AARHUS UNIVERSITY

Coversheet

This is the accepted manuscript (post-print version) of the article.

Contentwise, the accepted manuscript version is identical to the final published version, but there may be differences in typography and layout.

How to cite this publication

Please cite the final published version:

Michalak, P, Kang, L, Schou, MF, Garner, HR, Loeschcke, V. Genomic signatures of experimental adaptive radiation in *Drosophila*. *Mol Ecol*. 2019; 28: 600– 614. <https://doi.org/10.1111/mec.14917>

Publication metadata

Title:	Genomic signatures of experimental adaptive radiation in <i>Drosophila</i>
Author(s):	Michalak, P, Kang, L, Schou, MF, Garner, HR, Loeschcke, V.
Journal:	<i>Molecular Ecology</i>
DOI/Link:	https://doi.org/10.1111/mec.14917
Document version:	Accepted manuscript (post-print)

This is the peer reviewed version of the following article: Michalak, P, Kang, L, Schou, MF, Garner, HR, Loeschcke, V. Genomic signatures of experimental adaptive radiation in *Drosophila*. *Mol Ecol*. 2019; 28: 600– 614. <https://doi.org/10.1111/mec.14917>, which has been published in final form at <https://doi.org/10.1111/mec.14917>. This article may be used for non-commercial purposes in accordance with Wiley Terms and Conditions for Use of Self-Archived Versions.

General Rights

Copyright and moral rights for the publications made accessible in the public portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognize and abide by the legal requirements associated with these rights.

- Users may download and print one copy of any publication from the public portal for the purpose of private study or research.
- You may not further distribute the material or use it for any profit-making activity or commercial gain
- You may freely distribute the URL identifying the publication in the public portal

If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

If the document is published under a Creative Commons license, this applies instead of the general rights.

Article type : Original Article

Genomic signatures of experimental adaptive radiation in *Drosophila*

Short title
Experimental adaptive radiation

Pawel Michalak^{1,2,3§}, Lin Kang¹, Mads F. Schou^{4,5}, Harold R. Garner¹,
Volker Loeschcke^{4§}

¹ Edward Via College of Osteopathic Medicine, Blacksburg, VA 24060, USA

² One Health Research Center, Virginia-Maryland College of Veterinary Medicine, Blacksburg, VA 24060, USA

³ Institute of Evolution, University of Haifa, Haifa, 3498838, Israel

⁴ Department of Bioscience, Aarhus University, Aarhus, DK-8000, Denmark

⁵ (Present address) Department of Biology, Lund University, Lund, SE-22362, Sweden

§Correspondence: pawel@vbi.vt.edu; volker@bios.au.dk

Emails: pmichalak@vcom.vt.edu (PM), linkang@vt.edu (LK), mads.schou@biol.lu.se (MFS), sgarner@vcom.vt.edu (HRG), volker@bios.au.dk (VL)

Abstract

Abiotic environmental factors play a fundamental role in determining the distribution, abundance, and adaptive diversification of species. Empowered by new technologies enabling rapid and increasingly accurate examination of genomic variation in populations, researchers may gain new insights into the genomic background of adaptive radiation and stress resistance. We investigated genomic variation across generations of large-scale experimental

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/mec.14917

This article is protected by copyright. All rights reserved.

selection regimes originating from a single founder population of *Drosophila melanogaster*, diverging in response to ecologically relevant environmental stressors: heat shock, heat knock down, cold shock, desiccation, and starvation. When compared to the founder population, and to parallel unselected controls, there were more than 100,000 single nucleotide polymorphisms (SNPs) displaying consistent allelic changes in response to selective pressures across generations. These SNPs were found in both coding and noncoding sequences, with the highest density in promoter regions, and involved a broad range of functionalities, including molecular chaperoning by heat shock proteins. The SNP patterns were highly stressor-specific despite considerable variation among line replicates within each selection regime, as reflected by a principal component analysis, and co-occurred with selective sweep regions. Only ~15% of SNPs with putatively adaptive changes were shared by at least two selective regimes, while less than 1% of SNPs diverged in opposite directions. Divergent stressors driving evolution in the experimental system of adaptive radiation left distinct genomic signatures, most pronounced in starvation and heat shock selection regimes.

Introduction

Phenotypic traits that are tightly connected to the Darwinian fitness are targeted by natural selection and may evolve. Adaptive evolution of a simple independent trait is limited by the amount of expressed heritable genetic variance, but for most complex traits, the process of evolution typically involves micro-evolution of a suite of underlying subordinate morphological and physiological traits (Falconer & Mackay 1996; Lynch & Walsh 1998; Swallow & Garland 2005). This creates an opportunity for side-effects on other complex traits that also underlie Darwinian fitness, potentially producing evolutionary constraints in form of trade-offs, or alternatively parallel evolution of complex traits within a population (Walsh & Blows 2009). Because natural populations face multiple environmental stressors,

including thermal stress as well as water and food availability, several complex traits are typically targeted by selection simultaneously (Hoffmann & Parsons 1997). Experimental evolution has proved to be a powerful approach to improve our understanding of the process of genomic adaptation (Burke *et al.* 2010; Schlotterer *et al.* 2015; Teotonio *et al.* 2009). We now know more about the prevalence of soft sweeps in sexually reproducing organisms (Burke 2012; Burke *et al.* 2010; Tobler *et al.* 2014; Turner *et al.* 2011), the reversibility of evolution (Teotonio *et al.* 2009), maintenance of genetic diversity by selection in smaller populations (Schou *et al.* 2017), and the repeatability of evolution (Graves *et al.* 2017). However, our knowledge on how the pathways and mechanisms of genomic adaptation in one complex trait, may constrain or promote the adaptive evolution of another complex trait is still very limited. This is likely due to the fact that inference on this matter is limited by lack of similar genetic background in previous studies focusing on separate environmental stressors (Sarup *et al.* 2011); in other words, another experimental design is needed.

In 1996 one founder population of *Drosophila melanogaster* was used to establish a system of experimental adaptive radiation, involving five different environmental stressors with five replicate populations each: Desiccation resistance selection (DS); Starvation resistance selection (SS); Cold-shock resistance selection (CS); Heat shock resistance selection (HS) and Heat knock down resistance selection (KS), in comparison with Unselected control (UC, also represented by five replicates). Every second generation, the 300 (50%) most stress tolerant individuals for a given stressor were selected, and after 21 generations of selection all replicate populations had increased their respective stress tolerance (Bubliy & Loeschcke 2005). When correlated phenotypic responses across the different stressors were assessed, no trade-offs emerged and several stressors showed correlated responses (Bubliy & Loeschcke 2005), a pattern recently confirmed at the proteomic level (Sorensen *et al.*

2017). This impressive phenotypic assessment suggests that the prevalence of trade-offs constraining adaptive evolution in stress tolerance traits is low for the traits considered, but the underlying genomic data from this system is yet to be explored.

The focus of this study is to survey allele frequency differentiation among populations with divergent selection regimes based on ecologically relevant stressors. To this end, the above-mentioned selection regimes were continued for a total of 65 generations, allowing us to follow the genomic signatures of adaptive evolution beyond the time of phenotyping and compare generation 45 (F45) to generation 65 (F65). First, we identified candidate variants, genes and larger pathways for the adaptive evolution of the five environmental stressors, extending what we know of the genetic architecture underlying adaptation to environmental stressors such as temperature. We then compare these candidates across the stressors to examine the patterns of parallel/correlated evolution among these complex traits, which is possible because of the common laboratory ancestor population. Finally, we increased our knowledge related to mechanisms of adaptive evolution, by investigating the presence of soft sweeps and shifts in the presence of transposable elements.

Materials and Methods

Overview of flies and experimental selection regimes

The *D. melanogaster* lines used in this study originate from those first described and used in Bublly and Loeschcke (Bublly & Loeschcke 2005) and since used in other studies focusing on different levels of biological organization (Malmendal *et al.* 2013; Sorensen *et al.* 2007; Sorensen *et al.* 2017). Initially, a diverse mass-bred laboratory population of *D. melanogaster* was established and kept on standard oatmeal-sugar-yeast-agar *Drosophila* medium at 25 °C. Five selection regimes/groups and one control group were established

from the mass-bred population in generation F4, with each group consisting of five independent replicate lines (Fig. 1). The groups were unselected control (UC), Desiccation resistance (DS), Starvation resistance (SS), Cold-shock resistance (CS), Heat shock resistance (HS), and Heat knockdown resistance (KS). To avoid trans-generational effects influencing the experiment (Hercus & Hoffmann 2000; Watson & Hoffmann 1996), selection was performed every other generation to allow recovery in intermediate generations. In each generation of selection, the 50% with the highest stress tolerance was selected from 300 pairs. A sixth group selected for longevity was also established, but since it did not involve an ecological stressor, genomic variation in this group was investigated in a separate study (Michalak *et al.* 2017). The selection procedures are described in detail elsewhere (Bubliy & Loeschcke 2005; Malmendal *et al.* 2013), and the phenotypic data is highlighted by Figures 1 and 2 in Bubliy and Loeschcke 2005.

Sample preparation, DNA extraction and sequencing

Samples for sequencing were obtained from base population in F4 (5 replicates) and from each replicate line of the selection regimes in generations F45 and F65 (Fig. 1). Each sample consisted of 44 flies pooled by replicate population. The samples were frozen in ethanol (-80°C) in F4 corresponds to generation 0 of the selection regimes, and the first generation of selection, whereas F6 is the second generation of selection. At generation F45, the replicate population had been through 21 generations of selection and one subsequent generation without selection. At generation 65, the replicate populations had been through 10 additional generations of selection, as well as one subsequent generation without selection. DNA was extracted using the CTAB method. TruSeq DNA libraries were prepared and sequenced on the HiSeq 4000 platform following Illumina's protocols, and 2x100 bp paired-end reads were generated. Each sample was assigned to a group according

to the selection regime and generation (e.g. SS45). The summary of sequencing can be found in Supporting Table S1.

Mapping and genotyping

The *Drosophila melanogaster* genome (dm3) and corresponding annotations (RefSeq) from UCSC (<http://genome.ucsc.edu/>) were used as reference for mapping. Quality-control and filtering of raw sequence reads were done using FastqMcf (Aronesty 2013). The remaining reads were mapped to the reference using BWA (Li & Durbin 2009) using default parameters. GATK (DePristo *et al.* 2011) with default parameters (except for using ‘--sample_ploidy’ for pooled data and setting –heterozygosity to 0.01) was employed to generate genotypes in each sample. Genotypes with more than 2 alleles were discarded.

Only sites with genotyping quality greater than 30, minimum depth 10, and maximum depth 350 were used in the analysis.

Sweep region detection and heterozygosity estimation

Putative selective sweep regions were detected with Pool-hmm (Boitard *et al.* 2013), a hidden Markov model for finding selective sweep signatures from Pool-Seq data. The parameters used in Pool-hmm were “-n 88 -c 5 -C 400 -q 20 -e sanger -p -k 0.0000000001”, while “--theta” was set to be the θ estimated for each sample. Heterozygosity was estimated using a 100 kb sliding window with a step of 10 kb.

Identification of transposable element insertions

Transposable element (TE) insertions were identified by PoPoolation TE (Kofler *et al.* 2012), and transposable element sequences were downloaded from FlyBase (transposon sequence v9.42, <http://flybase.org/>). To minimize the effect of different sequencing depths

on TE identification, we approximated equal physical coverage by using the same read coverage for each sample. A randomly selected subset of mapped reads from each sample was used in TE identification, each containing the same number of reads (111,728,172), corresponding to the sample with the lowest number of mapped reads (11A in Supporting Table S1). TE frequencies were compared between groups using average numbers per replicate within a group, as well as the total numbers of unique insertions across all five replicates per group.

Polymorphic sites of interest

We focused on polymorphic sites in which allele frequency changes in selection lines from the same selection regime were in the same direction, possibly reflecting convergent adaptations. For a certain selection regime, we required (Rule 1) the allele frequencies in control lines to remain the same across generations (i.e., the average allele frequency differences among lines of F4, F45, and F65 to be less than 10%). Then we required (Rule 2) the frequency change between selection lines F45 (relative to control lines) and the change between selection lines F65 (relative to selection lines F45) to be in the same direction, and (Rule 3) the changes to be greater than 10%. Specifically, Rule 3 requires allele frequencies to change in a consistent manner from F45 to F65 compared to F4, i.e., the average frequency of selection lines of F45 to be >10% higher (lower) than the maximum (minimum) average frequency of control lines through all generations, and the average frequency of selection lines of F65 to be greater (lower) than that of selection lines of F45. If conditions specified by Rules 1-3 are met, a unidirectional ‘change-in-frequency’ in a ‘polymorphic site of interest’ (PSI) is identified (Fig. 2). To identify PSIs, Rules 1-3 were applied to all polymorphic sites in each selection regime, and allele frequency changes in selection lines of F45 and F65 compared to control lines were added up. A total of 108,388 PSIs were

found. We also applied more stringent criteria to PSI's based on neutrality tests using an R package poolSeq (Taus *et al.* 2017) and Cochran–Mantel–Haenszel (CMH) test.

Synchronized files containing allele frequencies for all SNPs were generated with PoPoolation2 (Kofler *et al.* 2011) and effective population size (N_e) for each group was estimated using the function “estimateNe” from the poolSeq package based on synchronized files. The selection coefficient (s , Supporting Table S2) for each PSI and each group was estimated through “estimateSH” with parameter “Ne” set to the estimated N_e from the previous step (Supporting Table S3) and with parameter “N.ctraj” (the number of simulations performed for bias correction) set to 1,000, while the P-value was estimated by performing 1,000 simulations. The CMH test was performed as follows: First, forward simulations using mimicrEE (Kofler & Schlotterer 2014) were carried out to generate expected neutral changes during the experiment. For each simulation, a founder population with 1,967,223 SNPs from the Drosophila Genetic Reference Panel 2 (DGRP2) (Huang *et al.* 2014; Mackay *et al.* 2012) was used, while the number of simulated haplotypes was selected to correspond to the N_e estimation from the previous step (Supporting Table S3). We generated the simulation data for generations 45 and 65 with 5 replicates and similar sequencing coverage to our experimental data using a homogeneous coverage across the chromosomes. We repeated the simulation 10 times. CMH tests were performed by comparing allele frequencies in generations 45 and 65 with the frequencies in the founder population using the simulated data. The SNPs were grouped in five categories based on their starting minor allele frequencies: <10%, 10-20%, 20-30%, 30-40%, and 40-50%. We combined all P-values of the CMH test from the 10 simulations and then determined the P-value corresponding to 1% FDR (i.e., 1% of the combined P-values) for each category. The CMH tests were performed in the same manner for each selection regime. A PSI was called a ‘rigorous PSI’ (rig-PSI) when the following conditions were met: (i) estimated selection

coefficient in the control group was less than 0.05 and (ii) greater than 0.05 in at least one selection regime with a significant P-value (<0.05), and (iii) passed the CMH test at empirical FDR of 1% or lower according to the starting minor allele frequencies from F4. Thus, a list of 108,388 PSIs was narrowed down to 3,317 rig-PSIs.

Reconstruction of selected haplotype blocks

The selected haplotype blocks for all PSIs were generated using R package (<https://www.r-project.org/>) haploReconstruct (Franssen *et al.* 2017), with parameters set as follows: minimum frequency change (minfreqchange=0.2), minimum number of replicate (minrepl=2), maximum minor allele frequency (max.minor.freq=0.10), and window size (winsize=1Mb). Haplotype blocks with the number of markers less than five or density of marker less than 10 per Mb were filtered out.

Principal component analysis (PCA) and GO functional enrichment

We used smartpca with default parameters from EIGENSOFT (Price *et al.* 2006) to perform the PCA based on a covariance matrix. We included only PSIs in PCA, with the input genotype file prepared in EIGENSTRAT format, which uses 0, 1, and 2 to reflect the number of copy of reference allele for each sample at each location. To take the gene length into account, an unbiased analysis of gene set enrichment using GOWINDA (Kofler & Schlotterer 2012) was performed. The gene mode was used (--mode assumes that all SNPs within a gene are in linkage disequilibrium) with parameters of 1,000,000 for the simulation (--simulations), the minimum gene number in GO category of 2 (--min-genes) and all SNPs as the background (--snp-file).

Overlap of PSIs and differentially expressed proteins

The selection lines investigated here also had expression levels of 811 proteins quantified in a previous investigation (Sørensen et al. 2017). The samples were obtained after two additional generations of selection and originated from the selection regimes CS, DS, HS and KS as well as UC. This is a unique opportunity to investigate if changes in protein expression due to selection can be associated with changes at the DNA-level. We approached this by testing if there was a significant overlap of the genes having a differential protein expression compared to UC and the genes having a nucleotide variant qualifying as rig-PSI's. We considered proteins which passed an $FDR < 0.05$ and a $\log_2$ fold change > 0.15 to be significant. As an initial test of overlap between the two biological levels we disregarded information of position of PSI-rig in a given gene and instead tested for overlap between genes affiliated with at least one PSI-rig and a significant differential protein level. The background set of genes consisted of genes where a SNP was identified and tested, as well as where protein expression was quantified ($n = 774$). P-values were obtained by randomization ($n = 10,000$) followed by a one-tailed test of a higher observed overlap than expected. As this yielded a significant overlap in one selection regime, we analyzed different gene-affiliated regions separately, in order to understand if the overlap was driven by e.g. changes in the promoter region or an artefact of change in the protein sequence (CDS). In these analyses, only genes with an identified and tested SNP in the relevant region were included in the background set.

Results

SNP frequency changes in PSIs

A total of 65 lines/samples (see Fig. 1 for overview of samples) were sequenced with an average depth of 137X (96X to 167X) and average mapping rate of 94% (Supporting Table

S1). The base population had a lower heterozygosity compared to populations of generations F45 and F65, but the difference was minor (Supporting Table S4), and there was no significant difference in heterozygosity between F45 and F65 in any comparison. We found a total of 1,967,223 polymorphic sites, with their highest density in putative promoter regions (100 bp upstream of 5'-UTR) and the lowest in the coding DNA sequences (CDS) (Table 1). After applying Rules 1-3 to find the polymorphic sites of interest (PSIs; see Materials and Methods), which showed allele frequency changes in the same direction among selection lines relative to control lines, 108,388 sites remained (Table 1). The PCA for the PSIs show replicate lines under the same selection regime to cluster together, with HS and SS lines distinctively separated from the other selection regimes (Fig. 3). The SS group produced on average ~3.7 times more PSIs than other selective regimes but HS produced PSIs with the most extreme values. Out of 6,721 PSIs with change-in-frequency over 60%, 4,799 (71.4%) of them showed the highest change-in-frequency in the HS regime compared with only 523 (7.8%) of them in the SS regime. For the 336 most extreme PSI with change-in-frequency over 100%, as many as 333 (99.1%) of them exhibited the highest change-in-frequency in the HS regime, along with two in SS and one in CS.

Of the 108,388 PSIs, the majority (84.9%) were found in only one selection regime, and 15.1% were shared by at least two regimes (Supporting Fig. S1). Eight PSIs were shared by all five selection regimes, but none of them resulted in non-synonymous changes of amino acids. Six non-synonymous PSIs in four genes (*CG42588*, *InR*, *Or98b*, and *CG12413*) were shared by four selection regimes (Supporting Dataset S1). A total of 1,160 of PSIs (1.07%) were found in the promoter regions, which resulted in the highest density of PSIs (1.12/kb) across all genomic features, potentially underscoring the significance of regulatory changes

in response to the selective regimes (Table 1). The overlap between selective regimes in promoter regions affected by PSIs was low (Supporting Fig. S1), with only one PSI in the putative promoter of *klg* (chr3R:18724961, C>A) being shared by four regimes (CS, DS, HS, and SS). The gene *klg* encodes Klingon, a cell adhesion molecule affecting memory, behavior, and cell differentiation (Butler *et al.* 1997; Dubnau *et al.* 2003; Matsuno *et al.* 2009). GO enrichment analyses of PSI-affected promoters revealed only two terms significant at FDR < 0.05: peptidyl-serine phosphorylation (DS) and metabolic pathways (HS) (Supporting Table S5). These two terms overlap with the enriched proteins in a previous proteomic analysis of the same lines where protein serine/threonine phosphatase activity was one of ten significant DS terms, and carbohydrate metabolic process was one of 11 significant HS terms.

SNP frequency changes in rig-PSIs

Under more stringent filtering criteria, a total of 3,317 rig-PSIs were found, and 1,918 (57.82%) were found in HS, while only 115 (3.47%) were found in DS (Figures 4 and 5 and Supporting Fig. S2). Only 12 rig-PSIs (see Materials and Methods) were shared by three selection regimes, and 172 were shared by two regimes (Supporting Dataset S2). We also screened for rig-PSIs at the gene level in each regime and found 169 genes containing PSIs shared by at least two regimes (Supporting Table S6), and 21 of them were shared by three regimes, including genes such as *C3G* (DS, KS, and SS), *caps* (CS, HS, and KS), *Calx* (CS, KS, and SS), *ush* (CS, HS, and SS), *mod(mdg4)* (CS, KS, and SS), *seq* (DS, KS, and SS), and *SNF4Agamma* (CS, KS, and SS), a gene previously reported to be involved in behavioral and cellular responses to starvation (Johnson *et al.* 2010). There were no genes with rig-PSIs shared by four or more regimes. Numbers of rig-PSIs among selection regimes are likely confounded by the presence of linkage blocks containing multiple rig-

PSIs. Indeed, based on the haplotype reconstruction, we identified 314 haplotype blocks under selection, with the highest number of blocks (104) found in SS and the lowest (40) in DS (Supporting Table S7). Of the 314 blocks, 88 of them contained more than one rig-PSI with density over 1 per Mb (Fig. 5 and Supporting Table S7), such as a ~4.5Mb 2L arm region (chr2L:15830143-20468342) and a ~1.1Mb 3R arm region (chr3R:24096490-25191891) in HS, as well as a ~0.4Mb X chromosome region (chrX:12160111-12593045) in CS and a ~0.6Mb 3L arm region in KS (chr3L: 7775940-8362982). Only 32.2% of identified haplotype blocks (by length) were shared between HS and KS, compared to 54.8% between HS and SS, and 67.7% between KS and SS (Supporting Table S7). The haplotype block with a peak cluster of rig-PSIs along 2L arm region (chr2L:15830143-20468342) in HS was not found in KS, but was partly shared with SS. However, another block with a peak cluster of rig-PSIs along 3R arm found in HS was also in part shared by KS (chr3R:24137576-24311581). Despite the fact that both HS and KS involve heat stress, important differences between the two regimes do exist. For example, the HS lines had gone through a hardening (pre-treatment) process, while the KS lines did not (Bublić & Loeschcke 2005). The hardening procedure itself may cause a distinct response (Hangartner & Hoffmann 2016; McColl *et al.* 1996) and thus potentially explain why we found little overlap between HS and KS.

For the 164 non-synonymous rig-PSIs, 82 were found in HS, while seven found in DS (Supporting Dataset S2). Interestingly, the top 50 most significant frequency changes non-synonymous rig-PSIs included 46 from HS, two from KS, one from CS, and one from DS. Only nine were shared in more than one regime, including one shared by three regimes (CS, HS, KS) resulting in an amino acid change within *CG42588* (chr3L:12810454; C>A). One non-synonymous rig-PSI from *mthl7* (chr3L:8173506; T>A), a gene associated with stress (starvation) response and determination of adult lifespan (Lin *et al.* 1998), was found in SS

and KS. Other non-synonymous rig-PSIs in genes such as *C3G*, *seq*, *Plc21C*, and *Kdm4B* were found in two regimes (Supporting Dataset S2). An enrichment analysis of all non-synonymous rig-PSIs showed such enriched GO terms as galactosyltransferase activity and cofactor biosynthetic process (Supporting Table S8). When the analysis was expanded to all 3,812 non-synonymous PSIs, the 5% (191) most significant frequency changes had a minimum change-in-frequency of 63%, and similar to what we found in rig-PSIs, 141 (73.8%) of them showed the highest change-in-frequency in the HS regime, which may indicate a rapid response to heat shock stress. The five PSIs with the change-in-frequency over 100% in five genes (*qua*, *CG31029*, *grp*, *Ppn*, and *CG34295*) were all found in HS (Supporting Dataset S1). The 191 PSIs clustered in such genes as *Ppn*, *tam*, *intr*, *Apc*, and *CG34295* (Supporting Fig. S3). The most significant GO term enrichment (FDR < 5%) for those PSIs was galactosyltransferase activity (Supporting Table S9).

Divergence in direction of change

The change-in-frequency of PSIs may show different directions in different selection regimes i.e., it may show increase of frequency in one regime and decrease of frequency in another. Only four rig-PSIs did show divergent direction changes. Three of them were in introns of *Ac13E* (chrX:15441117; A>T, between DS and SS), *Hmgcr* (chr3R:19564626; C>G, between CS and SS), and *CG31145* (chr3R:19482387; A>G, between CS and SS), and one located in intergenic region (chrX:15163514; A>T, between DS and SS). For all PSIs, 1,041 of them showed divergent direction changes in frequency in at least two regimes, and 26 of them were located in coding regions producing non-synonymous changes (Supporting Dataset S3). Seven of them were found to have divergent directions between SS and HS and six were found to have divergent directions between SS and KS. The low prevalence of divergent direction changes in both PSI and rig-PSI, may potentially

arise if the variants responding to selection had a low initial frequency in the founder population, such that only one direction of change allowed passing the PSI filtering process. However, the initial frequency distribution of PSIs (and rig-PSIs) was qualitatively similar to that of the PSIs (and rig-PSIs) shared among regimes, and no bias was observed in the distribution of the starting frequency of PSIs, showing that a low initial frequency was not the cause of relatively low prevalence of divergent direction (Supporting Fig. S4).

Allelic fixations

To examine if the selection pressures could result in allelic fixation, we looked for rig-PSIs and PSIs with fixed homozygous genotypes in the selection regimes of generation 65, being heterozygous genotypes (minor allele frequency greater than 10%) in all control lines. We found 63 such rig-PSIs: 3 of them were non-synonymous changes in genes *CG3280*, *CG33203*, and *CG11581* (Supporting Dataset S4 and Supporting Table S10). A total of 6,148 PSI fixations were observed, 217 (3.53%) of which were non-synonymous (Supporting Table S10). To test whether fixation occurs more likely within selective sweep regions, we compared the percentage of fixed PSIs in the sweep regions of generation 65 identified via Pool-hmm (Boitard *et al.* 2013) with the percentage of all PSIs in the same sweep regions. More than half of those fixations (3,196 out of 6,148 or 52%) were found in selective sweep regions of the selection regimes, significantly higher when comparing to the percentage of all other PSIs in the same sweep regions, which is 42% (43,115 out of 102,240, Fisher Exact Test $P < 2e-16$). Of the 217 PSIs, two of them were shared by four selection regimes: chr2R:6184201 of gene *CAP* (fixation of A in CS, HS, KS, and SS) and chr3R: 24659889 of gene *Apc* (CS, DS, HS, and SS), thirteen of the fixations were found in three selection regimes (Supporting Dataset S5), and 122 (56%) of them were in sweep

regions. GO terms and pathways such as response to iron-sulfur cluster assembly were found to be enriched among the PSIs fixed in sweep regions (Supporting Table S11).

Overlap of PSI's and differentially expressed proteins

At the gene level, only the CS regime showed a significant overlap (five genes) between genes with differentially expressed proteins and genes with identified PSI-rig (Supporting Table S12). When we considered rig-PSIs in each gene region separately, the significant overlap in the CS regime was driven by rig-PSIs in intron regions producing an overlap of four out of the five genes (Supporting Table S13). Correlating the protein levels of the four genes (*Scsalpha*, *Lasp*, *Flo2*, and *Elp2*) with the rig-PSI frequencies did not result in a significant relationship (Fig. 5 and Supporting Fig. S5).

Heat-shock response genes

The previous analyses of transcriptome and proteome in this experimental adaptive radiation system emphasized the role of heat-shock protein genes in divergence between selected and non-selected control lines (Sorensen *et al.* 2007; Sorensen *et al.* 2017). We thus specifically looked into SNP/PSI patterns in heat-shock response-related genes (heat-shock co-chaperones and cognates) and found 54 PSIs but no rig-PSI in 14 such genes (Supporting Dataset S6). Nearly half of them (26) were in CDSs and 10 were in UTR regions. Four PSIs from four genes (*Hsp26*, *Hsp60B*, *Hsp70Bbb*, and *Hsc70-1*) resulted in non-synonymous changes. Of the 4 non-synonymous PSIs, chr2L:729149 (C>A, *Hsp60B*) and chr3L:13967849 (C>T, *Hsc70-1*) were found in CS and SS, chr3L:9369713 (T>G, *Hsp26*) was identified in HS, and chr3R:8329034 (G>C, *Hsp70Bbb*) was located in SS (Supporting Dataset S6). Yet another PSI was located in the 3-UTR of *Hsp22* (chr3L:

9367885, A>G) and found in four selection regimes (CS, DS, HS, and SS). None of the PSIs in those heat-shock response genes were shared by regimes that involved high temperature stress, namely HS and KS. In fact, most of the 54 PSIs were found in SS regime (37 PSIs), followed by 11 in CS and 10 in HS.

Transposable elements profiling

The overall number of transposable elements (TEs) decreased over time in both selection and control lines. The average number of TE across lines of generation 4 was 8,620, while the numbers for lines of generation 45 and generation 65 were 7135 and 6753, respectively (Fig. 6A). The pattern held across major TE families (Fig. 6B-E). When we compared the numbers of TE insertions per replicate in generations 45 or 65 between selection and control groups, on average significantly more TEs were found in DS45 compared to UC45, and significantly less TEs were found in HS65 compared to UC65 (Fig. 6A). However, when we used the total numbers of unique TE insertions per group, the degree of decrease in the number of TE over generations was minor (Supporting Table S14), suggesting that the TE loss was largely random with respect to the insertion position. Even though more TEs were found in generation 4, and generation 45 tended to have more TEs than generation 65, the differences between generations 45 and 65 were small and less consistent (Supporting Table S14). Interestingly, we found that the TE number differences among generations and selective regimes were very low in CDSs compared with other genomic regions (Fig. 6F). The average percentage decrease of TE numbers between generation 45 and generation 65 was 1.07% and 4.42% for TEs in the CDS region and in all other regions, respectively (P-value=0.00077, Wilcoxon rank sum test). There was no significant difference (P-value=0.5412, Wilcoxon rank sum test) between TE frequencies in the CDS region and

those in non-CDS regions in the founding population (UC4), suggesting that the difference in TE frequencies between later generations is unlikely to be explained by sampling alone.

Discussion

Previous research has shown that the experimental adaptive radiation system of *D. melanogaster* has indeed diverged under distinct selective regimes, producing superior phenotypes in the selected traits, with predominantly positive correlated responses among traits (Bubliy & Loeschcke 2005) and accompanied by significant alterations in transcriptomes (Sorensen *et al.* 2007) and proteomes (Sorensen *et al.* 2017). In this study, we explore how these changes are reflected at the genome-wide level of DNA sequence variation. Despite the fact that the biological replicate lines showed a certain degree of variability (Fig. 3), the selective regimes produced very distinct patterns of genomic signatures, permitting unequivocal separation of the groups based on SNP profiles alone.

Similar to transcriptome and proteome results (Sorensen *et al.* 2007; Sorensen *et al.* 2017), HS exhibited the most extreme response consistent with the operation of directional selection at the sequence level, and thereby contain important data and candidate loci for ongoing climate change research on thermal adaptation of ectotherms (Araujo *et al.* 2013; Garcia-Robledo *et al.* 2016; Hoffmann & Sgro 2011; Kellermann *et al.* 2012). Another selective regime that quantitatively stood out was SS, well separated by SNP PCA1 and enriched in polymorphic sites with allele frequency dynamics consistent with adaptive evolution (PSIs). SS lines had nearly three times as many PSIs as HS lines, but the magnitude of allelic changes was much higher in the latter, and hundreds of the most extreme PSI values were predominantly produced in HS. One might speculate that this difference was related to more extensive pleiotropic interactions and fitness trade-offs

involved in responses to starvation compared with heat shock, assuming all else being equal. Alternatively, this difference may result from different heritability values of heritability for the two traits, or different distributions of effect sizes of the allelic variants.

Unlike previous studies that implied only few genes associated with resistance to cold ((Misener *et al.* 2001; Qin *et al.* 2005; Telonis-Scott *et al.* 2009) but see (Sorensen *et al.* 2017)), we found a rich and distinct pattern of SNP variation characterizing CS. A recent study on experimental evolution in hot and cold environments (Tobler *et al.* 2014) found candidate SNPs enriched within 42 heat tolerance genes and six cold tolerance gene. Using the same method, we found that HS PSIs were significantly enriched in 49 heat tolerance genes and CS PSIs were significantly enriched in four cold tolerance genes (Supporting Table S15). However, only six heat tolerance genes and no cold tolerance gene were shared between this study and Tobler *et al.* (2014). DS clustered most closely with unselected control lines (UC) and had the lowest number of PSIs, suggesting that desiccation exerted the weakest effects on genomic differentiation relative to other selective regimes. This result seems to be at odds with another ‘evolve-and-resequence’ experiment in which *D. melanogaster* were selected for increased desiccation resistance for 48 generations and showed extensive hallmark signatures of adaptive evolution (Kang *et al.* 2016). However, a major difference between these experiments lay in the intensity of selection, as the 15-30% most resistant flies were selected every generation in Kang *et al.* (2016), while in this study 50% most resistant flies were selected every other generation. Nevertheless, of the 13,404 PSIs found in DS in this study, 1,373 (10.2%) were identified as fixed in all desiccation lines but heterozygous in all control lines in (Kang *et al.* 2016), of which 50 caused non-synonymous changes (Supporting Dataset S7). A similar study of selection for desiccation resistance showed a low overlap (0.5% to 41.5%) of candidate SNPs among replicates

(Griffin *et al.* 2017). We found only 37 PSIs (Supporting Dataset S8) considered candidate SNPs in any of the Griffin *et al.*'s (2017) replicates, suggesting largely alternative selection responses at the SNP level between the experiments. Griffin *et al.* (2017) selected the top 10% of flies and therefore likely enforced an even stronger selection intensity than our study and Kang *et al.* (2016), but for only 13 generations of selection, compared to the 21-31 of this study and 48 generations of Kang *et al.* (2016). Long-distance linkage disequilibrium can arise early in experimental evolution studies due to low frequency variants (or haplotype blocks) under selection, which will influence the list of candidate SNPs due to hitchhiking effects (Franssen *et al.* 2015). These haplotype blocks will however break up across generations and ease the identification of causative variants (Kofler & Schlotterer 2014). It therefore seems likely that one major cause of the differences among the three studies is that each study describes a different time point in the process of evolutionary adaptation. Several other factors will also contribute to differences in the evolutionary path across studies, including genetic background, the acclimation environment experienced prior to artificial selection, the environment experienced after artificial selection, where successful reproduction and survival of offspring is crucial and finally the presence of simultaneous adaptation to the laboratory environment (Harshman & Hoffmann 2000).

Recent genome-wide analyses of variation in *D. melanogaster* have identified some genes within major chromosomal inversions as candidates for having undergone recent adaptive changes (Bergland *et al.* 2014; Kolaczowski *et al.* 2011). However, we found very low frequencies (0-1%) of the major inversions in our data based on a set of 473 inversion-specific diagnostic SNP markers (Kapun *et al.* 2014). The observed high density of PSIs in promoter regions likely contributes to *cis*-regulatory changes, consistent with the previously described alterations in transcriptomes and proteomes (Sorensen *et al.* 2007; Sorensen *et al.*

2017). Interestingly, the selection regimes (except KD and partly HS) led to significant gene down-regulation at the transcriptome level (Sorensen *et al.* 2007). A wide range of genes seem to be affected by SNP variation in promoter sequences, scattered among diverse functional groups related to metabolism and innate immunity. Heat shock protein genes encoding molecular chaperons, likely candidates for gene products related to stress resistance (Sorensen *et al.* 2003), have been found among differentially expressed genes (Sorensen *et al.* 2017). Even though we did not find differential polymorphisms in their promoter sequences, we identified dozens of SNPs likely responding to selection in other parts of *Hsp* and *Hsc* genes, including coding sequences. Some of the PSIs were shared by as many as four selective regimes (*Hsp22*). Shared genetic and physiological mechanisms across distinct environmental stressors may result in mechanistic trade-offs constraining adaptive evolution (Hoffmann 2014). Traditionally, life history theory assumes that genetic trade-offs arise from pleiotropy (Reznick *et al.* 2000), when a gene affects two or more traits. However, more complex genomic architectures involving epistasis have also been postulated to underlie trade-offs (Roff & Fairbairn 2007). We report that only 15.1% (16,367), of all PSIs were shared by at least two selective regimes of which only 6.4% had divergent allelic trajectories between regimes, suggesting that pleiotropic constraints are rare and unlikely to be a major factor limiting the adaptive potential to cope with environmental stress. A notable example of allelic frequency changes driven in opposite directions between HS and CS was provided by a non-synonymous substitution in *CCHa1-R*, a gene encoding a neuropeptide receptor.

Quantification of 811 proteins in a large subset of the selection lines investigated here, found 575 of these to be significantly up or down-regulated in at least one of the selection regimes (Sørensen *et al.* 2017). When we compared the genes encoding these proteins with the genes carrying rig-PSIs, we did not observe a significant overlap, with the exception of the CS

regime where introns in several differentially regulated genes carried rig-PSIs. For none of these rig-PSIs could the estimated variant frequency be directly linked to protein abundance. However, regulation of these genes is unlikely to be purely *cis*-acting and interactions between different *cis*-acting variants and with *trans*-acting variants will also affect final transcript abundance (Huang *et al.* 2015). This makes it challenging to estimate the contribution of the identified rig-PSIs to regulation of transcription, and the final protein abundance, which is also sensitive to protein degradation regulation and the post-transcriptional machinery (de Sousa Abreu *et al.* 2009; Vogel & Marcotte 2012). Nevertheless, we found 30 rig-PSIs that were also identified as eQTL in (Huang *et al.* 2015), including five that cause non-synonymous changes in three genes: *CG14506*, *CG31898*, and *CG33346* (Supporting Dataset S9).

Transposable elements are increasingly being recognized as agents of adaptive change and a rich source of evolutionary novelties (Aminetzach *et al.* 2005; Ashburner *et al.* 2005; Brookfield 2005; Brosius 2003; Joly-Lopez *et al.* 2012; Sinzelle *et al.* 2009).

‘Domesticated’ TEs can be redeployed to function as transcription factors, as well as to play various roles in heterochromatin formation, genome stability, centromere binding, chromosome segregation, meiotic recombination, TE silencing, programmed genome rearrangement, V(D)J recombination, and translational regulation (Feschotte 2008; Sinzelle *et al.* 2009). Transposition into a functional genomic region typically has deleterious effects due to direct disruptions, chromosomal rearrangements caused by ectopic recombination, and epigenetic modifications (Hollister & Gaut 2009; Montgomery *et al.* 1987; Nuzhdin 1999; Petrov *et al.* 2003), but can also be beneficial and associated with selective sweeps (Schlenke & Begun 2004). We observed TE frequencies to decrease in later generations, but since unselected control lines were also affected we conclude that these changes were

largely due to random drift. This decrease in TE frequency between generations was significantly slower in CDSs, which is counterintuitive given that purifying selection should be more effective in coding sequences, unless these insertions are actually beneficial, for example by modulating RNA abundance and alternative splicing (Kelley *et al.* 2014).

Conclusion

Although there is ample evidence of adaptive radiation in plants and animals, the genetic basis of adaptive changes even in response to basic abiotic stressors remains poorly understood to date. We examined a large-scale experimental system of *Drosophila melanogaster* evolving under distinctive abiotic selective regimes and found that SNPs whose allelic changes were consistent with adaptive divergence produced regime-unique SNP profiles, thus permitting unambiguous separation of environmental stressors. Unlike transcriptome and proteome data, genomic signatures suggested a low level of “common response” among the selection regimes, even for temperature-dependent regimes, and equally important there was a low frequency of divergent selection between regimes. This result is likely due to the pre-existing patterns of genetic variation in the initial population, as well as particulars of each selection regime. This study also highlights challenges related to the identification of true targets of selection in experimental evolution studies in which controlling the linkage disequilibrium (LD) landscape of founder populations is technically impractical, and haplotype blocks can be reconstructed only partially (Franssen *et al.* 2017). It is imperative that future studies aim at better control of LD or at least its minimization at the experimental design level. Our experimental setup is expected to have reduced LD, and thus increased the true discovery rate of selection targets, due to larger population sizes, multiple replicates (six experimental blocks replicated five times each), and alternating generations with and without selection (Kessner & Novembre 2015).

List of abbreviations

CDS: coding sequence; CS: Cold-shock resistance selection; DS: Desiccation resistance selection; GO: Gene Ontology; HS Heat shock resistance selection; KS: Heat knock down resistance selection; PCA: principal component analysis; PSI: polymorphic site of interest; rig-PSI: 'rigorous' polymorphic site of interest; SNP: single nucleotide polymorphism; SS: Starvation resistance selection; TE: transposable element; UC: unselected control; UTR: untranslated region

Availability of data and materials

All primary and analyzed data were deposited at NCBI's SRA under accession SRP151560. Custom scripts and sync files containing information about the identity of PSIs, rig-PSIs, and haplotypes can be found at <https://goo.gl/bprkvy>.

Authors' contributions

Project was conceived by VL. Data analysis and manuscript writing was done by PM, LK, MFS, HRG and VL.

Acknowledgements

We thank D. Andersen for excellent technical help. The creation of the long-term selection lines used in this study and their sequencing were supported by center and frame grants from the Danish Natural Science Research Council and by the Carlsberg Foundation to VL. Funding was also provided by the Edward Via College of Osteopathic Medicine (VCOM) and the Bradley Foundation to PM, HRG and LK.

References:

- Aminetzach YT, Macpherson JM, Petrov DA (2005) Pesticide resistance via transposition-mediated adaptive gene truncation in *Drosophila*. *Science* **309**, 764-767.
- Araujo MB, Ferri-Yanez F, Bozinovic F, *et al.* (2013) Heat freezes niche evolution. *Ecology Letters* **16**, 1206-1219.
- Aronesty E (2013) Comparison of Sequencing Utility Programs. *The Open Bioinformatics Journal* **7**, 1-8.
- Ashburner M, Golic KG, Hawley RS (2005) *Drosophila : a laboratory handbook*, 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
- Bergland AO, Behrman EL, O'Brien KR, Schmidt PS, Petrov DA (2014) Genomic evidence of rapid and stable adaptive oscillations over seasonal time scales in *Drosophila*. *PLoS Genet* **10**, e1004775.
- Boitard S, Kofler R, Francoise P, *et al.* (2013) Pool-hmm: a Python program for estimating the allele frequency spectrum and detecting selective sweeps from next generation sequencing of pooled samples. *Mol Ecol Resour* **13**, 337-340.
- Brookfield JF (2005) Evolutionary forces generating sequence homogeneity and heterogeneity within retrotransposon families. *Cytogenet Genome Res* **110**, 383-391.
- Brosius J (2003) The contribution of RNAs and retroposition to evolutionary novelties. *Genetica* **118**, 99-116.
- Bubliy OA, Loeschcke V (2005) Correlated responses to selection for stress resistance and longevity in a laboratory population of *Drosophila melanogaster*. *J Evol Biol* **18**, 789-803.
- Burke MK (2012) How does adaptation sweep through the genome? Insights from long-term selection experiments. *Proc Biol Sci* **279**, 5029-5038.
- Burke MK, Dunham JP, Shahrestani P, *et al.* (2010) Genome-wide analysis of a long-term evolution experiment with *Drosophila*. *Nature* **467**, 587-590.
- Butler SJ, Ray S, Hiromi Y (1997) klingon, a novel member of the *Drosophila* immunoglobulin superfamily, is required for the development of the R7 photoreceptor neuron. *Development* **124**, 781-792.
- de Sousa Abreu R, Penalva LO, Marcotte EM, Vogel C (2009) Global signatures of protein and mRNA expression levels. *Mol Biosyst* **5**, 1512-1526.
- DePristo MA, Banks E, Poplin R, *et al.* (2011) A framework for variation discovery and genotyping using next-generation DNA sequencing data. *Nat Genet* **43**, 491-498.
- Dubnau J, Chiang AS, Grady L, *et al.* (2003) The staufen/pumilio pathway is involved in *Drosophila* long-term memory. *Current Biology* **13**, 286-296.
- Falconer DS, Mackay TFC (1996) *Introduction to quantitative genetics*, 4th edn. Longman, Essex, England.
- Feschotte C (2008) Transposable elements and the evolution of regulatory networks. *Nat Rev Genet* **9**, 397-405.
- Franssen SU, Barton NH, Schlotterer C (2017) Reconstruction of Haplotype-Blocks Selected during Experimental Evolution. *Mol Biol Evol* **34**, 174-184.
- Franssen SU, Nolte V, Tobler R, Schlotterer C (2015) Patterns of linkage disequilibrium and long range hitchhiking in evolving experimental *Drosophila melanogaster* populations. *Mol Biol Evol* **32**, 495-509.
- Garcia-Robledo C, Kuprewicz EK, Staines CL, Erwin TL, Kress WJ (2016) Limited tolerance by insects to high temperatures across tropical elevational gradients and the implications of global warming for extinction. *Proc Natl Acad Sci U S A* **113**, 680-685.
- Graves JL, Jr., Hertweck KL, Phillips MA, *et al.* (2017) Genomics of Parallel Experimental Evolution in *Drosophila*. *Mol Biol Evol* **34**, 831-842.

- Griffin PC, Hangartner SB, Fournier-Level A, Hoffmann AA (2017) Genomic Trajectories to Desiccation Resistance: Convergence and Divergence Among Replicate Selected *Drosophila* Lines. *Genetics* **205**, 871-890.
- Hangartner S, Hoffmann AA (2016) Evolutionary potential of multiple measures of upper thermal tolerance in *Drosophila melanogaster*. *Functional Ecology* **30**, 442-452.
- Harshman LG, Hoffmann AA (2000) Laboratory selection experiments using *Drosophila*: what do they really tell us? *Trends Ecol Evol* **15**, 32-36.
- Hercus MJ, Hoffmann AA (2000) Maternal and grandmaternal age influence offspring fitness in *Drosophila*. *Proc Biol Sci* **267**, 2105-2110.
- Hoffmann AA (2014) Evolutionary limits and constraints. In: *The Princeton guide to evolution*. (ed. Losos JB), pp. 247-252. Princeton Univ. Press., Princeton, New Jersey.
- Hoffmann AA, Parsons PA (1997) *Extreme environmental change and evolution* Cambridge University Press, Cambridge ; New York.
- Hoffmann AA, Sgro CM (2011) Climate change and evolutionary adaptation. *Nature* **470**, 479-485.
- Hollister JD, Gaut BS (2009) Epigenetic silencing of transposable elements: a trade-off between reduced transposition and deleterious effects on neighboring gene expression. *Genome Res* **19**, 1419-1428.
- Huang W, Carbone MA, Magwire MM, *et al.* (2015) Genetic basis of transcriptome diversity in *Drosophila melanogaster*. *Proc Natl Acad Sci U S A* **112**, E6010-6019.
- Huang W, Massouras A, Inoue Y, *et al.* (2014) Natural variation in genome architecture among 205 *Drosophila melanogaster* Genetic Reference Panel lines. *Genome Res* **24**, 1193-1208.
- Johnson EC, Kazgan N, Bretz CA, *et al.* (2010) Altered metabolism and persistent starvation behaviors caused by reduced AMPK function in *Drosophila*. *PLoS ONE* **5**.
- Joly-Lopez Z, Forczek E, Hoen DR, Juretic N, Bureau TE (2012) A gene family derived from transposable elements during early angiosperm evolution has reproductive fitness benefits in *Arabidopsis thaliana*. *PLoS Genet* **8**, e1002931.
- Kang L, Aggarwal DD, Rashkovetsky E, Korol AB, Michalak P (2016) Rapid genomic changes in *Drosophila melanogaster* adapting to desiccation stress in an experimental evolution system. *BMC Genomics* **17**, 233.
- Kapun M, van Schalkwyk H, McAllister B, Flatt T, Schlotterer C (2014) Inference of chromosomal inversion dynamics from Pool-Seq data in natural and laboratory populations of *Drosophila melanogaster*. *Mol Ecol* **23**, 1813-1827.
- Kellermann V, Overgaard J, Hoffmann AA, *et al.* (2012) Upper thermal limits of *Drosophila* are linked to species distributions and strongly constrained phylogenetically. *Proc Natl Acad Sci U S A* **109**, 16228-16233.
- Kelley DR, Hendrickson DG, Tenen D, Rinn JL (2014) Transposable elements modulate human RNA abundance and splicing via specific RNA-protein interactions. *Genome Biol* **15**, 537.
- Kessner D, Novembre J (2015) Power analysis of artificial selection experiments using efficient whole genome simulation of quantitative traits. *Genetics* **199**, 991-1005.
- Kofler R, Betancourt AJ, Schlotterer C (2012) Sequencing of pooled DNA samples (Pool-Seq) uncovers complex dynamics of transposable element insertions in *Drosophila melanogaster*. *PLoS Genet* **8**, e1002487.
- Kofler R, Pandey RV, Schlotterer C (2011) PoPoolation2: identifying differentiation between populations using sequencing of pooled DNA samples (Pool-Seq). *Bioinformatics* **27**, 3435-3436.

- Kofler R, Schlotterer C (2012) Gowinda: unbiased analysis of gene set enrichment for genome-wide association studies. *Bioinformatics* **28**, 2084-2085.
- Kofler R, Schlotterer C (2014) A guide for the design of evolve and resequencing studies. *Mol Biol Evol* **31**, 474-483.
- Kolaczekowski B, Kern AD, Holloway AK, Begun DJ (2011) Genomic differentiation between temperate and tropical Australian populations of *Drosophila melanogaster*. *Genetics* **187**, 245-260.
- Li H, Durbin R (2009) Fast and accurate short read alignment with Burrows-Wheeler transform. *Bioinformatics* **25**, 1754-1760.
- Lin YJ, Seroude L, Benzer S (1998) Extended life-span and stress resistance in the *Drosophila* mutant methuselah. *Science* **282**, 943-946.
- Lynch M, Walsh B (1998) *Genetics and analysis of quantitative traits* Sinauer, Sunderland, Mass.
- Mackay TF, Richards S, Stone EA, *et al.* (2012) The *Drosophila melanogaster* Genetic Reference Panel. *Nature* **482**, 173-178.
- Malmendal A, Sorensen JG, Overgaard J, *et al.* (2013) Metabolomic analysis of the selection response of *Drosophila melanogaster* to environmental stress: are there links to gene expression and phenotypic traits? *Naturwissenschaften* **100**, 417-427.
- Matsuno M, Horiuchi J, Tully T, Saitoe M (2009) The *Drosophila* cell adhesion molecule Klingon is required for long-term memory formation and is regulated by Notch. *Proceedings of the National Academy of Sciences of the United States of America* **106**, 310-315.
- McColl G, Hoffmann AA, McKechnie SW (1996) Response of two heat shock genes to selection for knockdown heat resistance in *Drosophila melanogaster*. *Genetics* **143**, 1615-1627.
- Michalak P, Kang L, Sarup PM, Schou MF, Loeschcke V (2017) Nucleotide diversity inflation as a genome-wide response to experimental lifespan extension in *Drosophila melanogaster*. *BMC Genomics* **18**, 84.
- Misener SR, Chen CP, Walker VK (2001) Cold tolerance and proline metabolic gene expression in *Drosophila melanogaster*. *Journal of Insect Physiology* **47**, 393-400.
- Montgomery E, Charlesworth B, Langley CH (1987) A test for the role of natural selection in the stabilization of transposable element copy number in a population of *Drosophila melanogaster*. *Genet Res* **49**, 31-41.
- Nuzhdin SV (1999) Sure facts, speculations, and open questions about the evolution of transposable element copy number. *Genetica* **107**, 129-137.
- Petrov DA, Aminetzach YT, Davis JC, Bensasson D, Hirsh AE (2003) Size matters: non-LTR retrotransposable elements and ectopic recombination in *Drosophila*. *Mol Biol Evol* **20**, 880-892.
- Price AL, Patterson NJ, Plenge RM, *et al.* (2006) Principal components analysis corrects for stratification in genome-wide association studies. *Nat Genet* **38**, 904-909.
- Qin W, Neal SJ, Robertson RM, Westwood JT, Walker VK (2005) Cold hardening and transcriptional change in *Drosophila melanogaster*. *Insect Molecular Biology* **14**, 607-613.
- Reznick D, Nunney L, Tessier A (2000) Big houses, big cars, superfleas and the costs of reproduction. *Trends Ecol Evol* **15**, 421-425.
- Roff DA, Fairbairn DJ (2007) The evolution of trade-offs: where are we? *J Evol Biol* **20**, 433-447.
- Sarup P, Sorensen JG, Kristensen TN, *et al.* (2011) Candidate genes detected in transcriptome studies are strongly dependent on genetic background. *PLoS One* **6**, e15644.

- Schlenke TA, Begun DJ (2004) Strong selective sweep associated with a transposon insertion in *Drosophila simulans*. *Proc Natl Acad Sci U S A* **101**, 1626-1631.
- Schlotterer C, Kofler R, Versace E, Tobler R, Franssen SU (2015) Combining experimental evolution with next-generation sequencing: a powerful tool to study adaptation from standing genetic variation. *Heredity (Edinb)* **114**, 431-440.
- Schou MF, Loeschcke V, Bechsgaard J, Schlotterer C, Kristensen TN (2017) Unexpected high genetic diversity in small populations suggests maintenance by associative overdominance. *Mol Ecol* **26**, 6510-6523.
- Sinzelle L, Izsvak Z, Ivics Z (2009) Molecular domestication of transposable elements: from detrimental parasites to useful host genes. *Cell Mol Life Sci* **66**, 1073-1093.
- Sorensen JG, Kristensen TN, Loeschcke V (2003) The evolutionary and ecological role of heat shock proteins. *Ecology Letters* **6**, 1025-1037.
- Sorensen JG, Nielsen MM, Loeschcke V (2007) Gene expression profile analysis of *Drosophila melanogaster* selected for resistance to environmental stressors. *J Evol Biol* **20**, 1624-1636.
- Sorensen JG, Schou MF, Loeschcke V (2017) Evolutionary adaptation to environmental stressors: a common response at the proteomic level. *Evolution* **71**, 1627-1642.
- Swallow JG, Garland JT (2005) Selection Experiments as a Tool in Evolutionary and Comparative Physiology: Insights into Complex Traits—an Introduction to the Symposium1. *Integrative and Comparative Biology* **45**, 387-390.
- Taus T, Futschik A, Schlotterer C (2017) Quantifying Selection with Pool-Seq Time Series Data. *Mol Biol Evol* **34**, 3023-3034.
- Telonis-Scott M, Hallas R, McKechnie SW, Wee CW, Hoffmann AA (2009) Selection for cold resistance alters gene transcript levels in *Drosophila melanogaster*. *Journal of Insect Physiology* **55**, 549-555.
- Teotonio H, Chelo IM, Bradic M, Rose MR, Long AD (2009) Experimental evolution reveals natural selection on standing genetic variation. *Nat Genet* **41**, 251-257.
- Tobler R, Franssen SU, Kofler R, *et al.* (2014) Massive habitat-specific genomic response in *D. melanogaster* populations during experimental evolution in hot and cold environments. *Mol Biol Evol* **31**, 364-375.
- Turner TL, Stewart AD, Fields AT, Rice WR, Tarone AM (2011) Population-based resequencing of experimentally evolved populations reveals the genetic basis of body size variation in *Drosophila melanogaster*. *PLoS Genet* **7**, e1001336.
- Vogel C, Marcotte EM (2012) Insights into the regulation of protein abundance from proteomic and transcriptomic analyses. *Nat Rev Genet* **13**, 227-232.
- Walsh B, Blows MW (2009) Abundant Genetic Variation + Strong Selection = Multivariate Genetic Constraints: A Geometric View of Adaptation. *Annual Review of Ecology, Evolution, and Systematics* **40**, 41-59.
- Watson MJO, Hoffmann AA (1996) Acclimation, Cross-Generation Effects, and the Response to Selection for Increased Cold Resistance in *Drosophila*. *Evolution* **50**, 1182-1192.

Figure Legends:

Fig. 1. Schematic presentation of the experimental design. The process of sample divergence and sampling for pool sequencing is presented as a phylogenetic tree. Four generations after establishing a mass-bred population, 30 replicate populations were established and split into five selection regimes and one control group. Selection in the selection regimes was performed every other generation to allow recovery in intermediate generations. Samples for pool sequencing was obtained from the mass-bred population in generation 4, and from the selected and control replicate populations in generation 45 and 65.

Fig. 2. Schematic of a polymorphic site of interest (PSI) undergoing changes consistent with directional selection operating in selection regimes. Each dot represents a single biological replicate. The average allele frequencies within groups are marked by horizontal lines. Rule 1: In control groups, average allele frequency (AF) differences among lines of F4, F45, and F65 are less than 10%; Rule 2: The frequency changes between selection lines F45 relative to control lines (in this case, F4 that has the maximum AF) and the change between selection lines F65 relative to selection lines F45 are in the same direction (increasing in this case); Rule 3: The AF change between selection lines F45 relative to control lines (F4) is greater than 10%.

Fig. 3. Principal Component Analysis Plot based on all Polymorphic Sites of Interest (PSIs): (A) PC1 vs PC2 and (B) PC3 vs PC4. Arrows indicate the directions of change in generation 65 (closed symbols) compared to generation 45 (open symbols). Groups were unselected control (UC), Desiccation resistance (DS), Starvation resistance (SS), Cold-shock resistance (CS), Heat shock resistance (HS), and Heat knock down resistance (KS).

Fig. 4. Venn plots of rig-PSIs shared among selection regimes for (A) all rig-PSIs, and (B) non-synonymous rig-PSIs. Groups were unselected control (UC), Desiccation resistance (DS), Starvation resistance (SS), Cold-shock resistance (CS), Heat shock resistance (HS), and Heat knock down resistance (KS).

Fig. 5. Manhattan plots showing all chromosomes, PSIs (grey) and rig-PSIs (colored) with P-values of the CMH test on the y axis for all selection regimes. Horizontal colored blocks indicate haplotype block regions under selection, and the blocks containing two or more rig-PSIs have thick edges highlighted in contrasting colors. Horizontal dash line indicates the FDR 0.01 threshold. For clarity, only one out of the five cutoffs is shown (we used different P-value cutoffs for different subsets of SNPs based on their starting minor allele frequencies, with this FDR 0.01 threshold corresponding to the category ` $<10\%$ ` that was based on 56% simulated SNPs falling into that category; see Materials and Methods for details). Four genes (*Elp2*, *Scsalpha*, *Lasp*, and *Flo2*) showed a significant overlap between rig-PSI variant frequency and the protein expression in the CS regime.

Fig. 6. Box plots comparing transposable element (TEs) numbers among groups and generations (color orange for F45 and color blue for F65), according to (A) All TEs, different TE orders (B) LTR, (C) non-LTR, (D) TIR, and (E) All other TE orders, or (F) TEs in coding sequences. Groups were unselected control (UC), Desiccation resistance (DS), Starvation resistance (SS), Cold-shock resistance (CS), Heat shock resistance (HS), and Heat knock down resistance (KS).

Tables:

Table 1: Genomic distribution of all polymorphic sites and polymorphic sites of interest (PSIs)

Region	Number of sites	Percentage	Density (per kb)	PSIs	PSI percentage	PSI density (per kb)
3'-UTR	84,297	4.29%	12.75	4,573	0.23%	0.69
5'-UTR	52,504	2.67%	13.08	2,744	0.14%	0.68
CDS (Synonymous)	190,784	9.70%	11.69	11,801	0.60%	0.69
CDS (Non-Synonymous)	74,555	3.79%		3,812	0.19%	
Non-coding RNA	17,930	0.91%	16.01	1,011	0.05%	0.90
Intergenic	595,074	30.25%	18.13	32,954	1.68%	1.00
Intron	932,852	47.42%	17.91	50,333	2.56%	0.97
Promoter*	19,227	0.98%	18.58	1,160	0.06%	1.12
Total	1,967,223	100.00%	16.34	108,388	5.51%	0.90

*: Promoter is defined as the 100bp region upstream of 5'-UTR

A

B