Gene expression evolution is predicted by selection, genetic covariance and network topology

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Abstract

Changes in gene expression play a fundamental role in the process of adaptation. However, single gene expression levels cannot evolve independently but form highly correlated groups, often corresponding to functional modules. Any evolutionary response is therefore the product of direct selection acting on a respective gene and indirect selection acting on genetically correlated partners. Here, we studied the mechanisms behind evolution in expression in response to a change in climate conditions using whole transcriptomes and fitness data of the red flour beetle *Tribolium castaneum* before and after adaptation in seven independent selection lines. Genetic covariance between expression levels, as well as with fitness, could predict evolutionary responses and allowed us to quantify contributions from direct and indirect selection. These predictions were highly consistent with observed changes after 20 generations of adaptation and indicate a pivotal role of indirect selection. Consequently, genes with central positions in gene networks experienced the strongest selection and exhibited larger evolutionary changes in expression. Our genomic analysis revealed that selection on expression levels drives parallel allele frequency changes in the respective genes. More connected genes and those carrying expression quantitative trait loci (eQTLs) showed a higher degree of parallel evolution. We demonstrated how a quantitative genetics approach combined with transcriptomics and network analysis provides deep insights into the evolution of gene expression: Network structure can greatly enhance expression evolution through indirect selection when concerted selection pressures act on the functional properties of expression modules.

Introduction

Understanding the genetic basis of adaptation is a central goal in evolutionary biology. While significant progress has been made in mapping molecular variation onto phenotypic variation, we still face challenges in understanding how molecular variation translates into fitness variation to enable evolutionary changes. Molecular studies of the genetic basis of trait variation have revealed the key role played by gene expression in phenotypic variation and unraveled networks of locally and distantly acting gene regulation affecting variation

in expression (Brem et al., 2002; Wray, 2007; Stern and Orgogozo, 2008). Yet, although the role of gene expression in adaptation is largely accepted (Fay and Wittkopp, 2008) and has been amply documented (*e.g.*, Whitehead and Crawford, 2006; Fraser et al., 2010; Pritchard et al., 2017; Mack et al., 2018; Kelly et al., 2023), only few recent studies provide direct measurements of the relationship between gene expression and fitness in insects (McGuigan et al., 2014; Koch and Guillaume, 2020a), plants (Groen et al., 2020), or vertebrates (Ahmad et al., 2021). Such measurements provide estimates of phenotypic selection gradients on whole-transcriptome data from which evolutionary responses in gene expression can be predicted (Falconer and Mackay, 1996). This approach has been utilized to find genes whose expression variation is most important for fitness variation under particular stress (*e.g.*, drought in maize, Groen et al., 2020). However, we do not yet know how expression traits may actually respond to such selection pressures. The predictability of gene expression evolution thus remains untested.

Predicting evolutionary responses from phenotypic selection gradients may be biased by the presence of confounding environmental co-variation between gene expression and fitness. Gene expression can be strongly environmentally sensitive and noisy, responding plastically to abiotic stressors (Lòpez-Maury et al., 2008; Hodgins-Davis et al., 2012; des Marais et al., 2013). Although this plasticity may allow for rapid physiological and metabolic adjustments to new environmental conditions (Gibson, 2008; Ghalambor et al., 2015; Koch and Guillaume, 2020a) and enable adaptive changes when genetically grounded (des Marais et al., 2017), it may also result in non-genetic causes of phenotypic covariation with fitness. A response to selection may then be wrongly expected when phenotypic covariation obscures genetic covariation with fitness (Kruuk et al., 2002; Morrissey et al., 2010; Bonnet et al., 2017). More accurate predictions of the selection responses of phenotypic traits, including gene expression traits, are based on estimates of genetic selection gradients provided by the application of the Robertson-Price equation (or Secondary theorem of natural selection Robertson, 1966; Price, 1970). The method requires to estimate the genetic covariation of additive genetic variation of the trait and fitness and has been advocated to study evolution in wild populations (Morrissey et al., 2010, 2012) among other applications.

One important aspect of the estimates of genetic covariation with fitness is that they

include the effects of direct and indirect selection on the trait of interest, where indirect selection stems from direct selection on the traits correlated to the focal trait. The estimate of total selection provided by the Robertson-Price relation averages out the potential constraining and enhancing effects of selection on multiple traits (Walsh and Blows, 2009). Evolutionary changes might then occur because a trait is the direct target of selection, or because it is genetically correlated to traits under selection, or both. Disentangling effects of direct and indirect selection, although challenging, is especially relevant for gene expression phenotypes. It would help us gain detailed insights into mechanisms of how and why certain expression levels change, and understand their exact role in adaptation. Since genetic covariances between gene expression levels are prevalent (Ayroles et al., 2009; McGraw et al., 2011; Blows et al., 2015), the evolutionary response of a single gene's expression may thus be more influenced by indirect than direct selection.

The evolutionary importance and potential constraints associated with covariances between expression levels are largely unknown. For now, experimental evolution studies demonstrated rapid evolution of gene expression during adaptation to new environments, suggesting that standing variation in expression is relatively unconstrained (Yampolsky et al., 2012; Ghalambor et al., 2015; Huang and Agrawal, 2016; Koch and Guillaume, 2020b), and mutational accumulation experiments showed that the influx of mutational variation in gene expression can be large (Rifkin et al., 2005). However, studies of the genetic variance-covariance structure of gene expression show that species divergence in expression is constrained by genetic interactions (Fraser et al., 2002) and correlations (Innocenti and Chenoweth, 2013) within gene networks. Gene expression variation also seems predominantly under stabilizing selection (Josephs et al., 2015; Kita et al., 2017), potentially constraining genetic variation at pleiotropic genes, but other studies suggest that the constraining effect of stabilizing selection on expression variation might be weak (Steige et al., 2017; Glassberg et al., 2019). All in all, the potential for evolution of gene expression is likely large but might remain constrained by the regulatory and functional network context of variation in expression (Jordan et al., 2004; Innocenti and Chenoweth, 2013; Collet et al., 2018).

Here, we took advantage of individual whole transcriptome (RNA-seq) and fitness data from two time points within replicated experimental evolution lines of *Tribolium casta-*

neum (red flour beetle) adapting to a stressful hot-dry treatment. Combining expression and fitness data (offspring number) of 486 female individuals in a paternal half-sib breeding design enabled us to estimate genetic covariances between expression levels and fitness in the first generation and to make predictions about evolutionary changes. A previous study demonstrated that adaptation involved significant changes in gene expression (Koch and Guillaume, 2020b). Here, our goal was to evaluate the predictability of gene expression evolution by assessing whether long-term (20 generations) evolutionary changes were consistent with short-term predictions (one generation). We further wanted to understand in detail how and why expression evolved and quantified the contributions of direct and indirect selection to evolutionary changes. We built on previous studies that developed methods to estimate the genetic covariance matrix of gene expression in such high dimensionality data by using a Bayesian sparse factor analysis (Runcie and Mukherjee, 2013) and disentangle the contributions of direct and indirect selection to the expected evolutionary change (Stinchcombe et al., 2014).

Additionally, constructing gene co-expression networks enabled us to explore how expression network structure changes under stress. Combining it with selection estimates gave us insights into how selection was acting on different network components and how this was then ultimately related to improving fitness under stressful conditions. Using this approach, we could address crucial questions regarding the genetic architecture of gene expression adaptation: Are there few genes that are direct targets of selection with the majority of genes showing correlated response? Is selection acting on many gene expression levels simultaneously, and can position in gene expression networks predict their response to selection?

Finally, we wanted to link evolutionary changes in expression levels with genetic changes. Having seven replicate selection lines we tested for parallel allele frequency changes (AFC) and whether these changes were associated with genes under selection for expression changes. Furthermore, we identified eQTLs, SNPs associated with variation in expression patterns, and tested whether these showed parallel AFC in the different selection lines.

Results

Evolutionary changes are consistent with predictions

Significant additive genetic variance (V_A) in fitness is the key requirement for any evolutionary change and gives the evolutionary potential for adaptation (Fisher, 1930). The additive genetic variance of mean-standardized relative fitness ($I_A(w) = V_A(w)/\bar{w}^2$; w: relative fitness, $\bar{w}$: mean relative fitness) showed significant evolutionary potential for increased fitness in the hot-dry treatment (HD) with $I_A(w) = 0.033$, higher than in the control condition (CT) ($I_A(w) = 0.019$). This is consistent with the expectation that additive genetic variance in relative fitness $V_A(w)$ should be higher in novel conditions compared to ancestral conditions where strong selection in the past has removed genetic variation. It also agrees with the previously observed adaptation in the selection lines (Koch and Guillaume, 2020b). Similarly, evolutionary changes in the mean expression level of a gene requires additive genetic variation in expression. Genes greatly varied in their proportion of additive genetic (V_A) to phenotypic variance (V_P), as shown by the distribution of their heritability coefficients ($h^2 = \frac{V_A}{V_P}$; Figure S1). Median h^2 in HD was 0.64, very close but significantly lower than in CT (0.66, $P < 0.0001$, permutation test), indicating a large capacity to respond to selection.

We evaluated the evolvability of gene expression in HD with estimates of the genetic covariance of gene expression with relative fitness: $s_g = cov_a(w, z)$, hereafter called genetic selection. The s_g coefficient is also the predicted response to selection of the trait z (here gene expression) over one generation (Robertson, 1966; Price, 1970). We confronted those predictions with the changes in gene expression observed after 20 generations of adaptation in HD to assess the predictability of our estimates of genetic selection.

We found that we were able to predict changes in gene expression based on the genetic covariances of single gene expression levels with fitness. We detected a significant correlation between the transcriptome-wide responses predicted by the genetic covariance with fitness (s_g) in HD and the observed evolutionary changes in expression ($\rho = 0.22$; bootstrap 95% CI: [0.20, 0.24]; permutation test: $P < 0.0001$). 55 gene showed a significant s_g and 75 genes showed significant r_g (genetic correlation, *i.e.*, variance-standardized s_g) after correction for multiple testing. When we focused on the set of genes with sig-

nificant s_g, the correlation with observed evolutionary changes becomes even stronger ($N = 55$: $\rho = 0.64$, bootstrap 95% CI: [0.38, 0.83]; permutation test: $P < 0.0001$). Genes under positive genetic selection ($s_g > 0$) had on average significantly increased expression levels (0.067 log2-Fold change; bootstrap 95% CI: [0.036, 0.102]), and genes under negative genetic selection ($s_g < 0$) showed reduced expression (-0.094 log2-Fold change; bootstrap 95% CI [-0.133, -0.053]; Figure 1A). The predictability of the direction and, to some extant, the magnitude of the response to selection in the HD environment was thus remarkably good (Figure 1B).

Predicted response is mainly caused by indirect selection

Separation of direct and indirect selection can be done if the genetic co-variation among traits is also known (Stinchcombe et al., 2014), achieved by using a multivariate quantitative genetics approach to estimate the G-matrix, the genetic variance-covariance matrix (Lande, 1979; Lande and Arnold, 1983). Having computed estimates of G-matrices for gene expression traits, we could compare the expected response due to direct selection $R_\beta = \beta_g * diag(\mathbf{G})$ with s_g (predicted total response), where $diag(\mathbf{G})$ is the vector of additive genetic variance of gene expression. In general, we found largely weaker estimates of R_β than s_g (Figure 1C). Only 86 genes (0.7%) showed a larger magnitude of response to direct selection R_β than the predicted response s_g in HD. 25% of the genes had opposite signs of R_β than s_g. The proportion of the predicted response contributed by direct selection (R_β/s_g) in those genes where R_β and s_g were of the same sign was small with median proportion of 1%. These results indicate that indirect selection constitutes the majority of genetic selection s_g and that constraints on expression evolution due to opposing signs of direct and indirect evolution are small.

Gene co-expression networks and selection

The prevalent role of indirect selection underscores the importance of genetic correlations between expression levels and their interdependence. Analysis of gene co-expression networks is a powerful way to assess the importance of correlations among gene responses and identify clusters of co-regulated genes. A weighted gene co-expression network analysis (WGCNA; Langfelder and Horvath 2008) on gene expression levels in HD uncovered 26

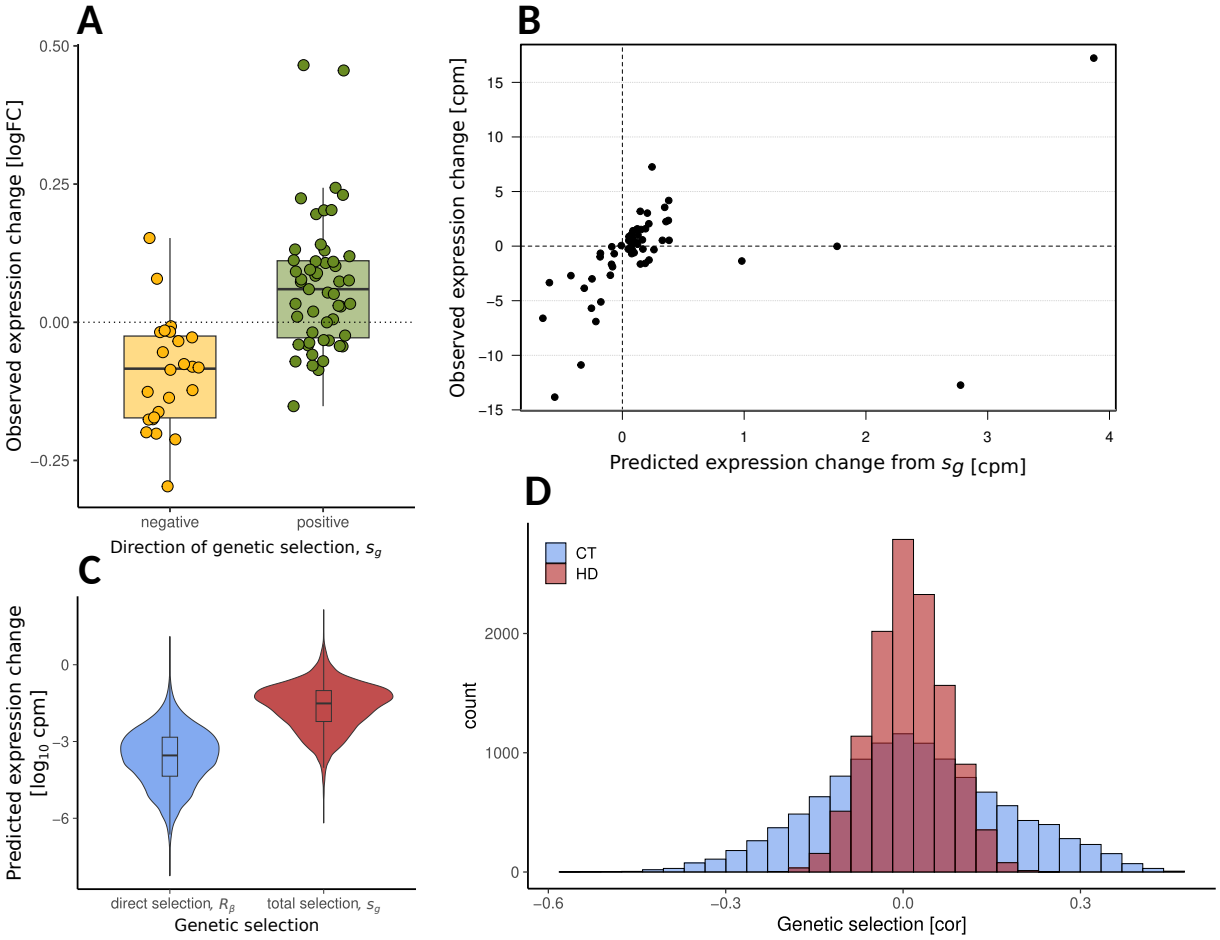

Figure 1: (**A**) Evolutionary response of gene expression ($\log_2$ fold change—logFC) in Hot-Dry conditions of the genes having significant genetic correlation with fitness in the first generation (genetic selection). The evolutionary response is the difference between selection lines after 20 generations of adaptation and non-adapted control lines in Hot-Dry conditions. (**B**) Relationship between predicted responses to genetic selection based on significant estimates of s_g and observed evolutionary changes after 20 generations of selection in Hot-Dry conditions (in counts per million gene expression levels—cpm). (**C**) Comparison of the absolute responses to direct (R_β) and total (s_g) selection in Hot-Dry conditions at the first generation for all 11,890 genes with expression data. (**D**) Distribution of the variance-standardized genetic selection coefficients (genetic correlation r_g) in the Control (CT) and Hot-Dry (HD) environments at generation 1.

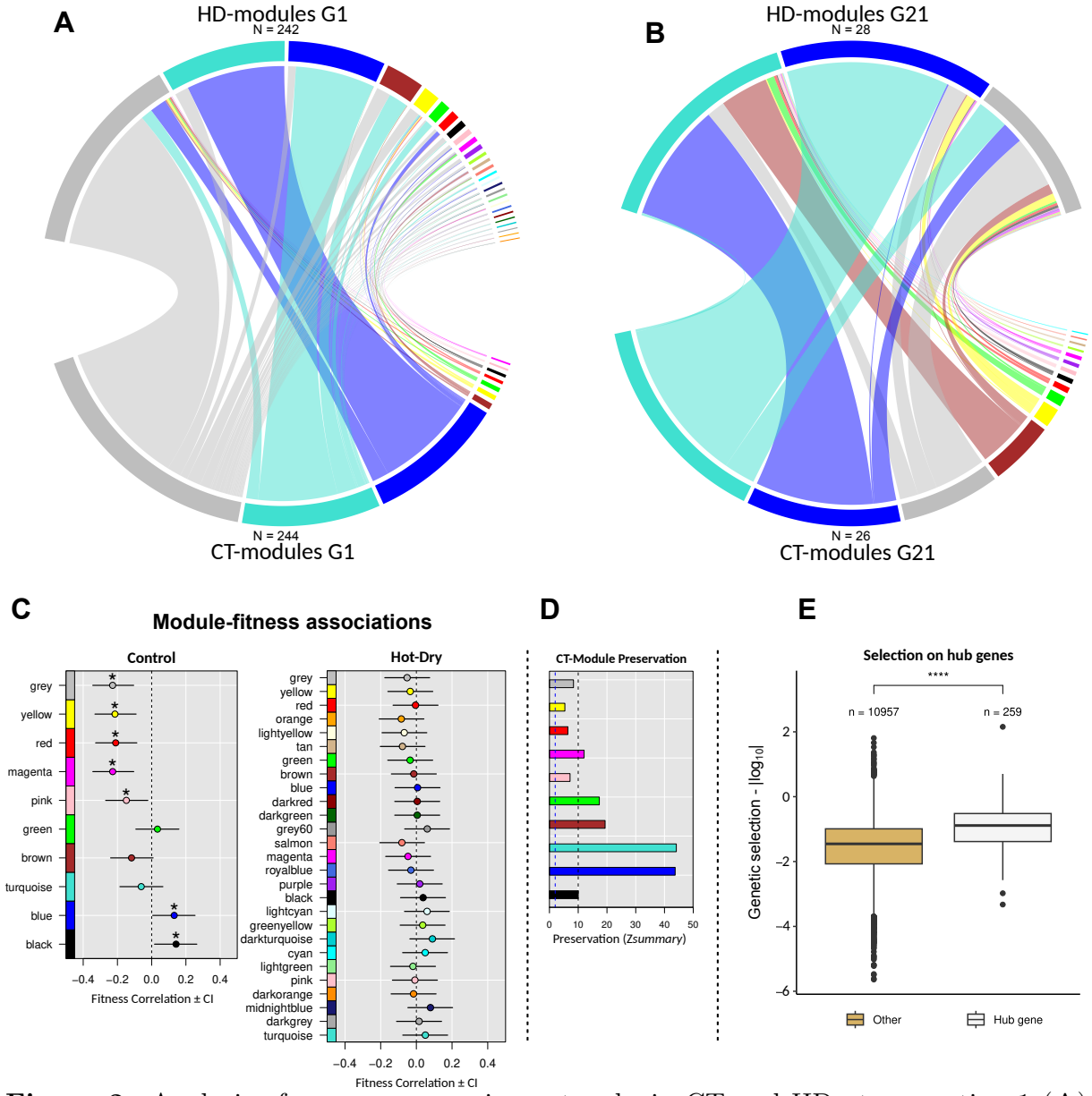

Figure 2: Analysis of gene co-expression networks in CT and HD at generation 1 (**A**) and generation 21. (**B**) The number of sampled sequences for each analysis is shown on the graphs. The significance and number of genes of module overlap are presented in Figures S2 and S3. The gray modules correspond to genes non-assigned to any cluster of co-expressed genes. (**C**) Fitness association of the gene co-expression networks in HD and CT, and (**D**) preservation of CT modules in HD at generation 1. (**E**) Difference in absolute $\log(s_g)$ of the hub versus non-hub genes.

modules of co-expressed genes (Figure 2A) and nine co-expression modules in CT. Overall, CT modules were preserved in HD, *i.e.*, there were no fundamental differences in the way gene expression levels are correlated with each other (Figure 2D). However, it seems that correlations between expression levels became weaker in HD leading to a splitting of the CT modules. We tested each module's fitness association by correlating the module's eigengene (corresponding to the first principal component of each module) with fitness. Six CT modules were significantly associated with fitness variation, but none of the HD modules were (Figure 2C). Additionally, when comparing selection coefficients of single gene expression levels between CT and HD, we observed a decrease in the number of significant coefficients from 729 in CT to 55 in HD, and a narrower distribution in HD relative to CT (Figure 1D), pointing to weaker genetic selection in HD. Therefore, it seems that the stress imposed by the HD conditions caused a weakening of expression-fitness and module-fitness associations, alongside a decrease of mean fitness in HD (from 49.1 in CT to 25.7 surviving offspring per female, see Koch and Guillaume, 2020a).

Reassessing the structure of gene co-expression networks after 20 generations of adaptation, we found that the number of modules in HD was drastically reduced to two modules in the adapted lines while the number of CT modules in the CT lines remained high at 14 (Figure 2B). We thus found the reverse pattern of integration of sub-networks into two large modules at generation 21 in HD. Moreover, the previous network fragmentation caused by exposing CT lines to HD conditions was somewhat recovered at generation 21 (module number increased from 14 to 19 for CT lines transplanted to HD; see Figure S5). Therefore, we can conclude that evolutionary changes which occurred during adaptation to HD conditions have led to a rewiring of gene co-expression networks.

Network position is associated with selection and evolutionary change

Despite a weaker module association with fitness at the gene network level, we found that the 260 hub genes in HD (10 most connected genes within each module, see Material and Methods) were associated with significantly higher absolute genetic selection s_g ($P < 0.0001$, permutation test, Figure 2E). Consistent with this observation, hub genes showed more evolutionary changes in expression compared to non-hub genes; they were over-represented among genes with significant evolutionary changes ($P = 1.1 \times 10^{-3}$, Fisher's

exact test) and had a larger absolute evolutionary change (logFC; $P = 10^{-4}$, permutation test) in generation 21. However, direct selection on hub genes in the first generation was significantly weaker compared to other genes ($P = 10^{-4}$, permutation test).

Selection on gene expression drives parallel allele frequency changes

To better understand the importance of our estimates of selection on expression levels in driving evolutionary changes, we looked for allele frequency changes (AFCs) in the coding sequences of the expressed genes between the first and the last generation in HD, and tested whether replicated lines exhibited parallel AFCs at the same genomic positions. Parallelism of AFCs among replicated lines should provide an indication of the role of selection in enabling evolutionary changes. Consistent AFCs across replicates have been previously interpreted as evidence for the role of selection, while lack thereof would be caused by genetic drift or highly redundant genetic architecture of quantitative traits (Yeaman et al., 2018; Barghi et al., 2019, 2020). Having both AFCs across multiple experimental lines and estimates of selection on gene expression levels, we can test for a relationship between selection on gene sequence variation and gene expression.

To uncover DNA sequence variation (single nucleotide polymorphisms, SNP), we developed bio-informatics pipelines (see Figure S6) to analyze 822 whole transcriptome RNA-seq samples. After quality check, we found 356,193 SNPs in 11,216 genes suitable for the analysis (31.76 SNP per gene on average; Figure S7), uniformly distributed on the genome (Figure S8), and 958 eQTLs (located in 564 genes) significantly associated with the expression level of 556 distinct other genes. All the eQTLs were in *trans*, *i.e.*, no eQTL was locally associated to its carrier gene expression level.

Genome-wide, 55,063 SNPs showed significant AFC in HD, when tested against the AFC distribution in CT (see Methods). We then defined the parallelism of a gene as the largest parallelism of its SNPs. This resulted in 8,906 genes being classified as exhibiting AFC in at least one line. A small set of 179 SNPs shifted in at least five lines and was considered highly parallel. This subset of SNPs belonged to 147 genes. Only one SNP shifted in all seven lines sampled in HD (see Figure S9A). The correlation between $log(s_g)$ and gene parallelism was positive and significant ($\rho = 0.215$, *adj.* $R^2 = 0.046$, p-value $< 2.2e\text{-}16$). Genes binned into different categories of AFC ("no shift"; "single

shift"; "partially or fully parallel shifts", Figure 3A) significantly differed in their mean s_g (all two-sided Mann-Whitney-U tests $P < 1 \times 10^{-4}$), and genes with significant s_g were significantly enriched in highly parallel SNPs (AFC in ≥ 5 lines; $P = 5 \times 10^{-3}$, permutation test on 2,245 SNPs in 75 genes minus five genes not carrying any SNP). These results suggest that selection on gene expression levels may play a key role in driving parallelism of AFCs among replicates.

On the other hand, we did not find enrichment of parallel AFCs in the genes with either largest or significant evolutionary changes in gene expression; we found no enrichment in parallelism among 2,424 SNPs located in the first 100 genes with largest evolutionary response ($P = 0.71$, permutation test), nor among 2,250 SNPs belonging to the 53 genes with a significant evolutionary response (from the 55 genes found in Koch and Guillaume 2020b and carrying at least one SNP; $P = 0.68$, permutation test). This suggests that although selection and parallelism of AFCs appear linked, the SNPs with parallel AFC did not seem to affect the evolution expression of the genes to which they belong.

Stronger signals of selection in hub genes and more pleiotropic eQTL carrier genes

Since in our case most selection seems indirect, we expect to find stronger signals of selection within more connected genes, *i.e.*, within hub genes or genes more pleiotropically connected to other genes. Hub genes and genes carrying eQTLs (*i.e.*, SNPs significantly associated to expression variation at other genes) are obvious candidates to also test for the effects of indirect selection on gene-level AFC parallelism. We found that the 259 hub genes in HD (ten per module; minus one gene not carrying any SNP) were significantly more parallel than the rest of the genome ($P = 1.9 \times 10^{-4}$, permutation test). Indeed, among these 259 hub genes, genes with partial (AFC in two to four lines) or full parallelism (AFC in ≥ 5 lines) were over-represented compared to genes with no significant shift (χ-squared = 21.34, df = 2, p-value = 2.323e-05). Additionally, we found that hub genes were more likely to carry an eQTL than expected by chance (χ-squared = 30.688, df = 1, p-value = 3.031e-08). This strengthens the vision that hub genes may be functionally linked with other expressed genes.

We then focused on the 564 genes carrying at least one eQTL. We found that eQTL

carrier genes were more highly parallel (in ≥ 5 lines; $\chi^2 = 11.974$, df $= 1$, p-value $= 5.396$e-04). Defining the pleiotropy of one eQTL as the number of distinct phenotypes (*i.e.*, gene expression levels) associated to it, we then asked whether more pleiotropic eQTL carrier genes were under stronger genetic selection. Using three pleiotropic categories, "non-eQTL carrier", "non-pleiotropic eQTL carrier" (*i.e.*, a single phenotype association) and "pleiotropic eQTL carrier" (≥ 2, max $= 41$; see Figure S10), we first found that expression of eQTL carrier genes was under significantly stronger genetic selection compared to non-eQTL carriers (Figure 3B; all two-sided Mann-Whitney-U tests $P < 1 \times 10^{-2}$). Second, genetic selection s_g was significantly higher among pleiotropic than non-pleiotropic eQTL carrier genes (Figure 3B), as expected from our previous finding on the predominant role of indirect selection (Figure 1C).

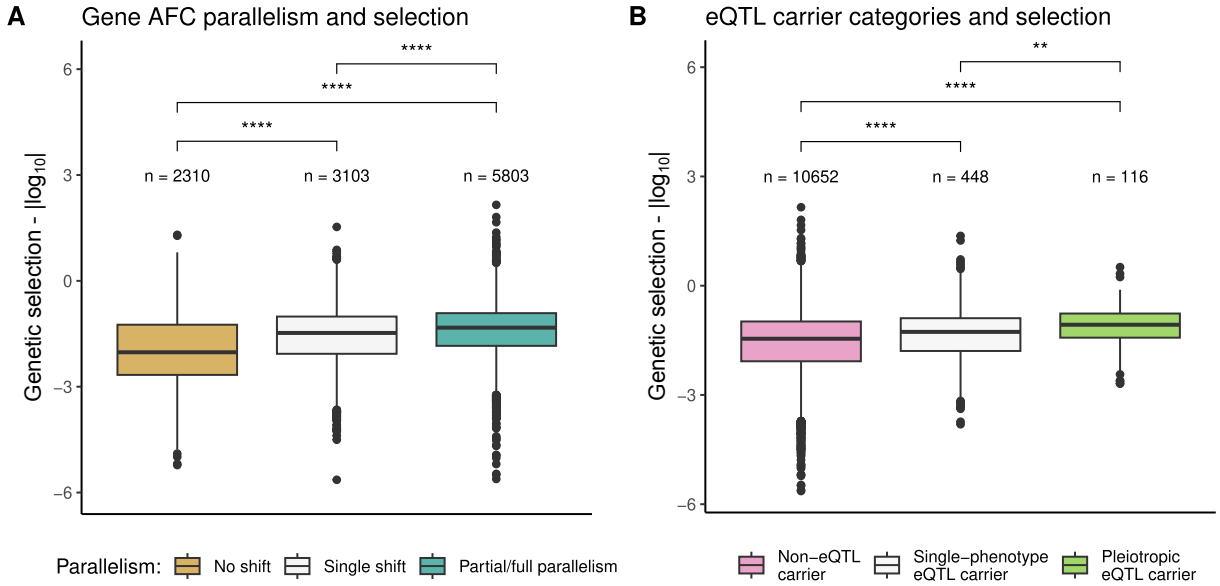

Figure 3: Genome-wide, gene AFC parallelism and eQTL carrier pleiotropy are significantly associated to selection at the gene expression level. For each panel, the distribution of genetic selection coefficients s_g (*i.e.*, covariances with fitness) is plotted by category in absolute log-scale, with all sample sizes and pairwise Mann-Whitney-U tests being shown (****: P < 0.0001, **: P < 0.01). (**A**) Gene parallelism categories: "No shift", "Single shift" and "Partial/Full parallelism". (**B**) eQTL carrier genes categories: "non-eQTL carrier", "non-pleiotropic eQTL carrier" (*i.e.*, a single phenotype association) and "pleiotropic eQTL carrier" (≥ 2, max $= 41$; see Figure S10).

Functional enrichment of eQTL carrier and hub genes

We performed a Gene Ontology (GO) category enrichment analysis to gain insights into the molecular functions of hub and eQTL carrier genes, with a 5% FDR. Apart from

broad functional categories like *cellular processes* or *metabolic processes*, eQTL carrier genes showed mostly enrichment in processes linked to *nuclear division* (GO : 0000280), *development* (GO : 0032502), *reproduction* (GO : 0032502) (*e.g.*, *gamete generation* (GO : 0007276), *regulation of reproductive processes* (GO : 2000241) or *meiotic cell cycle processes* (GO : 1903046)), and *stress response* (GO : 0006950). Enrichment in hub genes showed a similar pattern with *developmental processes* (GO : 0032502), *gamete generation* (GO:0007276) or *sexual reproduction* (GO : 0019953) being among the most significantly enriched categories. This underlines that processes in females are targeted towards increasing the number of offspring. Interestingly, both groups, hubs and eQTL carrier genes, showed one of the strongest enrichment for *epoxide hydrolase activity* (GO : 0004301), which was also the top enrichment category when we tested genes acting as both, hub and eQTL carrier genes. In insects, epoxide hydrolases are mostly known for their involvement in the metabolism of juvenile hormones (Daimon and Shinoda, 2013). Given the profound role of juvenile hormones as regulators of gene expression (Nijhout, 1994) and their involvement in development, reproduction and life-history trade-offs (Flatt et al., 2005), we should expect them to be targets of selection and occupy central positions in gene regulatory networks.

Discussion

We showed that gene expression evolution is predictable and demonstrated how a quantitative genetics approach can provide insights into the nature of selection driving these evolutionary changes. Our results emphasize the predominant role of indirect selection, suggesting that selection is acting on groups of genetically correlated expression phenotypes. Selection-driven evolutionary changes predominantly affect highly connected hub genes within co-expression modules, which may act as central regulators of gene expression in networks of co-expressed genes. Our study illustrates the importance of gene networks and genetic correlations for understanding and predicting gene expression evolution.

Gene expression evolution is driven by indirect selection

We showed that indirect selection is the predominant force driving evolutionary changes in gene expression. The predicted phenotypic response in expression, which integrates the effects of direct and indirect selection, was informative for multi-generation changes as demonstrated by our experimental evolution results. Disentangling the contributions of direct and indirect selection to predicted changes revealed that effects of direct selection were extremely small. This underscores that genes do not act or evolve in isolation. Instead, gene expression evolution can be better understood from a gene expression network perspective: Genes are evolving because they are highly interconnected and thus subject to high amounts of indirect selection stemming from direct selection acting on correlated partners.

Very few studies measured indirect selection acting on gene expression traits within species. Houle and Cheng (2021) showed how genetic correlation among gene expression traits may play an important role in the evolution of sexual dimorphism in expression in *Drosophila melanogaster* and Groen et al. (2020) predicted substantial constraints on responses to selection of expression traits in maize caused by indirect selection pressures of opposite direction. Among species, highly connected genes within regulatory networks were often found to show signs of evolutionary constraints during divergence according to their pattern of genetic co-variation (Fraser et al., 2002; Innocenti and Chenoweth, 2013). In contrast to these studies, our study shows how indirect selection can lead to larger, less constrained evolutionary changes, in most cases (0.7% of genes had a lower indirect than direct predicted response and 25% of genes had direct and indirect responses of opposite sign). More importantly, we could provide a test of the predictability of selection responses over multiple generations, which previous studies could not. More similar evolution experiments should be attempted to better understand the nature of multivariate selection on gene networks during adaptation to a new environment.

Network structure increases evolvability

The importance of genetic covariance among traits is often discussed in the context of evolutionary constraints and evolvability (Blows and Hoffmann, 2005; Walsh and Blows, 2009; Hansen and Pelabon, 2021). The presence of genetic covariance implies that a trait

cannot evolve independently, as selection will also affect correlated traits, potentially preventing responses to certain combinations of selective pressures. However, if selection acting on multiple traits is aligned with the direction of their genetic covariance, it can enhance selection responses and accelerate evolution. In this scenario, a single trait experiences not only the direct effect of selection but also the combined effects of selection acting on all its genetically correlated partners (Lande, 1979; Agrawal and Stinchcombe, 2009). Since gene expression traits are often organized into functional modules, exhibiting little correlation with genes outside their respective groups (Ihmels et al., 2002; Barabási and Oltvai, 2004), they are likely to experience concerted selection pressures if selection acts on the functional properties of the modules. Selection may thus be a driving force behind network structure (Petit et al., 2023). In line with that, several modules in the ancestral control conditions (CT) exhibited significant phenotypic correlations with offspring number, indicating selection acting on whole-module expression and alignment of covariation patterns with the direction of selection. We should thus expect that selection in the past has shaped the genetic covariance between expression levels to be aligned with selection acting on them (Petit et al., 2023; Watson et al., 2014). This alignment is expected from theory showing that correlational selection can orient genetic covariance with the direction of selection leading to stronger responses to selection (Arnold et al., 2008; Chebib and Guillaume, 2021). However, in the hot-dry treatment (HD), the phenotype-fitness correlation dwindled, showing a potentially strong effect of the stress environment on the network structure. Our results suggest that the metabolic and physiological stress responses in the first generation of selection affected gene expression in such a way as to mis-align gene expression modules with the direction of selection. Yet, evolution proceeded from that existing variation and led to observable changes in gene expression and network structure, potentially re-aligning co-expression modules with new selection pressures. Our results thus also support the idea that the topology of gene networks is dynamical (Luscombe et al., 2004) and reflects the topology of direct and indirect selection pressures (Burban et al., 2021; Petit et al., 2023).

Insights into how selection on expression levels induce genomic changes

We found a general association of the AFC parallelism of expressed genes with the intensity of selection on their expression levels but not with their evolutionary changes in gene expression. The genes which evolved most did not show stronger AFC parallelism. Also, all eQTLs were associated with expression variation at other genes, and not with local expression. We thus did not find a within-gene association between sequence variation and constitutive changes in gene expression. However, we showed that gene expression levels did evolve over the span of the experiment. These results surely indicate presence of selection on regulatory variation outside expressed sequences that we do not capture with our RNA-seq approach. It would indicate selection acting on two levels, variation within expressed sequences and variation in transcription regulation. An additional explanation for a lack of change in expression levels despite allele frequency changes could be a change in gene product properties that is expression independent, as for instance a change of enzyme activity or affinity favoured by selection in the new environment (*e.g.*, Brown et al., 2009). Future DNA sequencing should allow us to better understand the importance of regulatory variation as well as selection on allele specific expression levels.

Nevertheless, hub genes, with the highest connectivity, showed the highest amount of indirect selection, and were enriched in evolutionary changes at the sequence and phenotypic levels. They were also enriched in variants associated with expression variation (eQTL). Also, eQTLs with higher pleiotropy, *i.e.*, influencing a larger number of expression phenotypes, were under stronger selection. If anything, this shows how indirect selection induced evolutionary changes and confirms that selection primarily acts through indirect effects by targeting highly connected genes potentially affecting expression levels of many genes in expression modules.

Our main result, with selection and evolution affecting hub genes disproportionately, is in contrast to previous studies. Analyses of within population genetic variation reported purifying selection on highly connected genes (Josephs et al., 2017; Mähler et al., 2017), a negative association between selection strength and connectivity (Groen et al., 2020), and predominantly stabilizing selection on gene expression variation (Josephs et al., 2015; Kita et al., 2017). Selection acting on expression networks is likely to be condition dependent (Gibson, 2008; des Marais et al., 2017) and may differ from stabilizing selection when a

population is adapting to a novel environment, as shown here.

Conclusion

In summary, our study highlights the fundamental influence of indirect selection and genetic covariances on gene expression evolution. Through the integration of gene expression, fitness, and allele frequency data, we gained insights into selection acting on expression and the mechanisms underlying evolutionary changes. Our findings emphasize the importance of considering genetic correlations and network structures for predicting and understanding gene expression evolution.

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Conflicts of interest

The authors declare that there is no conflict of interest.

Data accessibility

All the supplementary data and scripts, including the complete dataset for the genomics analysis, are available on Zenodo at https://doi.org/10.5281/zenodo.11080749. The complete transcriptomics pipeline and analysis scripts related to the genomics analysis are freely available on Github at https://github.com/charlesrocabert/Koch-et-al-Predictability-of-Gene-Expression. Sequence reads of the G1 samples are available on GEO (90 samples) and ENA (396 samples) platforms with accession numbers GSE133570 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE133570) and PRJEB76655 (https://www.ebi.ac.uk/ena/browser/view/PRJEB76655) respectively. Sequence reads of the G21 samples are accessible at GEO, with accession number GSE156256 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE156256). Sample accession numbers and description are provided in a supplementary data table on the Zenodo repository.

Contributions

E.L.K. and F.G. designed research; E.L.K., C.R., C.B.R., and F.G. performed research; E.L.K., C.R., and C.B.R. analyzed data; and E.L.K., C.R., and F.G. wrote the paper.

Material and Methods

We used the *Tribolium castaneum* Cro1 strain (Milutinović et al., 2013), collected from a wild population in 2010 and adapted to lab standard conditions (33°C, 70% relative humidity) for more than 20 generations. Beetles were kept in 24h darkness on organic wheat flour mixed with 10% organic baker's yeast. We sterilized the flour and yeast at 80°C for 24 hours before use. At the start of the experiment (generation G1), we measured fitness in control (=standard) conditions and in a stress treatment with increased temperature (37°C) and decreased humidity (30% r. h.), the hot-dry treatment (HD). To estimate genetic variances in both conditions, we performed a split-brood paternal half-sib breeding design with 147 half-sib families from six replicate lines (see Koch and Guillaume, 2020a; Koch et al., 2020). We created the half-sib families by mating a virgin male to three virgin females at the age of four weeks, in three subsequent rounds of mating per male. This resulted in three full-sib families per male. This nested paternal half-sib-breeding design enabled us to estimate additive genetic variances and co-variances (Falconer and Mackay, 1996). Mating and egg laying of each single female took place during four days in control conditions in 15 mL tubes with 1g of medium. A second round of egg laying was performed for four days to produce a second tube for each female. Each tube was then randomly assigned to either the control (CT) or hot-dry (HD) treatment, resulting in full-sib families (F1) split between CT and HD. Four F1 females and two F1 males per full-sib family and condition were separated at the pupal stage and transferred to 10 mL tubes with 1 g of medium and remained there until they were used for the fitness assay eight weeks later.

Fitness assay in generation 1

We measured fitness as the reproductive output per female by counting the number of surviving adult offspring of 2,937 virgin females (1,518 in Control and 1,419 in Hot-Dry). We mated each virgin female with one unrelated male from the same condition in 15 mL tube with 1 g medium. The male was removed after 24 h. Females were removed from the tubes after one week of egg laying, and 9 g of medium was added to provide food for the developing offspring. Females were flash-frozen in liquid nitrogen and stored at -80°C until sequencing. The number of adult offspring was counted after five weeks.

Experimental evolution

We used seven replicate lines per condition originating from the Cro1 ancestral population and used for the fitness assay in G1. We let each line adapt for 20 non-overlapping generations in CT and HD. Each line consisted of 120 breeding individuals. Each new generation was set by randomly selecting 120 pupae and placing them into a new vial with 80g of fresh medium.

Reciprocal transplant after 20 generations

At generation 21, we performed a common garden reciprocal transplant experiment to assess adaptation by measuring fitness as in G1 (see Koch and Guillaume, 2020b). Adult beetles from all selection lines in CT and HD were transferred to CT conditions, in which they stayed for one generation before creating full-sib families within each line from both CT and HD origins. Each full-sib family was then split again between CT and HD at the egg stage (generation 22). With this design, we could assess fitness differences of full-sib families in both conditions from lines originated from either CT or HD. Fitness was assessed by mating a virgin male and a virgin female of the same selection line, but from different families. After four days of mating and egg laying, males and females were removed from the medium and flash-frozen in liquid nitrogen to be stored at -80°C until sequencing. The number of adult offspring produced within four days was counted after four weeks in CT and five weeks in HD to account for a difference in development time.

Sampling, RNA extraction, library preparation, sequencing and data processing

RNA-seq was performed on 486 female beetles with known fitness at generation 1 (244 from CT and 242 from HD) and on 158 females with known fitness at generation 21 (90 from CT and 68 from HD). Generation 1 samples originated from a half-sib/full-sib cross (see above) with approximately 21 sires, 3 dams/sires and 8 full-sib females/dam, which were separately grown in CT and HD in batch of 4 females per full-sib family. In generation 21, we sampled four females per family per environment from seven full-sib families and lines.

The same extraction and library preparation protocols were followed for each set of

individuals sequenced separately at generation 1 and 21. Single individuals were homogenized in Tri-Reagent® (Zymo Research, California, USA) using an electric bead mill. RNA was extracted with the RNA Mini Prep kit (Zimo Research, California, USA) following the instructions of the manufacturer. RNA-quality was checked with the Bioanalyzer 2100 (Agilent, Waldbronn, Germany) and concentrations were measured with a Qubit® Fluorometer (Life Technologies, California, USA). Libraries were created with 500 ng RNA for each individual separately with the LEXOGEN mRNA-Seq Library Kit following the manual (LEXOGEN GmbH, Vienne, Austria). Library quality was checked on a TapeStation (Agilent, Waldbronn, Germany) and concentrations were determined by qPCR. Libraries were diluted to the same molarity. Concentration of dilutions were checked again by qPCR and libraries were pooled (36 libraries per pool). The treatments were randomized during RNA-extraction, library preparation, and sequencing. The single-end sequencing was performed in five runs on the Illumina NextSeq 500 (Illumina, Inc, California, USA) using the 75 cycles High Output Kit. Each run resulted in 550-600 million reads that passed the internal sequencer filter. After quality control using FastQC (`www.bioinformatics.bbsrc.ac.uk/projects/fastqc`), reads were mapped against the reference genome (`ftp://ftp.ensemblgenomes.org/pub/release30/metazoa/gtf/tribolium_castaneum/Tribolium_castaneum.Tcas3.30.gtf.gz`) with STAR v.2.5 (Dobin et al., 2013) (adapters were trimmed and the first 10 bases were hard trimmed, minimum average quality Q10, minimum tail quality 10, minimum read length 20). We then used FeatureCounts (Liao et al., 2014) to count the number of reads that mapped to each gene in the reference genome. Mapping as well as read counting was performed within the data analysis framework SUSHI (Hatakeyama et al., 2016). We normalized read counts to count-per-million (cpm) with the R package edgeR (Robinson et al., 2010) using TMM normalization. Only genes with a minimum of 1 cpm in at least two samples were kept. We did this for each condition separately resulting in slightly different gene numbers in CT and HD at generation 1 (CT: 11,812 genes, HD: 11,890 genes) and 11,527 genes for generation 21.

RNA-seq single nucleotide polymorphism detection

We used GATK-4.2.3.0 (Van der Auwera and O'Connor, 2020) to detect genomic variants from a total set of 822 whole transcriptome RNA-seq samples, following GATK best practices and previous approaches (Adetunji et al., 2019; Jehl et al., 2021). Individuals raised in different conditions at G21 (see Koch and Guillaume, 2020b) were added to the set of 486 samples to improve genotype imputation (see below). Mapped RNA-sequences (BAM files) were edited to add read groups (samtools 1.14, Danecek et al. 2021), and MAPQ values equal to 255 were re-calibrated to 60 to match GATK format. Duplicated reads were marked (picard Duplicates function, Toolkit 2019), and N-cigar reads were split using GATK SplitNCigarReads function. Variant detection was done for each sample with GATK HaplotypeCaller using the parameters -ERC GVCF –pcr-indel-model NONE –dont-use-soft-clipped-bases. The joint variant call was performed using GATK GenomicsDBImport and GenotypesGVCFs functions with parameter –max-genotype-count 1500. Bi-allelic single nucleotide polymorphisms (SNPs) were then selected using GATK SelectVariants with parameters –select-type-to-include SNP –restrict-alleles-to BIALLELIC –exclude-non-variants. Missing genotypes (DP=0) were labeled as missing ("./.") using bcftools-1.14 (Danecek et al., 2021), and SNP tags were re-calculated using bcftools +fill-tags function. We then removed low-quality SNPs using GATK VariantFiltration function with six filters: QD $<$ 2, FS $>$ 30, SOR $<$ 40, MQ $<$ 40, MQRankSum $<$ -12.5 and ReadPosRankSum $<$ -8.0. SNPs were annotated on the reference genome using snpEff-5.1 software (Cingolani et al. 2012, default interval size of 5kb) resulting in 566,296 quality-checked SNPs (Data S2). Finally, we filtered out SNPs with a call rate lower than 50% using GATK VariantFiltration function, leading to a VCF file containing 358,142 SNPs (see Data S3).

Imputation of missing genotypes

Around 38% of the genotypes are missing in the dataset, compromising non-missing markers' overlap between generations in each line. To impute missing genotypes, we used Beagle-5.4 software (Browning et al., 2021) with the VCF file as a sole input. Imputed markers were then merged with the original VCF file and SNP tags were re-calculated using bcftools +fill-tags function (Data S4). Importantly, we tested Beagle-5.4 accuracy

by building a toy dataset from all SNPs with a call rate of 100% (7,844 SNPs), and by generating missing markers using the same distribution as in the complete VCF file. In all cases, more than 98% of genotypes were accurately imputed (see Appendix S1 and Data S5).

eQTL associations

We used the imputed VCF file to calculate eQTL associations from the half-sib/full-sib cross at generation 1 in HD. SNPs with a minor allele frequency (MAF) lower than 0.05 were filtered out, yielding a set of 141,387 SNPs (see Data S6). After applying a quantile normalization on the expression of each gene (reference: standard normal distribution), genome-wide association analyses (GWAA) were performed on a gene-wise basis to detect eQTLs for each gene expression level as a phenotype by using a linear mixed model implemented in the software GEMMA-0.98.5 (option -lmm; Zhou and Stephens 2012). GEMMA was run with the SNP-based kinship matrix (genomic relatedness matrix) as random factor. The kinship matrix was calculated with GEMMA prior to running the association analysis. As GEMMA does not accept VCF files in input, the VCF file was converted into a .bed file with PLINK2 (Chang et al., 2015). Phenotypes (gene expression data) were further added to a .fam file (PLINK2) containing the pedigree structure of the data. For each GWAA, a GC correction was applied on p-values (Bacanu et al., 2000), and a FDR of 5% was applied to detect and collect significant eQTL associations. We found 958 eQTLs significantly associated to 556 gene expression levels. The same analysis was run by using relative fitness measurements as a phenotype, no significant associations were found. Results are gathered in Data S7.

Allelic frequency changes

We used the imputed VCF file to perform the analysis of allele frequency changes (AFCs) within lines between generations 1 and 21, in CT and HD separately. In CT and HD, four lines (L1, L3, L5, L6) were sequenced at generations 1 and 21. Additional lines were sequenced at G21 only: (L4, Mx1, and Mx3) in CT and (L2, Mx1, and Mx2) in HD. To calculate AFCs, we used allele frequencies at generation 1 when available (L1, L3, L5, L6), or we used a common pool at generation 1 (all available lines merged in a single pseudo-

population). To do so, we split the imputed VCF file into one file per line and generation for each environment, as well as two additional files with all samples at generation 1 for CT and HD. SNP tags were then re-calculated using bcftools +fill-tags function. Allele frequencies were polarized by MAF at generation 1 to match the eQTL analysis. For each line and each SNP, the allelic frequency change (AFC) was then the difference between allele frequencies at generation 21 and 1. The resulting dataset is available in Data S8.

Detection of significant allele frequency changes and parallelism

To detect significant allele frequency shifts in each line of HD environment, we used the distribution of AFCs in CT environment as reference distribution for a non-parametric bootstrap procedure. We tested whether the tail probability of each SNP's AFC within each HD line at G21 was larger than 97.5% based on its confidence interval derived from a sample of 10,000 bootstrapped AFC distributions from the full AFC distribution in CT (*i.e.*, from the pooled lines). The number of AFCs randomly sampled in each bootstrap replicate equated the number of SNPs within each HD line. This allowed us to test for significant AFC in all seven HD lines sequenced in G21. We then defined AFC parallelism as the number of lines in which a SNP was showing a significant AFC. Parallelism thus varies between 0 and 7. The full results are available in Data S9.

Differential gene expression analysis

Evolutionary responses of gene expression after 20 generations of adaptation in HD were measured and analyzed in Koch and Guillaume (2020b). Briefly, we compared gene expression between CT and HD lines in HD condition in the reciprocal transplant at G21 described above. Expression was measured by RNA-seq in 56 individuals from seven replicate CT lines and seven replicate HD lines. Differential gene expression was assessed with normalized and standardized read counts with the R package *limma* (Law et al., 2014; Ritchie et al., 2015). We used the *duplicatecorrelation* function in *limma* to account for non-independence between samples from the same line (*i.e.*, sequencing batch and maternal effects). See Koch and Guillaume (2020b) for more details. This analysis yielded 55 genes with a significant evolutionary response of their transcript abundance in HD at generation 21 after correction for multiple testing (Koch and Guillaume, 2020b). From

the same study, we retrieved the complete set of up- and down-regulated genes in HD lines compared to their counterparts in CT lines in HD.

Quantitative Genetics analyses of selection on gene expression

We estimated genetic selection acting on gene expression from the genetic covariance of the trait with relative fitness $s_g = cov_A(z, w)$ (Robertson, 1966; Price, 1970) in 486 individuals sampled at the first generation of the experiment. We implemented a Bayesian sparse factor analysis of genetic covariance matrices (BSFG) method as described in Runcie and Mukherjee (2013). BSFG uses a linear mixed model and sparse factorization to estimate the additive genetic covariance matrix $\mathbf{G}$ and the residual covariance matrix $\mathbf{R}$ among traits. We utilized BSFG to estimate the genetic covariance between the expression level of each gene and relative fitness and between pairs of genes. To run BSFG, we used the MATLAB (MATLAB version 2014b) code provided at `www2.stat.duke.edu/~sayan/bfgr/Instructions/soft.html`. We included a batch effect (sequencing runs) as fixed factor and mother identity as random factor to account for potential maternal and common environment effects. Since it was not possible to analyze the whole data in one single analysis, we divided the whole data set randomly into ten smaller subsets (each consisting of 1181 genes in CT, 1189 genes in HD). We repeated this subdivision five times, resulting in five subdivisions each consisting of ten different subsets. Relative fitness was included as a trait in all subsets. All of these subsets were analyzed three times (three replicates) with 50,000 iterations of BSFG each. The first 20,000 iterations were discarded as burn-in and the remaining chain thinned for every 30th sample resulting in 1000 posterior samples for each replicate. Convergence was assessed by using Gelman-Rubinstein diagnostics $\hat{R}$ (Gelman and Rubin, 2007; Brooks and Gelman, 1998) for the three replicates. MATLAB output was further analyzed in R (version 3.4.2, R Core Team, 2017). The posterior distribution of $\mathbf{G}$ was calculated from the output of BSFG as described in Runcie and Mukherjee (2013). The genetic correlation of transcript expression with fitness (r_g) was computed as $r_g = \frac{s_g}{\sqrt{V_{A_w} \cdot V_{A_z}}}$, where V_{A_w} and V_{A_z} are the additive genetic variances of relative fitness and of the transcript expression level, respectively. Additive genetic variances correspond to the diagonal elements of $\mathbf{G}$. The off-diagonal elements hold the pairwise genetic covariance between gene expression levels. We further decomposed genetic se-

lection s_g into its direct and indirect selection components following Stinchcombe et al. (2014). Accordingly, we computed the gradient of direct genetic selection on gene expression levels as $\beta_g = \mathbf{G}^{-1}s_g$ from the posterior distributions of $\mathbf{G}$ and s_g. As s_g is the sum of direct and indirect selection gradients, the difference $(s_g - \beta_g)$ indicates how selection acting on correlated genes may affect evolutionary changes in gene expression at a given gene. Constraints can be indicated if the predicted response s_g is opposite to the direct selection acting on a gene expression level. Another sign of constraints can be a smaller total predicted response compared to the direct selection. We compared the direction of s_g and β_g and quantified the proportion of the predicted response that was due to direct selection as β_g/s_g.

We obtained five estimates of s_g and β_g for each gene, one for each subdivision of the dataset. Each estimate and its significance are evaluated from the pooled posterior distributions of the three independent runs of BSFG. The estimate's value is the mode of the joint posterior and significance is reached if the 95% HDI does not overlap with zero. We report the average mode of s_g and β_g over the five subdivisions for genes with at least one significant value in HD. We used the R package *MCMCglmm* (Hadfield, 2010) to compute the posterior modes, highest density intervals (HDI).

Weighted gene co-expression network analysis (WGCNA)

To get more insights into the genetic architecture of transcriptomes, we applied a weighted gene co-expression network analysis (WGCNA) as implemented in the R package *WGCNA* (Langfelder and Horvath, 2008). We were interested to explore how regulatory network structures were modified in stressful conditions and to identify modules that were associated with fitness. Dependent on the pairwise correlations of their expression levels, genes are grouped into modules, *i.e.*, clusters of highly connected genes. Genes within each cluster have highly correlated expression levels but low correlation with genes outside their modules. We constructed networks for CT and HD conditions separately. We then tested for association between the identified modules with fitness by correlating each module's eigengene (its first principal component) with offspring number. To explore how the network structure was impacted by stressful conditions, we tested whether genes in the CT-modules remained connected or whether rewiring took place and new mod-

ules were formed. We calculated the module preservation statistic *Zsummary* using the *modulePreservation* function in WGCNA to evaluate the preservation of CT modules in HD conditions. *Zsummary* is a composite statistic that combines preservation of density as well as connectivity of a module. Values below two indicate no preservation, values between two and ten moderate and above ten strong preservation. For hub gene identification we used the *intramodularConnectivity* function of the WGCNA Rpackage and selected the top ten genes of each model showing the highest intramodular connectivity. We then tested whether these hub genes were enriched in eQTLs, and whether they significantly differed from other genes in selection and evolutionary responses.

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Supporting information

Appendix S1. Testing imputation capabilities of Beagle-5.4 software with the raw VCF file.

Script S1. BSFG analysis.

Script S2. WGCNA analysis.

Data S1. Datasets resulting from the global genomics analysis of the output of the transcriptomics pipeline.

Data S2. VCF file containing the 566,296 quality-checked SNPs.

Data S3. VCF file containing the 358,142 SNPs with a call rate higher or equal to 50%.

Data S4. VCF file containing the imputed genotypes.

Data S5. Results of the imputation tests.

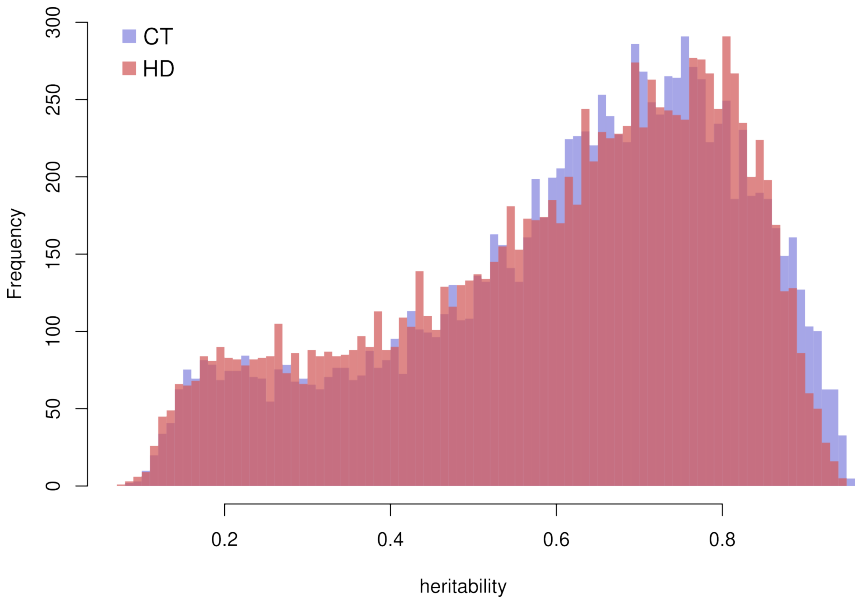
Data S6. VCF file containing imputed genotypes where SNPs with a minor allele frequency lower than 0.05 have been filtered out (MAF $\leq$ 0.05).

Data S7. List of the 1,273 significant eQTL associations (for gene expression levels or relative fitness as phenotypes).

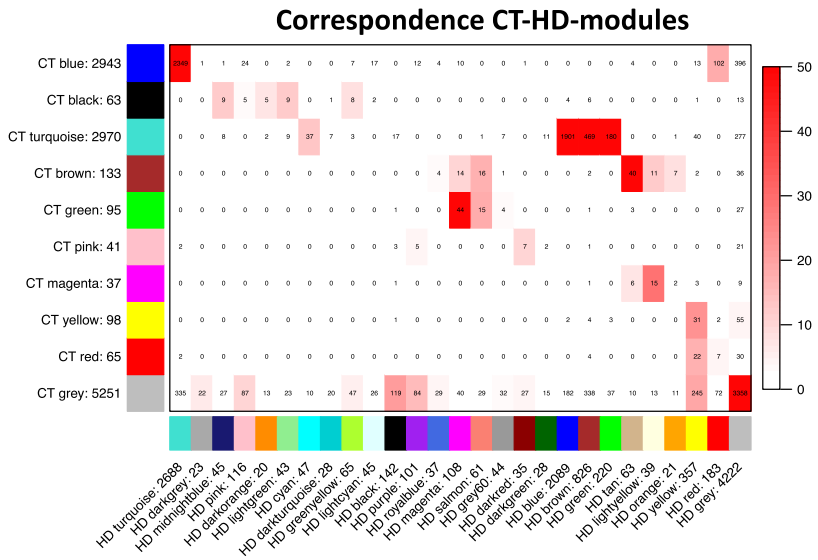
Data S8. List of allele frequency changes (AFCs) for every markers in HD environment, for lines L1, L2, L3, L5, L6, Mx1 and Mx2.

Data S9. List of all SNPs indicating if their AFC is significantly higher in each line and their degree of parallelism.

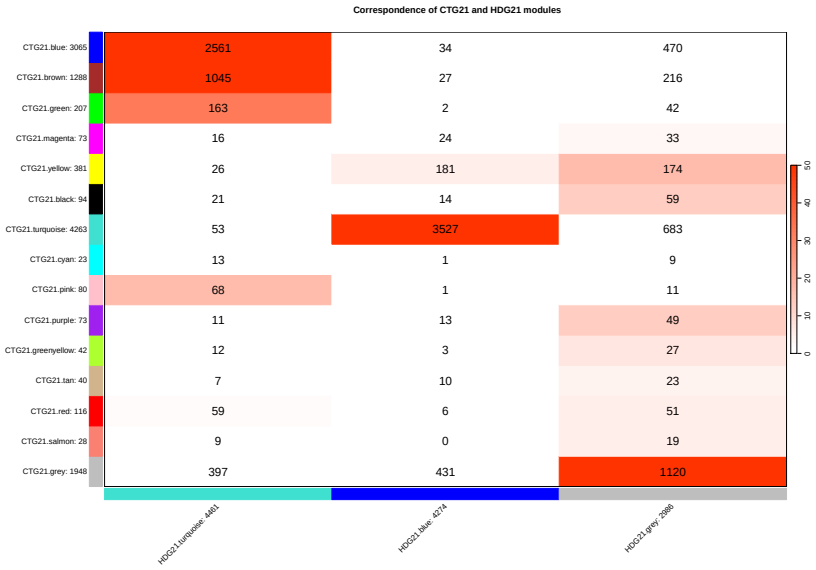
Supplementary figures



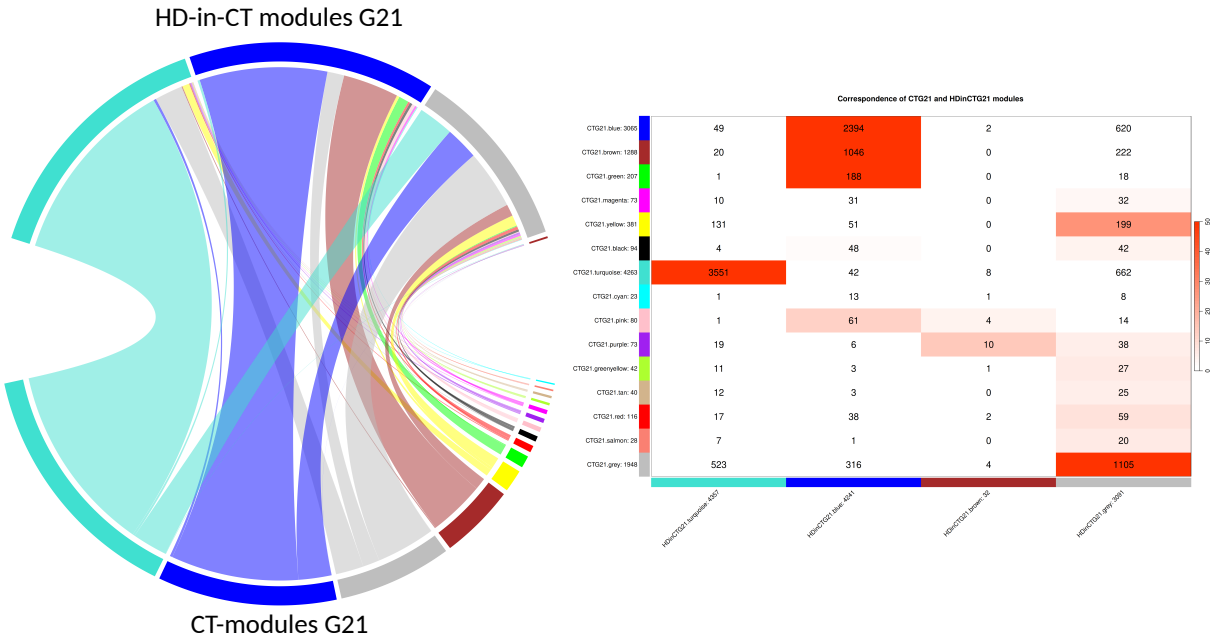
Supplementary Figure 1: Transcriptome-wide distributions of heritability h^2 of expression levels in CT (blue) and HD (red).



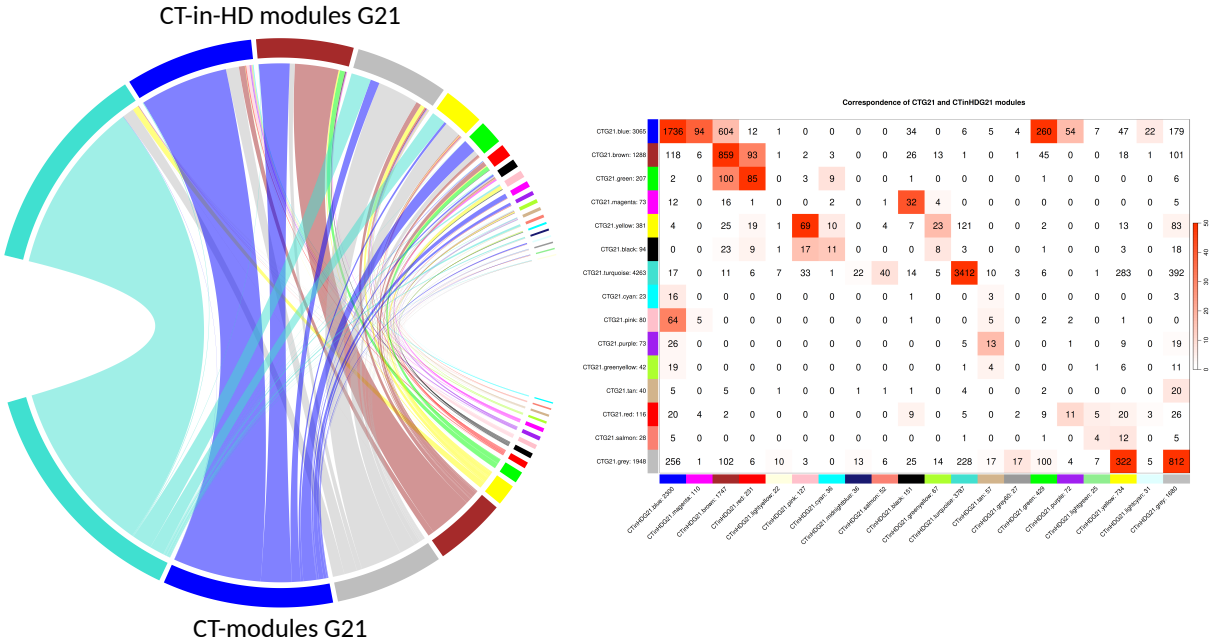
Supplementary Figure 2: Module overlap between gene co-expression networks of CT lines and HD lines at generation 1. The number of genes in each module and their overlap is shown. The intensity of red indicates significance of overlap tested with Fisher's exact test (-log10(p-value)).



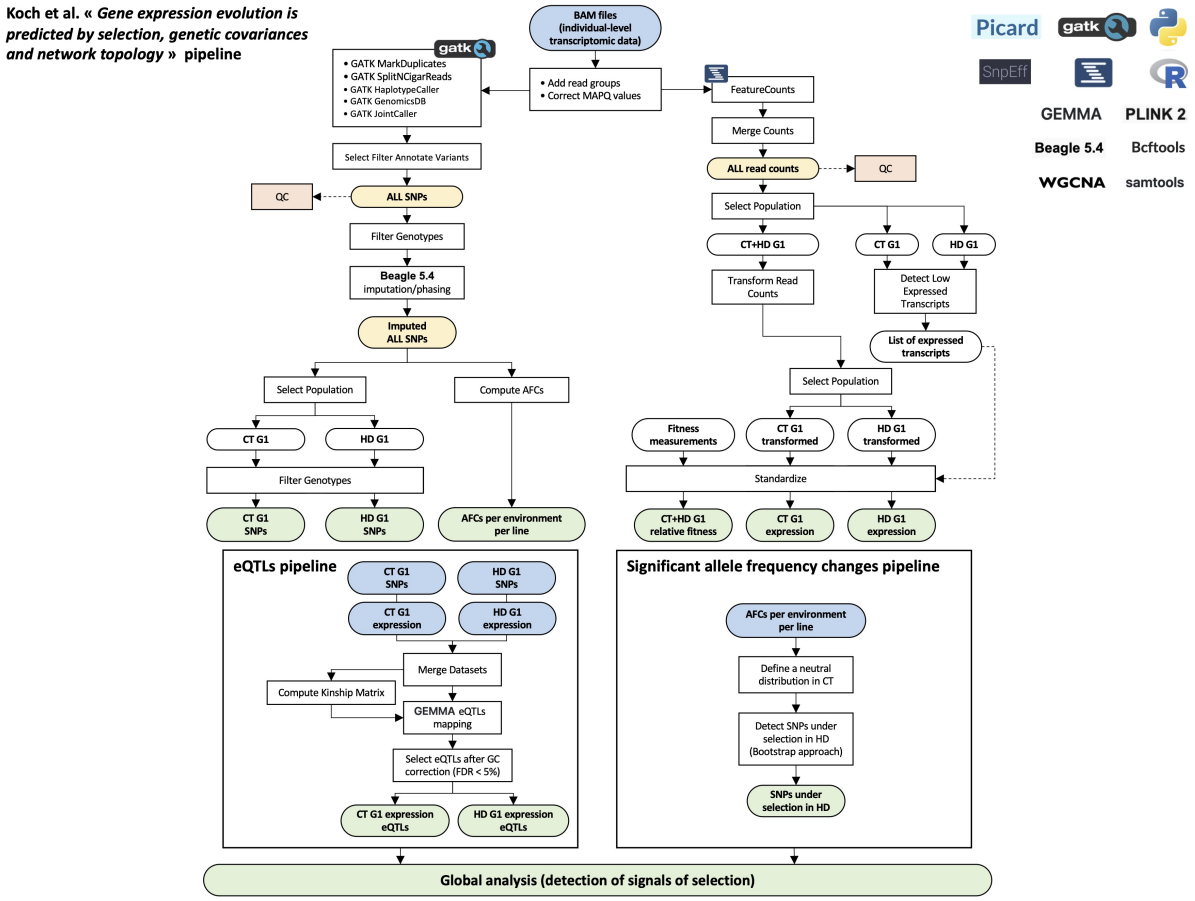
Supplementary Figure 3: Module overlap between gene co-expression networks of CT lines and HD lines at generation 21. The number of genes in each module and their overlap is shown. The intensity of red indicates significance of overlap tested with Fisher's exact test (-log10(p-value)).



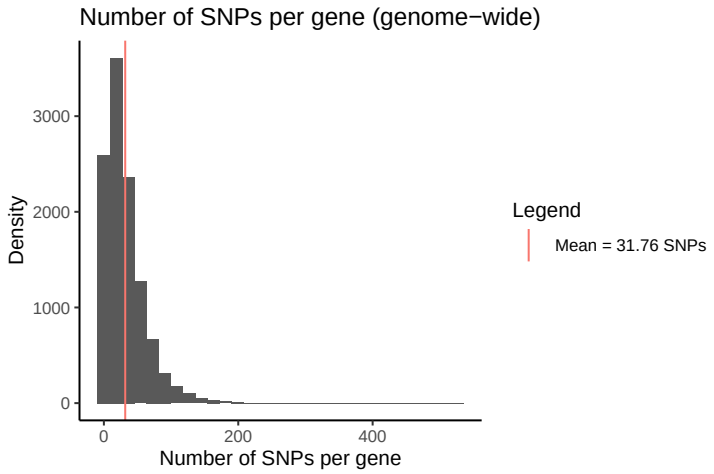
Supplementary Figure 4: Module overlap between gene co-expression networks of CT lines and HD lines who were transplanted in CT at generation 21. The number of genes in each module and their overlap is shown in the table on the right. The intensity of red indicates significance of overlap tested with Fisher's exact test (-log10(p-value)).



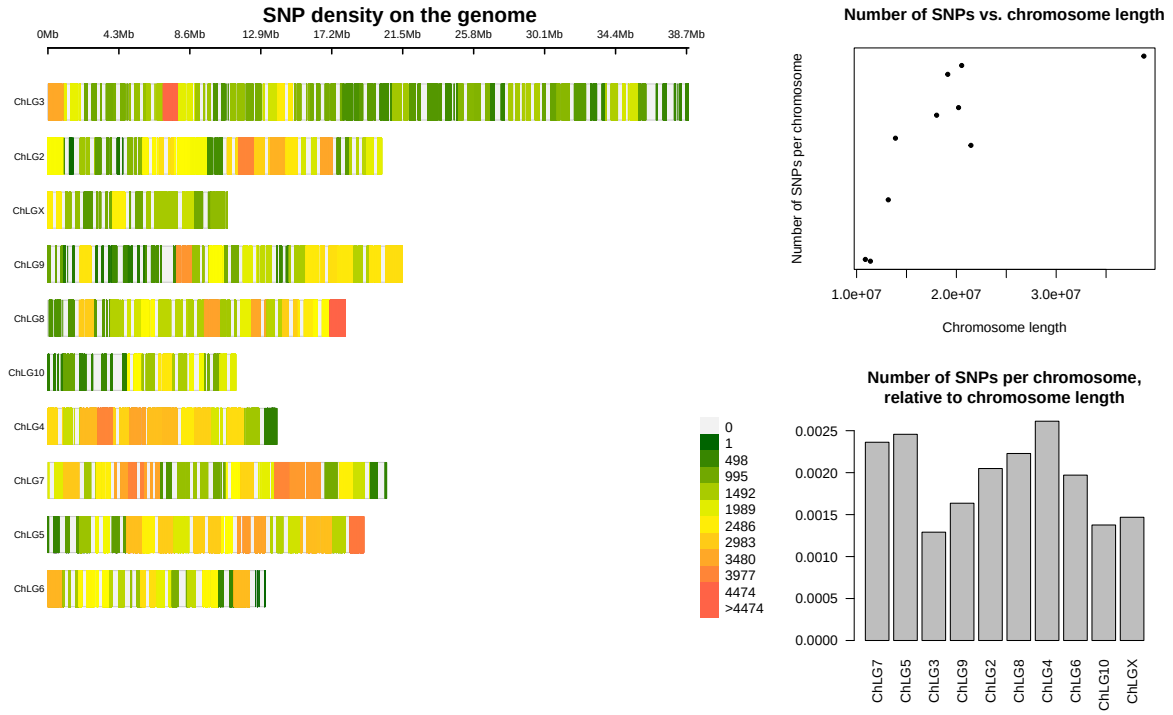
Supplementary Figure 5: Module overlap between gene co-expression networks in CT lines and CT lines who were transplanted in HD at generation 21. The number of genes in each module and their overlap is shown in the table on the right. The intensity of red indicates significance of overlap tested with Fisher's exact test (-log10(p-value)).



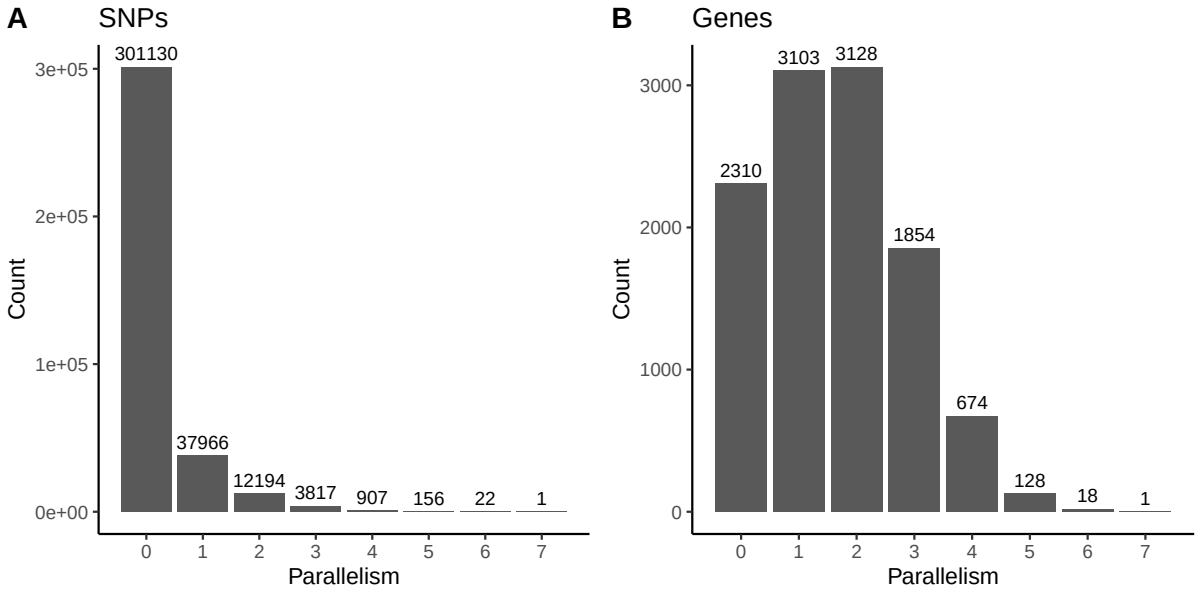
Supplementary Figure 6: Overview of the bioinformatics pipelines. The bioinformatics pipelines used to recover single nucleotide polymorphism, eQTLs and allele frequency changes (AFCs) are summarized in this diagram.



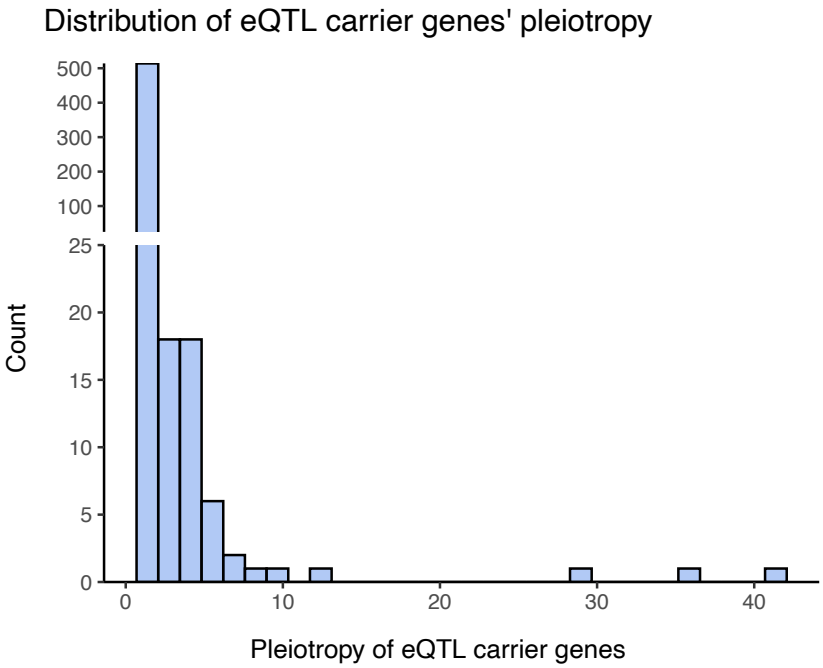
Supplementary Figure 7: Number of SNPs per gene in the genome. Histogram of the number of SNPs per gene on the entire genome. The mean number of SNPs per gene is indicated by a red vertical line (31.76 SNPs).



Supplementary Figure 8: Repartition of SNPs on the genome. Left panel: SNP density along the genome. **Top-right panel:** Number of SNPs per chromosome vs. chromosome length for the 10 chromosomes of *Tribolium castaneum*. **Bottom-right panel**: Number of SNPs per chromosome relative to chromosome length.



Supplementary Figure 9: Parallelism of SNPs and genes frequency shifts. Distribution of the parallelism of frequency shifts, from no shift (0) to fully parallel shifts in the seven lines for (**A**) SNPs, (**B**) genes. The count is shown in each category.



Supplementary Figure 10: Distributions of the pleiotropy of eQTL carrier genes. Distribution of the pleiotropy (*i.e.*, the number of significantly associated phenotypes) for all 564 eQTL carrier genes. Pleiotropy values range from 1 to 41. Vertical axis was re-scaled above $y = 25$ for readability.