0.001	0.351***
P02517	Hsp26	-0.038	0.194***	0.268***	0.082	0.030	0.203***
P02518	Hsp27	-0.033	0.128**	0.225***	0.126**	0.024	0.027
O02649	Hsp60	0.028	0.037	0.055**	0.095***	0.029	0.191***
O97125	Hsp68	-0.052	-0.073	-0.181***	0.066	-0.080	0.029
P02828	Hsp83	0.032	0.035	0.098***	0.033	0.067***	0.298***
Q24276	Hsp90 co-chap.	-0.164*	-0.124	-0.198*	-0.262**	-0.180*	0.067
P29843	Hsc70-1	-0.038	-0.047	-0.089***	-0.016	-0.076**	-0.060*
P29844	Hsc70-3	-0.025	-0.009	0.052	0.124***	0.009	0.173***
P11147	Hsc70-4	0.009	0.036*	0.060***	0.075***	0.040*	0.136***
P29845	Hsc70-5	0.050*	-0.002	0.034	0.012	0.065**	0.129***

Figures

Figure 1. A) Number and direction of the change of significantly differentially expressed proteins from each contrast between individual selection regimes as compared to controls. Note that the LS regime had fewer selection events than other regimes. B) Bars show the percentage of significantly differentially expressed proteins that are significantly regulated in opposite directions as a response to selection (i.e. shared by regimes and showing a negative correlation in the direction of regulation). The absolute number of significantly differentially expressed proteins (and enriched GO terms in brackets) shared among regimes (disregarding the direction of expression) are displayed on top of each bar. Note that the HS regime consistently shows the largest number of negative correlations with all other regimes. C) As B, but showing common proteins shared simultaneously by all selection regimes and by all selection regimes disregarding HS. Note that more proteins are shared by all regimes and with more consistent direction of regulation when disregarding the HS lines, pointing to this regime having a fundamental different response to selection than all other selection lines. The regimes were: Desiccation resistance selection (DS); Longevity selection (LS); Cold-shock resistance selection (CS); Heat shock resistance selection (HS); Heat knock down resistance selection (KS); and exposure to constant 30 °C during development (C30).

Figure 2. Plot of the first versus the second principal component (A) as well as the third principal component (B) (% variation explained in brackets) of global protein expression levels (637 proteins) among five biological replicates within each of seven environmental stress resistance selection regimes. The regimes were: Unselected control (UC); Desiccation resistance selection (DS); Longevity selection (LS); Cold-shock resistance selection (CS); Heat shock resistance selection (HS); Heat knock down resistance selection (KS); and exposure to constant 30 °C during development (C30).

Figure 3. Venn diagrams showing the overlap of significantly enriched GO terms among high temperature selection regimes (KS, HS and C30) (A) and among KS, DS and CS selection regimes (B). (A) The three high temperature regimes show very distinct responses, with little overlap. (B) Both DS and CS showed a considerable overlap with KS. The overlap explained all the significant GO terms of KS, except for one. While both DS and CS showed this considerable overlap with KS, these two regimes only shared two GO terms, and thus showed very distinct responses. The regimes were: Desiccation resistance selection (DS); Cold-shock resistance selection (CS); Heat shock resistance selection (HS); Heat knock down resistance selection (KS); and exposure to constant 30 °C during development (C30).

Figure 4. Plot of the first versus the second principal component (A) as well as the third principal component (B) (% variation explained in brackets) of heat shock protein expression levels (11 proteins) among five biological replicates within each of seven environmental stress resistance selection regimes. The regimes were: Unselected control (UC); Desiccation resistance selection (DS); Longevity selection (LS); Cold-shock resistance selection (CS); Heat shock resistance selection (HS); Heat knock down resistance selection (KS); and exposure to constant 30 °C during development (C30).

Figure 5. Protein expression correlations among the eleven detected heat shock response related proteins. The strength and direction of each Pearson product-moment correlation is indicated by the color as well as the shape of the ellipse. Insignificant correlations are crossed ($\alpha > 0.05$). The P-values were adjusted using the Holm-Bonferroni correction method.

Supplemental material

Suppl. Table 1. Proteins significantly differentially expressed in at least one contrast

between selection and control regimes. This table lists log2 fold change (FC) and ANOVA P-values for all individual contrasts, and thereby allows for comparison of significant proteins between individual selection regimes as well as their direction of change. To allow the interpretation of the common response to selection, the last two columns identify (with '1') proteins that are detected as significant in all regimes simultaneously (58 proteins) or in all regimes excluding the HS regime (86 proteins). The regimes were: Unselected control (UC); Desiccation resistance selection (DS); Longevity selection (LS); Cold-shock resistance selection (CS); Heat shock resistance selection (HS); Heat knock down resistance selection (KS); and exposure to constant 30 °C during development (C30).

Suppl. Table 2. Significant and non-significant GO-terms that are detectable among the

637 proteins found in the full proteomic dataset. All detectable GO-terms were evaluated for each selection regime, which allows for comparison of significant GO-terms between pairs of selection regimes and across all for identification of GO-terms related to the common stress response. Significantly enriched GO-term as identified by Gene-Set Analyses (GSA) for specific contrasts among regimes are marked with a '1' while '0' signifies a non-significant enrichment. Row only having '0' signifies GO-terms that are represented by the proteins identified in the full dataset, and thus, GO-terms that we have power to detect. GO-terms not represented in the table were not detectable in this study. Several '1' in a row signifies that the same GO-term was enriched in several different selection regimes as compared to controls and thus shared between regimes.