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Environmental variation partitioned into separate heritable components

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Author contributions

MØ, PDR, AAH and TNK framed the conceptual ideas. The experimental work was primarily conducted by MØ and TNK. All authors contributed to the data analysis. MØ, PDR, and TNK wrote the first draft of the manuscript. All authors reviewed the final manuscript.

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Data archival

All data will be archived in the Dryad digital repository if accepted

Abstract

Trait variation is normally separated into genetic and environmental components, yet genetic factors also control the expression of environmental variation, encompassing plasticity across environmental gradients and within-environment responses. We defined four components of environmental variation: plasticity across environments, variability in plasticity, variation within environments, and differences in within-environment variation across environments. We assessed these components for cold tolerance across five rearing temperatures using the *Drosophila melanogaster* Genetic Reference Panel (DGRP). The four components were found to be heritable, and genetically correlated to different extents. By whole genome single marker regression, we detected multiple candidate genes controlling the four components and showed limited overlap in genes affecting them. Using the binary *UAS*-GAL4 system, we functionally validated the effects of a subset of candidate genes affecting each of the four components of environmental variation and also confirmed the genetic and phenotypic correlations obtained from the DGRP in distinct genetic backgrounds. We delineate selection targets associated with environmental variation and the constraints acting upon them, providing a framework for evolutionary and applied studies on environmental sensitivity. Based on our results we suggest that the traditional quantitative genetic view of environmental variation and genotype-by-environment interactions needs revisiting.

Introduction

Phenotypic trait variation can be partitioned into genetic (V_G) and environmental (V_E) variation. However, the expression of environmental variation is under heritable genetic control (Ros et al. 2004; Ibáñez-Escriche et al. 2008; Ayroles et al. 2015; Morgante et al. 2015; Sørensen et al. 2015; Blasco et al. 2017). While environmental variation has been studied for many decades, the underlying genetic mechanisms controlling V_E are poorly understood, particularly as different forms of V_E are recognized (Hill and Mulder 2010) which are not all captured by the traditional view of genotype-by-environment (GxE) interactions. The different forms of V_E may be genetically independent, thereby having distinct impacts on evolutionary outcomes (Hill and Mulder 2010). Knowledge of the genetic basis of V_E is important for understanding evolutionary trajectories in variable and potentially stressful environments (Zhang and Hill 2005; Lande 2014), when undertaking breeding programs aiming at generating homogenous phenotypes across different production conditions (Mulder et al. 2008; Hill and Mulder 2010), and for identifying the impact of environmental conditions on genotype specific responses to diseases and treatments in humans (Hunter 2005; Vasseur and Quintana-Murci 2013).

The terminology around different components of V_E is unsettled. Much of the ambiguity pertains to genotypic differences in variation within environments, which has been described by different terms (Table 1; Hill & Mulder 2010; Struchalin *et al.* 2010; Rönnegård & Valdar 2012; Ayroles *et al.* 2015; Forsberg *et al.* 2015). In the work presented here we attempt to provide clear-cut definitions of some previously-described components of V_E as well as other components which have not been characterized to the same extent (Table 1).

The ability of genotypes to produce a multitude of phenotypes when exposed to different environmental conditions is termed phenotypic plasticity. Environmental variation resulting from plasticity (here termed V_{plast}) is perhaps the most studied type of V_E (DeWitt and Scheiner 2004; Valladares et al. 2006), and is usually assessed by measuring individuals of a given genotype at different environmental conditions (Fig. 1A and Table 1). Trait plasticity is often regarded as being separate from trait means, thus, being controlled by a different set of genes capable of responding to selection (Stearns 1989; Scheiner and Lyman 1991). It is considered particularly important from an adaptive perspective because V_{plast} may allow populations to rapidly utilise novel ecological opportunities in temporally and spatially heterogeneous environments or respond to stressful conditions, including those emerging as a consequence of anthropogenic climate change (Teplitsky et al. 2008; Hoffmann and Sgrò 2011; Anderson et al. 2012).

A second source of environmental variation is the within-environment variation (here termed V_{WE}), which is a measure of the extent to which a genotype can produce the same phenotype within an environment (Fig. 1B and Table 1). Like plasticity, within-environment variation is typically considered to be under genetic control (Ros et al. 2004; Ibáñez-Escriche et al. 2008; Ayroles et al. 2015; Morgante et al. 2015; Sørensen et al. 2015), and have recently been shown to respond to selection, without affecting trait mean (Blasco et al. 2017). This form of variation is also an important component of adaptive responses. For instance, in a fluctuating environment, stabilising selection will favour individuals, which produce high levels of variability in their offspring to maximise fitness (Zhang and Hill 2005; Devaux and Lande 2010). This is often referred to as a diversified bet hedging strategy, and represent a genetically controlled form of variation expressed within an environment (Simons and Johnston 1997; Marshall et al. 2008). Improving our knowledge of the underlying genetic basis of V_{WE} and its relationship to other components of environmental variation helps to

understand and predict species responses in a more variable future climate (Chown et al. 2010). In applied breeding programs, where a homogenous phenotype is often desirable, in-depth knowledge of the genetic basis of V_{WE} may have important economic value (Mulder et al. 2008; Hill and Mulder 2010).

Two other forms of environmental variation impacting phenotypic variation can be recognized, but are not commonly characterised. The first involves developmental stability across environments; that is variation of within-environment variation across environments (here termed V_{AE}). It measures the extent to which the expression of phenotypic variation within an environment is constant across the range of environments considered (Fig. 1B and Table 1). This can be thought of as GxE at the level of variance. The second involves variation of the plastic response (here termed $V_{v.plast}$), i.e., a measure of the extent to which the same genotype can consistently produce the same plastic response across environments (Fig. 1A and Table 1).

All four components of environmental variation are likely to be under genetic control, although for the latter two (V_{AE} and $V_{v.plast}$) this has not been documented. Control of within-environment variation (V_{WE}) is thought to be linked to heat shock proteins and several other classes of environmental stress responsive genes (Bergman and Siegal 2003; Sangster et al. 2008). The control of plastic responses is thought to reside with processes like acclimation and hardening (Schlichting and Pigliucci 1993; Beaman et al. 2016) and the genetic architecture of these processes have been investigated especially in *Drosophila* (Sørensen et al. 2005). However, it is currently unknown whether the different components of V_E have the same underlying genetic basis, given that quantitative genetic and genomic analyses of the different components of V_E have rarely been performed.

Here we investigated the genetic control of the four components of environmental variation for cold tolerance in *Drosophila melanogaster*. Cold tolerance is an important trait for understanding evolutionary processes in natural populations because the ability to withstand low temperatures limits the geographic distribution of many species (Sunday et al. 2011; Williams et al. 2015) and cold tolerance in particular is a good predictor of present and future geographical distributions (Kimura 2004; Overgaard et al. 2011; Araújo et al. 2013). Cold tolerance is strongly impacted by environmental variation; in ectotherms the trait is highly plastic (Schou et al. 2017b), and plays a key role in determining adaptation to thermal extremes (Hoffmann et al. 2003; Chown et al. 2010; Overgaard et al. 2011).

We conducted a comprehensive investigation of the genetic basis for each of the four components of environmental variation impacting cold tolerance, as well as the correlation among these, using 166 inbred lines from the *Drosophila melanogaster* Genetic Reference Panel (DGRP) (Mackay et al. 2012). Individuals from each DGRP line were reared at five thermal environmental condition; 17, 20, 23, 26, and 29 °C, for one generation and the cold tolerance of adult males was then assessed using the dynamic measure critical thermal minimum (CT_{min}; the temperature of chill coma onset under gradual cooling (Overgaard et al. 2012)). The four components of V_E were determined (Table 1) for each DGRP line, and to investigate the underlying genetic basis, we performed whole genome single marker regression for each component of V_E and validated our results by gene expression knockdown using the *UAS*-GAL4 system. This allowed four questions about the genetic control of different components of V_E affecting cold tolerance within and between environments to be answered: 1) Are the different components of V_E heritable, i.e. have adaptive potential? 2) Is genetic variation of the different components of V_E correlated? 3) Are the

components of V_E related to inherent (non-plastic) cold tolerance? and 4) What is the genetic architecture controlling the different components of V_E?

Materials and Methods

Fly stocks and maintenance

The DGRP consists of a collection of lines that through full-sib mating has been inbred to an expected inbreeding coefficient of F∼1 (Mackay et al. 2012), resulting in the large majority of genomic sites being homozygous within line. The study included 166 lines from the *Drosophila melanogaster* Genetic Reference Panel (DGRP) (Mackay et al. 2012) obtained from Bloomington *Drosophila* Stock Center (NIH P40OD018537), 40 *UAS*-RNAi lines and the two corresponding genetic background strains from Vienna *Drosophila* Resource Center (Dietzl et al. 2007), and the GAL4 line act5-*GAL4*/CyO, which was donated to us from colleagues at Copenhagen University, Denmark. All stocks were maintained at 23 °C, 50 %RH and a 12:12 h photoperiod on a standard oatmeal-sugar-agar-yeast medium for two generations in our laboratory prior to initiating the study.

Thermal rearing conditions

To assess the effect of thermal rearing condition on cold tolerance, the 166 DGRP lines were reared at five thermal conditions: 17, 20, 23, 26, and 29 °C. For each line, three replicates of approximately 20 adult flies (age 3-4 days), reproduced for 12 h at 23 °C in vials containing 7 mL medium after which the flies were discarded and the vials were transferred to incubators (KBWF 720 E5.3, Binder, Tuttlingen, Germany) at the respective rearing temperature. The

temperature within the incubators was continuously monitored using data loggers (iButton DS1923-F5 with software OneWireViewer x64 version 0.3.15.50, both from Maxim, Sunnyvale, CA, USA). Incubators maintained an average temperature (±SD) of 17±0.14 °C, 20±0.35 °C, 23±0.15 °C, 26±0.37 °C, and 29±0.39 °C.

Assessment of critical thermal minimum, CT_{min}

Individuals from each of the five rearing conditions were assessed for their ability to tolerate low temperatures determined using a dynamic measure of critical thermal minimum (CT_{min}), which is a standardised procedure of gradual cooling (Overgaard et al. 2012). CT_{min} is defined as the temperature at which a complete cessation of movement is observed as a result of the flies entering chill coma.

Within one day after eclosion, a maximum of 20 adult males were collected under CO_2 anaesthesia, transferred to a new vial with 3 mL medium, and returned to the respective temperature regime for 48 h. Assessment of CT_{min} was performed on approximately 10 male flies (age 60±12 h) per line per rearing temperature (for exact numbers see Supplementary Table S1). Flies were transferred to individual screw-cap glass vials (45 x 15 mm) and randomly placed in a metal rack, which was submerged into a water bath with ethylene glycol and water (1:3 vol.) pre-set to 23 °C. The temperature of the water bath was decreased at a rate of 0.1 °C/min, and the temperature at which all movement of a fly ceased (gently tapping the vials with a metal rod and shining a flashlight) due to chill coma was recorded. In all experiments, 12,612 flies were scored for CT_{min}. Because the rearing temperature affects developmental time and due to the scale of the experiment, the assays were performed on

multiple days, at different time of the day and in two different water baths. To minimize potential bias due to these experimental factors, all assays were performed with the exact same setup, in the same location, and throughout the experiment all flies were scored for CT_{min} by the same person. The temperature in the water bath was continuously monitored during the assays with iButton data loggers. In the temperature range comparable among all assays (23.0 to 8.5 °C), the average cooling rates (±SD) of temperature change in the two baths were -0.0970±0.0016 °C/min and -0.0971±0.0017 °C/min, respectively.

Measures of environmental variation

Four types of environmental variation were computed (Fig. 1, Table 1). In order to retain a replicated structure of the data, the CT_{min} from each DGRP line and rearing temperature were grouped into three groups of individuals according to day of assay, time of day, and water bath, in this order. This way we also accounted for some of the variation due to experimental factors potentially impacting V_E components, especially V_{WE} (Supplementary Fig. S1). Based on the three replicate groups, the four components of environmental variation were computed (i.e. obtaining three replicates per measure per line) using log-transformed CT_{min} to ensure normality of the data. To test the robustness of the V_E estimates, we performed 10,000 random samplings of three replicate groups of the same size as the experimental groups, with replacement of observations in each line and in each environment. The four V_E components were calculated based on each of these samplings. We found a high degree of concordance between the estimates based on the three experimental groups and estimates based on randomly sampled groups across all the DGRP lines (see Supplementary methods for details).

Phenotypic plasticity (V_{plast}) was estimated as the regression coefficient of a linear regression (i.e., slope) of CT_{min} as a function of rearing temperature (Fig. 1A). We chose a linear fit because previous studies have shown that the relationship between CT_{min} and developmental temperature is linear (Schou et al. 2017a), and because linear norm-of-reaction analysis is the most commonly used in studies of phenotypic plasticity (Valladares et al. 2006). The variation of the plastic response ($V_{v.plast}$) was computed as the standard error of the regression coefficient (Fig. 1A), as this defined the uncertainty of determination of the plastic response.

Line specific variation, both within (V_{WE}) and across (V_{AE}) environments, was estimated using the coefficient of variation (CV = SD/mean) (Fig. 1B). To enable the comparison of a single measure of within-environment variation with the other three components of environmental variation, we computed an average V_{WE} as the mean of the individual CVs from each of the five rearing conditions ($\overline{CV}$, Fig. 1B). As a measure of developmental stability, i.e. environmental variation across environments (V_{AE}), we computed the difference between the highest CV and the lowest CV across environments (ΔCV, Fig. 1B).

DGRP genotypes

Only segregating biallelic SNPs with a minor allele frequency ≥ 0.05, a Phred quality score ≥ 500, and a genotype call ≥ 0.8 were included. This resulted in a total of 1,725,755 SNPs distributed on the six chromosome arms (2L, 2R, 3L, 3R, 4 and X). Sequence data including information on major inversions and *Wolbachia* infection status were downloaded from the

DGRP2 facility (http://dgrp2.gnets.ncsu.edu). SNPs were annotated to genes using FlyBase annotation v5.49 (flybase.org). In addition, genes were linked to gene ontology (GO) categories (The Gene Ontology Consortium et al. 2000). Only GO terms with at least 10 directly evidenced genes were included. In total, SNPs were annotated to 10,517 genes and 1,117 GO terms.

Quantitative genetic parameters

The proportion of phenotypic variance explained by genetic variance (H^2) and additive genetic variance captured by SNPs (h^2) were estimated using the average information restricted maximum-likelihood (REML) procedure (Madsen et al. 1994; Johnson and Thompson 1995). H^2 was estimated as:

$$H^2 = \frac{\sigma^2_{g_L}}{\sigma^2_{g_L} + \sigma^2_e}$$

where $\sigma^2_{g_L}$ and σ^2_e were obtained by fitting $\mathbf{y} = \mathbf{Xb} + \mathbf{Zg_L} + \mathbf{e}$, where $\mathbf{y}$ was a vector of phenotypic observations (CT_{min} at 23 °C and slope SE (log transformed), slope (untransformed), ΔCV and $\overline{CV}$ (both square root transformed)), $\mathbf{b}$ was a vector of fixed effects (day, bath, time on day, and *Wolbachia* infection status for CT_{min}, and for environmental variation phenotypes only *Wolbachia* infection status was included), $\mathbf{g}_L$ was a vector of random line effects assuming the DGRP lines to be independent, i.e. $\mathbf{g}_L \sim N(0, \mathbf{I}_L\sigma^2_{g_L})$, where I_L was an identity matrix, and $\mathbf{e}$ was a random residual, $\mathbf{e} \sim N(0, \mathbf{I}\sigma^2_e)$. The estimate of h^2 was obtained as:

$$h^2=\frac{\sigma_g^2}{\sigma_g^2+\sigma_e^2}$$

where σ_g^2 and σ_e^2 were obtained by fitting $\mathbf{y} = \mathbf{Xb} + \mathbf{Zg} + \mathbf{e}$, where **y** was a vector of phenotypic observations (as defined above), **b** was a vector of fixed effects (as defined above, including five major polymorphic inversions, *I2Lt*, *I2Rns*, *I3Rp*, *I3Rk* and *I3RMo*), **g** was a vector of random genetic effects, $\mathbf{g} \sim N(0, \mathbf{G}\sigma_g^2)$, where **G** was the genomic relationship matrix, and **e** was a random residual, $\mathbf{e} \sim N(0, \mathbf{I}\sigma_e^2)$. The **G** matrix was computed as $\mathbf{G}=\mathbf{WW}'/m$ (VanRaden 2008), where *m* was the number of genetic markers, and **W** was a centred and scaled genotype matrix. Each column vector of **W**, $\mathbf{w}_i=\frac{\mathbf{a}_i-2p_i}{\sqrt{2p_i(1-p_i)}}$, p_i was the frequency of the minor allele at locus *i*, and $\mathbf{a}_i$ was the *i*th column vector of the allele count matrix, **A**, containing the genotypes encoded as 0, 1, 2, counting the number of the minor alleles. Standard errors of the heritability estimates were obtained using the inverse of Fisher's information measure multiplied by minus one (Lynch and Walsh 1998; Sorensen and Gianola 2002).

Phenotypic (ρ_p) and genetic correlations (ρ_g) were computed among inherent cold tolerance (defined as CT_{min} at 23 °C), V_{plast}, $V_{v.plast}$, V_{AE}, V_{WE}. The genetic correlation was approximated as the correlation between SNP effects from the whole genome single marker regression (see below). In both cases, the correlations were obtained as $\rho = \mathrm{cov}(x,y)/\sqrt{\sigma_x^2\sigma_y^2}$, with standard errors computed as $SE_\rho = \sqrt{\frac{1-\rho^2}{n-2}}$, where *n* was the sample size.

Whole genome single marker regression

For each component of V_E we performed whole genome single marker regression to test individual SNPs for association. In order to account for the fixed effects (i.e. experimental conditions, chromosomal inversions and *Wolbachia* infection) and polygenic inheritance among the DGRP lines, the response variables were the estimated genetic values (i.e., $\hat{\mathbf{g}}$) obtained from linear mixed models described in 'Quantitative genetic parameters'. Thus, the degree of association between the i^{th} SNP and the component of V_E was determined using a *t*-test of the regression of the genetic values on the i^{th} SNP. Significance level was set to p-value $< 1 \times 10^{-5}$. This arbitrary significance level was based on a range of other DGRP studies (Durham et al. 2014; Montgomery et al. 2014; Vaisnav et al. 2014; Gaertner et al. 2015). There are several reasons why we used the estimated genetic values as response variable in the association analyses. First, in the case of DGRP studies, the genetic values will approach line means, in fact performing quantitative genetic analyses on DGRP line means can result in broad sense heritability estimates approaching 1 (Mackay and Huang 2017). Secondly, there exists a very high correlation between the genetic values and the phenotypic line means (Supplementary Fig. S2). Thirdly, the marginal SNP analysis on genetic effects is efficient in terms of computational time because we do not need to include the fixed effects as these has been accounted for.

Functional categories, here GO categories, were tested for enrichment of associated SNPs, i.e., SNPs with *p*-values $< 1 \times 10^{-5}$. We used a count based set test approach (previously described by (Rohde et al. 2016; Sørensen et al. 2017), which counts the number

of genetic markers in a particular GO term that are associated with the trait. The enrichment score was computed as,

$$T_{\text{count}} = \sum_{i=1}^{m_f} \mathbb{I}(t_i > t_0)$$

where m_f is the number of markers within the GO term, t_i is the i-th single marker p-value, $t_0$is the significance threshold (here $t_0 = 1 \times 10^{-5}$), and $\mathbb{I}$ is an indicator function that takes the value one if the argument is satisfied. Under the competitive null hypothesis, i.e., individually associated SNPs are distributed randomly across the genome, the significance can be determined by obtaining an empirical distribution of T_{count}. Here, the empirical distribution was obtained using the circular permutation approach as described by Cabrera et al. (2012), where the genome was considered to be circular. Then, the set of SNP p-values were permuted by rotating with respect to their genomic position, i.e., a random number between one and the total number of SNPs was drawn, and the observed SNP p-value of the first SNP in the genome rotates to that of the random number-th SNP, and all other SNP p-values rotate to the same degree to the corresponding SNPs. Thus, the SNPs retain the original order but, at each permutation, gain new random p-values. This uncouples the association between SNP and GO term, while retaining the correlation pattern (due to linkage disequilibrium) among p-values. For each permutation (here 10,000) a new T_{count} statistic was computed based on the original position of the GO term, and a empirical p-value was obtained as a one-tailed test of the proportion of permuted test statistics that were larger than the observed.

A permutation approach was used to test if the number of genes and GO terms in common between traits was larger than expected. Initially, an incidence matrix with *n* rows (number of genes or GO terms) and *m* columns (the number of traits, i.e., five) was constructed: genes or GO terms associated with a trait was set to one, otherwise to zero. The true overlap between traits was compared to an empirical distribution obtained by permuting the elements within columns and computing the overlap (x10,000). The probability of the observed overlap was estimated under the null hypothesis of independent association among traits. An empirical *p*-value was obtained as the fraction of all permutations with an overlap equal/higher to the observed overlap among traits.

Across environment predictions

In order to support the estimated genetic correlations, we used the results from the whole genome single marker regression to predict the observed values of inherent cold tolerance and values of the four V_E components. This was achieved by fitting series of genomic best linear unbiased prediction (GBLUP) models (Meuwissen et al. 2001) and genomic feature best linear unbiased prediction (GFBLUP) models (Edwards et al. 2016; Sarup et al. 2016). The GBLUP models serves as a NULL model fitting all SNPs within one random genetic component assuming all SNP effects to be drawn from the same distribution:

$$\tilde{\mathbf{y}} = \boldsymbol{\mu} + \mathbf{Zg} + \mathbf{e}$$

where $\boldsymbol{\mu}$ is a vector of the overall mean, $\mathbf{Z}$ are design matrices linking adjusted phenotypic values to genetic values ($\mathbf{g}$) captured by all SNPs; $\mathbf{g} \sim \mathrm{N}\left(0, \mathbf{G}\sigma_g^2\right)$. $\tilde{\mathbf{y}}$ is a vector of adjusted

phenotypic values, i.e. $\tilde{\mathbf{y}} = \mathbf{g}+\mathbf{e}$, where the genetic and residual effects were obtained from the linear mixed model from the 'Quantitative genetic parameters'. The GFBLUP model includes an additional random genetic effect, that allows one to assess the importance (in terms of e.g., variance explained or predictive ability) of a selected set of SNPs;

$$\tilde{\mathbf{y}} = \boldsymbol{\mu} + \mathbf{Zf} + \mathbf{Zr} + \mathbf{e}$$

with same specifications, as in the GBLUP model, except **f** is a vector of line-specific genetic effects for the SNPs within the genomic feature, and **r** is a vector of line-specific genetic values for the SNPs not within the genomic feature (i.e. the remaining SNPs). The random genetic and residual effects were assumed to be independent normally distributed: $\mathbf{f} \sim N(0, \mathbf{G}_f\sigma_f^2)$, $\mathbf{r} \sim N(0, \mathbf{G}_r\sigma_r^2)$, $\mathbf{e} \sim N(0, \mathbf{I}\sigma_e^2)$, where $\mathbf{G}_f$ and $\mathbf{G}_r$ were computed as described in the paragraph above for the subset of SNPs.

Here, the feature group contained SNPs that in the whole genome single marker regression with a *p*-value bin, i.e., $p <$ 1E-5, 1E-4, 0.001. 0.01, 0.05, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8 and 0.9. Specifically, the feature groups were based on the *p*-values obtained from one trait (inherent cold tolerance or a component of V_E), and the predictions were performed for another trait. A total of 20 cross-trait predictions were performed.

For each genomic feature (i.e., *p*-value bin) a GFBLUP model was fitted, and the performance was measured as the predictive ability, which is the correlation between observed and predicted genomic effects (see below), compared to the predictive ability of the GBLUP model using 10-fold cross validation, i.e., 9:10 as training set (t), and 1:10 as a

validation set (v) with 100 training and validation sets. The predicted genetic effects for GFBLUP of lines in the validation set were computed as:

$$\hat{\mathbf{g}}_v=\left(\mathbf{G}_{f_{v,t}}\hat{\sigma}_f^2+\mathbf{G}_{r_{v,t}}\hat{\sigma}_r^2\right)\left[\mathbf{G}_{f_{t,t}}\hat{\sigma}_f^2+\mathbf{G}_{r_{t,t}}\hat{\sigma}_r^2+\mathbf{I}_{t,t}\hat{\sigma}_e^2\right]^{-1}\left(\tilde{\mathbf{y}}_t-\hat{\mu}_t\right)$$

Similarly, for the GBLUP model, the genetic effects were computed as:

$$\hat{\mathbf{g}}_v=\left(\mathbf{G}_{v,t}\hat{\sigma}_g^2\right)\left[\mathbf{G}_{t,t}\hat{\sigma}_g^2+\mathbf{I}_{t,t}\hat{\sigma}_e^2\right]^{-1}\left(\tilde{\mathbf{y}}_t-\hat{\mu}_t\right)$$

Validation of candidate genes

For each V_E measure we selected top 10-11 associated genes to be used in gene expression knockdown using the binary *UAS*-GAL4 system. For each rearing temperature, virgin females from the *UAS*-RNAi lines where crossed to actin-GAL4 (*y*^{1} *w***; P{Act5C-GAL4}25FO1/CyO,*) males to ubiquitous knockdown gene expression of individual candidate genes in the F_1 adult male offspring. CT_{min} of ca. 20 flies from each *UAS*-GAL4 cross and rearing condition were assessed. Each measure of V_E was then estimated based on seven replicates, each consisting of ca. 3 flies. The observed phenotype (i.e., the four V_E components) of the *UAS*-GAL4 F_1 males was compared to a corresponding control line with the same genetic background crossed to the GAL4 line, and significance was assessed using a one-tailed Mann-Whitney U-test. The *p*-values were corrected for multiple testing using Bonferroni correction. The standard error of the difference of means were determined as $\sigma_{M_1-M_2}=\sqrt{\frac{\sigma_1^2}{n_1}+\frac{\sigma_2^2}{n_2}}$, where M_1 and M_2 are means, σ_1^2 and σ_2^2 are the variance among replicate values, and n_1 and n_2 are number of replicates of the *UAS*-GAL4 line and its control, respectively.

Assessment of inbreeding effects using diallel crosses

To test whether differences in CT_{min} between DGRP lines was due to line-specific inbreeding effects, we evaluated potential inbreeding depression by comparing CT_{min} of parental lines and their hybrids. In addition, the contribution of dominance and overdominance affecting the inheritance of CT_{min} was evaluated. A set of half diallel crosses of selected DGRP lines was used (Supplementary Table S3). Five DGRP lines with high average CT_{min} and four lines with low average CT_{min} (averaged across the five rearing temperatures), all free of *Wolbachia*, were used in the crosses. Less than 8 h after eclosion, five to ten virgin males and females were collected under CO_2 anaesthesia and put into separate vials, and left for 48 h to recover before the crosses were established. Hereafter, they were transferred to a new vial for egg-laying for four time periods of 12 h at 23 °C using the same setup as in the main experiment. All possible crosses between the selected DGRP lines were performed, resulting in a total of 36 hybrid crosses (approximately equal number of crosses were done with males and females from each of the high and low CT_{min} lines). At an age of 60±12 h 8 males from the 36 hybrid crosses and 9 parental lines were tested using the same setup as in the main experiment.

Measures of additive and non-additive components were obtained by computing the general combining ability (GCA) and specific combining ability (SCA). The GCA/SCA ratio evaluates the contribution of additive vs. non-additive gene action responsible for the inheritance of the trait. GCA/SCA < 1 indicates larger effects of the non-additive gene action, whereas, GCA/SCA > 1 indicates greater importance of additive gene action (Griffing 1956).

Statistical software

All analyses were done within the R programming environment (R Core Team 2017). All quantitative genetic and genomic analyses were performed with the "qgg" package freely available at http://psoerensen.github.io/qgg/. In particular, the mixed models are efficiently solved using the average information REML implemented in DMU (Madsen et al. 1994). The AI-REML function in the "qgg" package provides an R interface to DMU, which can be downloaded from http://dmu.agrsci.dk/DMU/. Genes were linked to GO categories, using the BioConductor package 'org.Dm.eg.db' v. 2.14 (Carlson 2017). Estimates of GCA and SCA were obtained following the methodology of Griffing (Griffing 1956) for analysis of parental and hybrid genotypes (Method II) with a random effects model using the R package "DiallelAnalysisR" (Yaseen 2016).

Results

All four components of environmental variation had a heritable component

Plasticity (V_{plast}) was estimated as the slope of a linear regression of CT_{min} as a function of rearing temperature, and the variation of the plastic response ($V_{v.plast}$) was computed as the standard error of the slope (Table 1). Within-environment variation (V_{WE}) for CT_{min} was estimated using the coefficient of variation (CV = SD/mean). To enable the comparison of a single measure of within-environment variations (CVs) with the other three components of environmental variation, we computed a line mean V_{WE} (Table 1). As the measure of environmental variation across environments (V_{AE}), we computed the DGRP line specific difference between the minimum and maximum CV across environments (Table 1). The robustness of all four measures was confirmed with resampling as described above (Supplementary methods, Supplementary Fig. S1). We observed

large phenotypic differences among the DGRP lines in the four measures of environmental variation (Fig. 2) and in CT_{min} at the individual temperatures (Supplementary Table S1). The broad and narrow sense heritability estimates (H^2 and h^2) of the four components of environmental variation were all significant and varied considerably with V_{WE} and V_{AE} having low estimates, $V_{v.plast}$ an intermediate estimate, and V_{plast} having the highest estimate (Fig. 2). We observed significant positive phenotypic (ρ_p) and genetic correlations (ρ_g) among $V_{WE,}$ V_{AE} and $V_{v.plast}$, and between V_{plast} and $V_{v.plast}$ (Fig. 2), however, V_{plast} was only weakly positively correlated with the other measures of environmental variation (Fig. 2). The correlations between the rank orders of the DGRP lines for the four measures of environmental variation resembled these patterns, with the highest correlation between the rank order of V_{WE} and V_{AE} (Supplementary Fig. S3).

Single nucleotide polymorphisms (SNPs) associated with the four components of V_E

To disentangle the underlying genetic architecture of the four components of environmental variance, we applied whole genome single marker regression on individual genetic markers. At a nominal *p*-value threshold of $p < 1 \times 10^{-5}$ we found several SNPs associated with the four components of environmental variation (Supplementary Fig. S4 and Supplementary Table S4). In total, 148 genes (132 unique) were significantly associated with one or more of the four components of environmental variation (30 for V_{plast}, 18 for $V_{v.plast}$, 35 for V_{WE}, and 65 for V_{AE}). The majority of genes were not in common between V_E components, however, a significant overlap of 16 genes between V_{WE} and V_{AE} was found (Supplementary Table S4). In line with ρ_p we found significant positive genetic correlations (ρ_g) for the V_E measures (Fig. 2), supported by increased predictive ability across traits when causal variants were used in the prediction models (Supplementary Fig.

S5). To investigate whether there was overlap in the functional categories associated with each type of environmental variation, we performed a gene ontology (GO) enrichment test with sets of SNPs with a *p*-value < $1x10^{-5}$. We only found a significant overlap in GO terms between V_{AE} and V_{WE} (Supplementary Fig. S6) supporting the notion that there is some degree of similarity in the genetic architecture of these two V_E components, and that the rest are decoupled on a functional level as well.

Components of environmental variation are genetically distinct from inherent cold tolerance

To assess if components of environmental variation were linked to inherent (non-plastic) cold tolerance, we used CT_{min} of DGRP lines reared at 23 °C as a measure of inherent cold tolerance. While 23 °C is arbitrary, this is a benign temperature and normally used to rear the DGRP lines. We found a weak but significant negative phenotypic correlation between CT_{min} at 23 °C and V_{plast} supported by a negative genetic correlation (ρ_g) (Fig. 2). Thus, there was a tendency that for individuals reared at 23 °C the most cold tolerant DGRP lines showed the highest degree of plasticity. Despite these correlations, we found no overlap of genes associated with inherent cold tolerance and V_{plast}. Similarly, we found no overlapping genes between inherent cold tolerance and $V_{v.plast}$, V_{WE} and V_{AE} (Supplementary Table S4) and inherent cold tolerance was a poor predictor of the four components of environmental variation (Supplementary Fig. S5).

Functional validation of candidate genes

To phenotypically validate a set candidate genes, we selected 10-11 top candidate genes for each V_E component (Supplementary Figs. S7-10) and performed gene expression knockdown using the

binary *UAS*-GAL4 system (Supplementary Table S5). The knockdown lines were reared under the same five thermal regimes as the DGRP lines, and then characterized for CT_{min} to allow estimation of the different components of V_E for each candidate gene. Each *UAS*-GAL4 line was compared to a control line with the same genetic background. These comparisons provided evidence that 16 of the 40 tested candidate genes exhibited a significantly altered phenotypic response compared to the controls in the four components of V_E (Fig. 3 and Supplementary Table S5).

We further tested whether the genetic (ρ_g) and phenotypic (ρ_p) correlations between the four components of V_E and inherent cold tolerance (CT_{min} 23 °C) obtained using the DGRP (Fig. 2) were evident in the *UAS*-GAL4 lines, providing validation that the genes contributed to the genetic architecture of the traits in ways expected from the DGRP set. We compared the difference of each *UAS*-GAL4 line from its respective control (signed effect sizes) for all pairwise components of V_E and inherent cold tolerance (CT_{min} 23 °C) using both the 10-11 candidate genes in each of the four V_E components and all 40 candidate genes together (Supplementary Figs. S11-20). These comparisons showed that the highly significant correlations between V_{WE}, V_{AE}, and $V_{v.plast}$ in the DGRP lines were also found in the distinct genetic backgrounds of the *UAS*-GAL4 lines. This was regardless of whether only the candidate genes for the individual V_E components or all 40 candidate genes were used in the pairwise correlations (Supplementary Figs. S11-13). Similarly, the weak or non-significant correlations of ρ_g and ρ_p between V_{plast} and both V_{WE} and V_{AE}, as well between CT_{min} 23 °C and $V_{v.plast}$, V_{WE} and V_{AE} in the main experiment was confirmed using the *UAS*-GAL4 system (Supplementary Figs. S14-18). The positive correlation between V_{plast} and $V_{v.plast}$ and the negative relationship between inherent cold tolerance and V_{plast} was also confirmed with the *UAS*-GAL4 system (Supplementary Figs. S19-20). Overall, the pairwise relationships between the four components of V_E and inherent

cold tolerance in the *UAS*-GAL4 lines were highly consistent with the observations in the main experiment using the DGRP (Fig. 4).

Diallel crosses show additive gene action and no inbreeding effects

Because inbreeding effects may confound interpretations of correlation patterns, we performed diallel crosses with selected DGRP lines to test if inbreeding depression affected CT_{min} (Supplementary Table S3). Out of 36 line crosses, 6 F_1's had significantly lower average CT_{min} than the mean of the parental genotypes (one sample t-test, $t_{14} < -2.70$, $p < 0.05$). We found no F_1's with lower average CT_{min} than the best performing parental genotype, i.e. with lowest CT_{min} (two-sample t-test, $t_7 > -1.75$, $p > 0.11$). Overall, there was no significant difference in CT_{min} between parental genotypes and hybrid crosses ($\chi_1^2 = 1.48$, $p = 0.22$, as determined by maximum likelihood). Based on one-way ANOVAs, a significant effect of general combining ability (GCA) was found ($F_8 = 11.55$, $p < 0.001$), whereas no significant effect of specific combining ability (SCA) ($F_{36} = 1.38$, $p = 0.075$) was detected. The GCA/SCA ratio was 3.42, suggesting that inbreeding effects are minor.

Discussion

Here we undertook a comprehensive analysis of the genetic control of environmental variation (V_E) of cold tolerance in *Drosophila melanogaster*. This was obtained by partitioning environmental variation into four components: plasticity (V_{plast}), within-environment variation (V_{WE}), across environment variation (V_{AE}), and variation of plasticity ($V_{v.plast}$; Fig. 1 and Table 1). We investigated the genetic basis for each component, the independence of these components from each other and from inherent cold tolerance, and the genes governing these patterns.

We found considerable variation among the DGRP lines for within-environment variation (V_{WE}) (Fig. 2 and Supplementary Table S1) suggesting genetic control of the trait which is consistent with recent studies (Ayroles et al. 2015; Morgante et al. 2015; Sørensen et al. 2015). The finding that V_{WE} is under genetic control suggests that this trait will be impacted by selection and genetic drift. Such effects have implications for breeding programs where consistent trait values are often desirable (Hill and Mulder 2010; Rönnegård et al. 2013). For decades, V_E has been seen as separate from genetic variation in classic quantitative theory (Falconer and Mackay 1996). In traditional analyses, V_E has typically been lumped into a residual variance component as part of the variance that cannot be explained by other components in the model. Only recently, have researchers started to acknowledge that V_E also has a genetic basis (Ros et al. 2004; Ibáñez-Escriche et al. 2008; Ayroles et al. 2015; Morgante et al. 2015; Sørensen et al. 2015; Blasco et al. 2017). We expand on this with our novel proposed partitioning of V_E into four components, which has implications for evolutionary biologists, as well as animal and plant breeders alike.

We observed substantial genetic variation for plasticity (V_{plast}) (Fig. 2) in line with high heritability of plasticity in fitness related traits in *Daphnia magna* (Stoks et al. 2016), but inconsistent with some quantitative genetic models pointing to low genetic variation for plasticity, and thereby slow evolution of plastic responses (Berrigan and Scheiner 2004). The phenotypic and genetic correlations between V_{plast} and V_{WE} were rather low but significant (Fig. 2) suggesting that the genetic mechanisms affecting trait plasticity and variation within environments share some characteristics. This implies that it will be difficult to fully disentangle the effects of plastic and non-plastic effects as organisms respond to changing environments (Via et al. 1995; van Kleunen and Fischer 2005; Stoks et al. 2016).

We considered two novel components of environmental variation; across environment variation (V_{AE} calculated as ΔCV) representing the extent to which within-environment variation changes across environments, and variation in the plastic response ($V_{v.plast}$ calculated as standard error of the slope). The heritability estimate for $V_{v.plast}$ was relatively high, suggesting a large degree of genetic variation for this trait (Fig. 2). The estimated H^2 and h^2 for V_{AE} and V_{WE} were significant and similar, suggesting potential for evolutionary change in both within- and across environment variation. Selection for increased V_{AE} would be beneficial for a population of similar genotypes if the optimum fluctuates in time and space such that some individuals would benefit from large V_{WE}, whereas others would increase fitness by decreased V_{WE}, resulting in a large V_{AE}. As an example of genetic variation in V_{AE} likely to be under selection, differentiated diapause or diapausing egg formation in insects can be considered, i.e. the situation where a fraction of genotypes always show diapause, a fraction of individuals diapause only at certain conditions, and a fraction that never diapause (Tuljapurkar and Istock 1993; Simons and Johnston 1997). This leads to situations where genotypes produce different levels of phenotypic variation both within and across environments.

Partitioning of V_E into four components is novel and genetic variation in these components is not captured by classic analyses of environmental variation and genotype by environment (GxE) interactions. Genotype specific plasticity (V_{plast}) can be regarded as a type of GxE interaction, however, the three remaining proposed components of environmental variation are not covered by the classic GxE paradigm, as we investigate both environmental variation within an environment (V_{WE}) and environmental variation across environments (V_{AE}). This results in potential interactions between genotype and V_{WE} and between genotype and V_{AE}, which cannot be distinguished under a GxE approach. The GxE term does not capture genotypic effects on environmental variation due to variation in the plastic response ($V_{v.plast}$), as this encompasses both variation within and across

environments. Therefore, our partitioning provides new insights into the nature of genetic contributions to environmental variances and potential interactions among them.

We emphasize that the specific computations of the various V_E components are context dependent, i.e., using slope and SE of the slope for V_{plast} and $V_{v.plast}$, respectively, only makes sense for linear responses. In the case of a non-linear response, a different measure of the consistency of the plastic response ($V_{v.plast}$) such as the coefficient of determination (R^2) might be more appropriate. Similarly, other specific measures of the variation within and across environments might be employed instead of CV, e.g. SD, variance or similar (Table 1; see e.g. Morgante *et al.* 2015).

Phenotypic and genetic correlations between inherent cold tolerance (CT_{min} for flies developed at 23 °C) and $V_{v.plast}$, V_{WE}, and V_{AE} (Fig. 2) were rather weak and differed in direction, supported by a lack of overlapping genes between inherent cold tolerance and the four components of V_E identified by marker regressions. This was confirmed in the validation using the *UAS*-GAL4 system. Gerken et al. (2015) similarly found little overlap between genes controlling basal and plastic cold tolerance. Other studies with *D. melanogaster* show variable associations between trait means and environmental variation (Harbison et al. 2013; Ayroles et al. 2015; Morgante et al. 2015). In the case of a positive correlation between trait means and overall V_E, directional selection for increased trait means will simultaneously increase V_E. This has been suggested as a reason for the high environmental variation typically observed in fitness related traits (Morgante et al. 2015). On the other hand, traits with a negative correlation between trait mean and trait variance can be of particular interest, especially in breeding programs where selecting for both increased trait value and trait homogeneity can be of economic value (Mulder et al. 2008; Hill and Mulder 2010). We found some evidence of similarity in the genetic mechanisms controlling V_{WE} and V_{AE} as indicated by shared candidate genes (Supplementary Table S4) and the significant genetic correlation observed

between these two V_E components. Similarly, we found an overlap of GO terms between V_{WE} and V_{AE} (Supplementary Fig. S6), which could suggest some shared characteristics on a higher functional level between these two V_E components.

We identified no overlapping genes between the V_{plast} and inherent cold tolerance (CT_{min} 23 °C) (Supplementary Table S4), suggesting partly separate genetic mechanisms for plasticity and inherent cold tolerance. The existence of regulatory genes that control plasticity but not trait means is often debated (Schlichting and Pigliucci 1993; Via et al. 1995), but there is supporting evidence (Schlichting and Levin 1986; Scheiner and Lyman 1991). We did, however, find significant negative correlations between inherent cold tolerance and V_{plast} (ρ_p and ρ_g) i.e. genotypes with low cold tolerance will on average tend to have low plasticity. This finding argues against a trade-off between plasticity and trait value (Murren et al. 2015). The debate about whether plasticity (hardening or acclimation) constrains an organism's basal temperature tolerance is ongoing (Stillman 2003; Calosi et al. 2008; Chown et al. 2010), and although trade-offs have been observed in some taxa (Stillman 2003; Gerken et al. 2015), a recent meta-analysis across taxa found a lack of support for the trade-off hypothesis (Gunderson et al. 2015). The results presented here were not confounded by inbreeding effects, as the diallel crosses showed no evidence of overdominance and only a very small degree of dominance. Furthermore, the general- and specific combining ability (GCA/SCA) ratio was well above 1, implying a greater relative importance of additive gene action compared to non-additive gene action (Griffing 1956). In accordance with other authors (Morgante et al. 2015), we found no association between any of the four V_E measures and residual heterozygosity across the DGRP lines (results not shown).

The majority of genes associated with V_{plast} with known biological functions were involved in regulatory processes of development (*Adgf-A* (Dolezal et al. 2005)), components of the nervous system (*NetB* (Labrador et al. 2005) and *cno* (Pérez-Gómez et al. 2013)), immune responses (*dpr16* (Vogel et al. 2003)), and in DNA repair (*Pino* (Park and Song 2008)), where we functionally validated the latter two (Fig. 3). Regulatory genes are believed to be a crucial part of the genetic control of plasticity (Schlichting and Pigliucci 1993), and might therefore also be expected to be involved in expression variation in $V_{v.plast}$. Several of the candidate genes for $V_{v.plast}$ have been shown to be involved in regulatory processes such as neurotransmitter regulation (*CASK* (Zordan et al. 2005)), regulation of transcription (*EloA* (Gerber et al. 2004)), regulation within biological pathways such as MAPK (*alph* (Baril and Therrien 2006)) and Notch (*eIF-3p40* (Zhang et al. 2012)), and some of the candidate genes for $V_{v.plast}$ have been identified in *Drosophila* as general environmental stress responsive genes (King-Jones et al. 2006; Baril et al. 2009). Here, we showed functionally that *eIF-3p40* and *CASK* have an effect on $V_{v.plast}$ (Fig. 3). The gene *mub* was associated with both V_{AE} and V_{WE}, which we validated functionally for V_{AE} (Fig. 3). This gene has been shown to be involved in thermosensory behaviour (Hong et al. 2008). Similarly, we identified candidate genes that have previously been associated with variation in cold tolerance, e.g. *Dys* (Gerken et al. 2015), and *Cad87A* which has been associated with thermal stress (DeSalvo et al. 2008).

The validation data strongly support the results from the DGRP for the individual gene functions (Fig. 3). Furthermore, the overall patterns of phenotypic relationships among the four V_E components and between V_E components and inherent cold tolerance was similar in the *UAS*-GAL4 and DGRP lines; in fact, the phenotypic correlations among the V_E components obtained from each of the two distinct sets of genotypes was highly correlated (Fig. 4 and Supplementary Figs. S11-20), despite the *UAS*-GAL4 lines coming from distinct genetic backgrounds. This further strengthens our

confidence in the conclusions drawn on the basis of the DGRP comparison. It also represents a novel way of utilizing this gene expression knockdown system, beyond simply functionally validating individual genes.

In conclusion, CT_{min} and all four components of environmental variation were under genetic control. Some of these components of V_E were weakly genetically correlated to each other, suggesting some shared genetic architecture. Despite being correlated, there was little overlap in genes controlling different components of environmental variation, suggesting separate selection targets. Using genomic resources, we identified new candidate genes that are shared between the different components of V_E and genes specific to particular components.

We acknowledge that characterizing variances is difficult because they are inherently noisy, and that studies on variances (including our own) typically have limitations due to low sample sizes and power issues. However, we argue that the DGRP system has some merits in that the genetic variation within lines is practically non-existent, and this means that variance components can be estimated with relatively small sample sizes (see the discussion of resampling in Supplementary methods). In identifying candidate genes with single marker regressions, we did not use FDR correction, an approach also followed in many other DGRP studies. This can lead to the effects of individual SNPs being overestimated, but in our study we also undertook a functional validation of candidate genes. Further analyses on the genetic control of environmental variance components may need to focus on data from production animals where large datasets from defined environments are often available (Hill and Mulder 2010; Sanchez-Garcia et al. 2012; Rönnegård et al. 2013).

Empirical studies on the genetic basis of V_E are still in their initial phase, with most studies aiming simply to document its existence (Mulder et al. 2008; Ayroles et al. 2015; Sørensen et al. 2015). We have conceptualized the partitioning of overall environmental variation into separate identifiable components to an extent well beyond what has been attempted before, and shown substantial genetic variance for each component. We propose that our study can work as a hypothesis generating platform motivating future studies elucidating the nature of evolutionary forces maintaining variation for V_E components and their interactions.

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Table 1

Overview of the four components of environmental variation. Overview of four components of environmental variation as applied here to cold tolerance (CT_{min}); plasticity (V_{plast}), within-environment variation (V_{WE}), across environment variation (V_{AE}), and variation of the plastic response ($V_{v.plast.}$) and how they are computed. Sample studies or reviews are included. Alternative interpretations or terms used in the literature to describe the components are included where appropriate.

Component of environmental variation	Computation	Definition	References	Alternative terms/interpretations (and references)

Plasticity, V_{plast}	Regression coefficient (i.e. slope) of a linear regression of CT_{min} data as a function of rearing temperature.	The ability of a genotype to produce a multitude of phenotypes when exposed to different environmental conditions. Norm-of-reactions are widely used in the study of phenotypic plasticity.	Different indices of phenotypic plasticity are reviewed in e.g. Valladares et al. (2006) and DeWitt and Scheiner (2004).	Genotypic differences in plasticity can be regarded as a genotype-by-environment interaction (GxE).
Within-environment variation, V_{WE}	Coefficient of variation (CV = standard deviation/mean) within each environment. Line mean CV ($\overline{CV}$) was also computed.	How consistently a genotype produces the same phenotype within a given environment. Similar measures include micro-environmental variation.	Ayroles et al. (2015); Morgante et al. (2015); Sørensen et al. (2015).	"Genetic control of environmental variation" (Hill and Mulder 2010); "Intragenotypic variability" (Ayroles et al. 2015); "Genetic variance heterogeneity" (e.g. Struchalin *et al.* 2010; Forsberg *et al.* 2015); "Variance controlling QTLs (vQTL)" (Rönnegård and Valdar 2012).
Across environment variation, V_{AE}	Highest CV in any given environment minus lowest CV in any other given environment (ΔCV).	Developmental stability across environments; the consistency of within-environment variation across environments.	Tuljapurkar and Istock (1993).	This can be thought of as GxE at the level of variance.

Variation in plastic response, $V_{v.plast}$	Standard error of the slope.	A measure of the extent to which the same genotype can produce the same plastic response consistently.	Present study.	-

Figure legends

Fig. 1 Illustration of the four components of environmental variation. (**a**) Average values of any phenotype measured across an environmental gradient in individuals from three hypothetical genotypes differing in plasticity (V_{plast}), i.e. slope of the reaction norm, and thus in the contribution of plasticity to the overall environmental variation of the genotypes. Genotypes 1 and 2 have different phenotypic plasticity with genotype 1 being the most plastic. Genotype 3 is canalized and has no environmental variation as a result of plasticity. The shading represents the variation of the plastic response ($V_{v,plast}$), i.e. differences in the standard error of the slope. Here genotype 1 has the highest plasticity and simultaneously the highest variation in the plastic response, whereas genotypes 2 and 3 have the same slope SE despite having different slopes. (**b**) Environmental variance (measured as the coefficient of variation, CV) of three hypothetical lines (separate from panel (a)) across an environmental gradient. Points represent the within-environment variation (V_{WE}) measured as within-environment CV. Dashed grey lines represent a measure of the mean within-environment variation ($\overline{CV}$) across environments. On the right-hand side the difference in variation across environments (V_{AE}) measured as ΔCV is shown. The genotypes differ in both variation within-environments and across environments. Genotype 1 has a consistently high CV in all environments, i.e. the phenotypic variation within each environment is large, and consistently so across environments (low ΔCV). Genotype 2 has low CVs in some environments but high in others, i.e. an intermediate $\overline{CV}$, but a high variation in the expression of phenotypic variation across environments (high ΔCV). Genotype 3 has low CVs in all environments, i.e. low developmental instability both within and across environments.

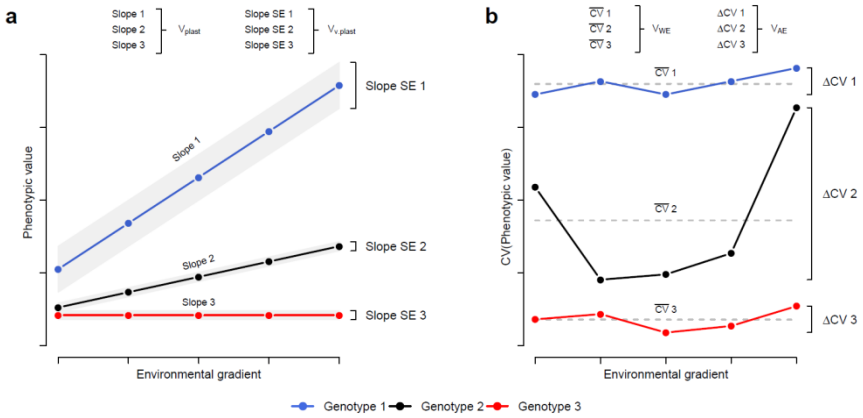


Fig. 2 Estimated genetic parameters and correlations between cold tolerance and the components of environment variance. Estimated genetic parameters for inherent cold tolerance (i.e., CT_{min} at 23 °C) and the components of environment variance (V_{plast}, $V_{v.plast}$, V_{AE}, V_{WE}). Diagonal elements show the DGRP line mean (±SE) sorted by increasing values within trait. Broad sense (H^2) and narrow sense (h^2) heritability estimates with their approximated SEs are shown. Plots above the diagonal show the phenotypic correlations (ρ_p, SE in parenthesis) between traits. Plots below the diagonal show the genetic correlations (ρ_g, SE in parenthesis). Here genetic correlations were approximated as SNP-correlations. Dashed lines are the regression lines visualizing the correlations. Genetic parameters in bold indicate values significantly different from zero, i.e. the estimate deviates more than 1.645 x SE from 0 ($p < 0.05$). Note that the y-axis across the top row changes because the correlations have been computed based on log(y), but CT_{min} values (i.e., top left panel) are shown in their original scale.

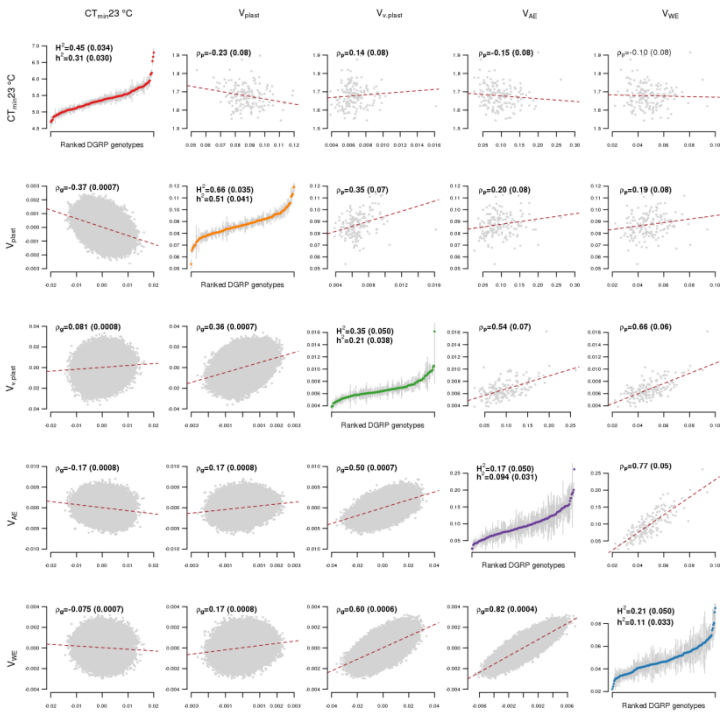


Fig. 3 Effects of gene expression knockdown of the candidate genes for the four environmental variation components. Effects of gene expression knockdown of the candidate genes for (**a**) V_{plast} in orange (11 genes), (**b**) $V_{v.plast}$ in green (10 genes), (**c**) V_{WE} in blue (10 genes), and (**d**) V_{AE} in purple (10 genes). Bars represent the absolute difference between the mean of a given gene assessed in the *UAS*-GAL4 lines and its respective genetic background. Asterisks denote significant difference from control as determined by a one-tailed Mann-Whitney *U* test between replicate values of each V_E component compared to the control (n=14): * *p*-value < 0.05, ** *p*-value < 0.01. The *p*-values has been corrected for multiple testing using the Bonferroni approach. Error bars represent standard error of the difference of means determined as $\sigma_{M_1-M_2} = \sqrt{\frac{\sigma_1^2}{n_1} + \frac{\sigma_2^2}{n_2}}$, where M_1 and M_2 are means, σ_1^2 and σ_2^2 are the variance among replicate values, and n_1 and n_2 are number of replicates of the *UAS*-GAL4 line and its control, respectively.

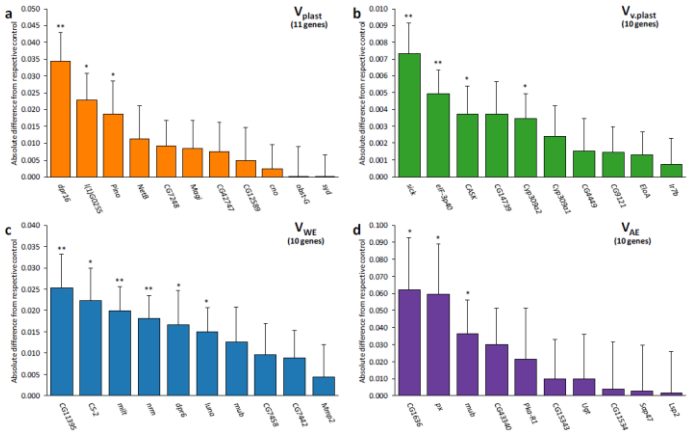
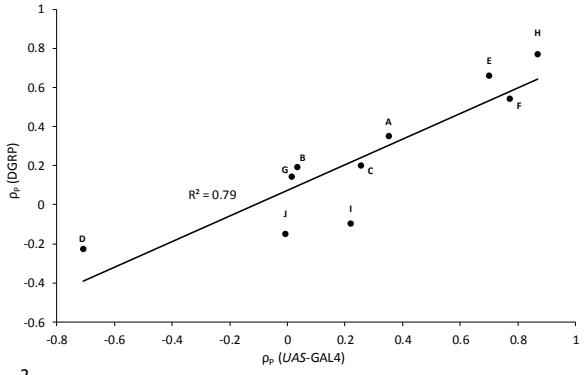


Fig. 4 Comparison of pairwise trait correlations in the DGRP with correlations obtained in the *UAS*-GAL4 genetic background. Comparison of phenotypic (ρ_p) correlations between the four V_E components and inherent cold tolerance (i.e. CT_{min} 23 °C) obtained from the DGRP (Figure 2) with the same pairwise correlations obtained when investigating top candidate genes for each component using the *UAS*-GAL4 system (ρ_P (*UAS*-GAL4)). Estimates of ρ_P (*UAS*-GAL4) were obtained as correlations for all combinations of the four V_E components and inherent cold tolerance, and using all 40 tested candidate genes in calculating the correlation, regardless of which V_E component these genes were associated with (see Supplementary Figs. S11-20). The coefficient of



determination, R^2, is shown. Letters denote correlations between the following combinations of V_E components and inherent cold tolerance: A: V_{plast} and $V_{v.plast}$, B: V_{plast} and V_{WE}, C: V_{plast} and V_{AE}, D: V_{plast} and CT_{min} 23 °C, E: $V_{v.plast}$ and V_{WE}, F: $V_{v.plast}$ and V_{AE}, G: $V_{v.plast}$ and CT_{min} 23 °C, H: V_{WE} and V_{AE}, I: V_{WE} and CT_{min} 23 °C, and J: V_{AE} and CT_{min} 23 °C.