An attempt to select non-genetic variation in resistance to starvation and reduced chill coma recovery time in *Drosophila melanogaster*

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Abstract

Phenotypic variance is attributed to genetic and non-genetic factors, and only the former are supposed to be inherited and thus suitable for the action of selection. Although increasing amounts of data suggest that non-genetic variability may be inherited, we have limited empirical data in animals. Here, we performed an artificial selection experiment using *Drosophila melanogaster* inbred lines. We quantified the response to selection for a decrease in chill coma recovery time and an increase in starvation resistance. We observed a weak response to selection in the inbred and outbred lines, with variability across lines. At the end of the selection process, differential expression was detected for some genes associated with epigenetics, the piRNA pathway and canalization functions. As the selection process can disturb the canalization process and increase the phenotypic variance of developmental traits, we also investigated possible effects of the selection process on the number of scutellar bristles, fluctuating asymmetry levels, and fitness estimates. These results suggest that, contrary to what was shown in plants, selection of non-genetic variability is not straightforward in *Drosophila* and appears to be strongly genotype-dependent.

Introduction

Phenotypic variance observed in quantitative traits is classically additively split into genetic (G), environmental (E) and G by E (GXE) interaction components. Among these, only the additive contribution to genetic variance is transmitted to the next generation. Non-genetic inheritance is suggested to explain part of the phenotypic variance that is observed in nature (Salinas et al 2013). Epigenetic marks play an important role in this non-genetic inheritance since DNA methylation patterns or chromatin conformation can be transmitted across generations (Herman & Sultan, 2016). In

addition, it is becoming clear that maternally or paternally transmitted small RNAs can play an important role in the maintenance of gene expression patterns (Watanabe et al. 2011; Conine et al. 2013; Holoch and Moazed 2015). Moreover, microbes may also participate in non-genetic inheritance, often exhibiting vertical transmission and affecting host gene expression (Vastenhouw et al. 2006).

While it is clear that non-genetic inheritance contributes to phenotypes, and despite some attempts, it has remained difficult to quantify this contribution. For example, the part of phenotypic variation that can be explained by changes in DNA methylation was estimated using epiRILs (epigenetic recombinant inbred lines) in *Arabidopsis thaliana* (Johannes et al. 2009; Zhang et al. 2013; Cortijo et al. 2014). The authors showed that a significant percentage of variance can be explained by the epialleles. Using the same epiRILs, the non-genetic heritability of several traits (such as leaf area or flowering time) was estimated to be low but significant (Zhang et al. 2013; Kooke et al. 2015). Epialleles were thus demonstrated to play a part in the evolution of the organisms (Zhang et al. 2013; Kooke et al. 2015). No equivalent experiments have been done in animals, including in *Drosophila*.

With this work, we intend to test the hypothesis that non-genetic inheritance can play a part in the phenotype and that it can be selected. If this is the case, it will provide us with new mechanisms to understand how species adapt to different environments. To test this hypothesis, we performed artificial selection experiments on *Drosophila melanogaster* inbred lines, which harbour low levels of genetic variability. *Drosophila melanogaster* is one of the best studied model organisms in quantitative genetics, and a great number of selection experiments have been performed for a large number of traits (Harshman and Hoffmann, 2000). Here, we selected for a decrease in chill coma recovery time (CCRT) and an increase in starvation resistance (ST). Indeed, chill coma recovery time and starvation resistance are often recorded as displaying high levels of heritability (Ayrinhac et al. 2004; Hoffmann et al. 2005), and the underlying mechanisms are starting to be elucidated (Slocumb et al. 2015; Hardy et al. 2018). If non-genetic inheritance is not transmitted to the next generation, the selection procedure should fail. Because the selection procedure can be considered a stress condition, we estimated developmental

instability using bristle number and fluctuating asymmetry measures, which can indicate a break of the canalization process. We also measured the expression of candidate genes known to be implicated in the stress response, such as *Hsp*, and genes implicated in the epigenetic pathways involved in TE silencing.

As previously shown in other organisms, we found that response to selection was strongly genotype dependent (Groot et al., 2017; Herman & Sultan, 2016). However, and contrary to what has been shown in plants (Cortijo et al. 2014), the extent of the response to selection was weak in our *D. melanogaster* lines. It was previously shown that morphological and fitness alterations can occur through the selection process and remain after selection relaxation in Drosophila (Rutherford and Lindquist 1998; Solars et al. 2003). This is not an obvious result in our experiment. However, we did detect expression changes for some genes following the selection process, such as *thor*, *Hsp27*, and *ago3*.

Materials and methods

Drosophila melanogaster lines

Samples of natural populations of *D. melanogaster* were collected from a single population at Gotheron, France (44º56'0"N / 04º53'30"E) in June 2014 using fruit bait. Thirty isofemale lines were established directly from gravid females from the field. Brother-sister matings were subsequently performed for 30 generations, resulting in 30 inbred lines, which are supposed to harbour very low intra-line genetic variability (Falconer and Mackay 1996). We randomly chose three of these lines to continue with the experiments, denominated 6.6, 10.1 and 15.4. An *outbred* line was built from one virgin pair sampled from each of the 30 isofemale lines. The progeny arising from these flies constituted generation 0. This outcome makes a control line displaying initial genetic variability, which we expect to respond to selection. Indeed, this initial genetic pool is made of a variety of alleles, either

favourable or unfavourable to starvation resistance or chill coma recovery time. The selection process applied on this outbred line allows for the removal of unfavourable alleles, and therefore makes the genetic pool evolve towards the intended direction. Since inbred lines display non-genetic variability, whereas the outbred line displays both genetic and non-genetic variability, response to selection is expected to be stronger in the outbred line compared to the others.

Flies were maintained in the laboratory at 24°C in a standardized culture medium for *Drosophila*.

Chill coma and starvation assays

CCRT: We only considered two- to five-day-old flies, as previous studies found that CCRT depended on age (David et al. 1998). Flies were first sexed on ice within a 5°C chamber, and 50 females were then transferred into empty plaques (one female per well) and placed in chambers containing melting ice. After 16 hours, individuals were promptly removed from cold to room temperature (24°C), and CCRT was measured individually by recording the time until the fly could stand on its legs (Gibert et al. 2001).

ST: Flies were put into starvation vials (1.5% agar medium) with no nutritional value, only allowing the flies to obtain ingestible moisture (Bubliy and Loeschcke 2005; Harshman et al. 1999; MacMillan et al. 2009). Two- to five-day-old females were sampled using an insect aspirator, without anaesthesia. Five tubes were established, each containing 10 females and kept at 25°C with 70% relative humidity. The number of dead flies was recorded three times per day.

Selection experiments

Artificial selection was applied during 10 generations, without relaxation, on samples of 50 females.

For CCRT decrease, a selection pressure of 20% was applied; i.e., the first 10 recovering females were used as the breeders for the next generation. Ten other pairs were randomly selected from a pool of flies to make up the control lines. Each selected line had its own control, maintained in the same conditions (temperature, density and culture medium), except that it was neither subjected to chill nor to starvation, aiming to minimize the effects of microenvironmental variations. Individuals who took longer than 120 minutes to recover from chill coma were excluded from the analysis.

For ST resistance, approximately 50% of surviving females (L50) from the five replicates were used to make the following generation. As selection was performed only on females, at each generation, males were randomly taken from the pool of males in the same numbers as females. After each treatment, flies were placed into vials containing fresh food and survival was measured 24 hours later. All control lines were kept with the same number of flies as the selected lines at each generation. Thus, we ensured that density was a not a variable to be considered in the analyses.

Morphological alterations and fitness estimates

All measures were recorded 10 generations after the end of the selection process, that is at generation 20. Thirty females per line were analysed, for a total of 480 individuals. All visible morphological alterations (deformed body parts) were recorded (Table S1). We also recorded the number of scutellar bristles (SCT), which is considered a strongly canalized trait (Rendel 1959, Sgrò et al. 2010). Indeed, changes in SCT numbers would be reflective of canalization alterations.

Fluctuating Asymmetry (FA) analyses: We counted the number of sternopleural bristles from both the left and right sides of individual flies, and estimated FA as the absolute value of their

difference (Van Valen 1962; Palmer and Strobeck 1986).

Fitness estimates: Twenty two- to four-day-old mated females were placed into four vials (five flies per tube) to lay eggs for 48 hours, and then placed into new vials for 48 hours. Vials were maintained at 24°C in a standardized culture medium. Hatching adults were counted daily for 8 days. We used the total number of adults in the progeny as a proxy for the fitness of the line.

Isolation of RNAs and quantification of transcripts by qPCR

We chose to work on ovaries because it is the female tissue the most closely related to trans-generational transmission. Pools of 70 pairs of ovaries from two- to five-day-old mated females were dissected in PBS 1% and split into three biological replicates. Total RNA was extracted using the RNeasy kit (Qiagen) plus QIAzol Lysis Reagent and subsequently treated with DNase (DNA-free kit; Ambion). One microgram of total RNA was then converted into cDNA using the Superscript III reverse transcriptase (Invitrogen) primed with oligo$(dT)_{20}$.

cDNAs were 50-fold diluted and then quantified using SYBR green quantitative PCR (qPCR) in a LightCycler apparatus (Roche Diagnostics). We chose genes that were shown to be implicated in the canalization process and stress response (*Hsp26*, *Hsp27*, *Hsp68*, *Hsp83*, *technical knockout* (*tko*)), in epigenetic regulation (*argonaute 3* (*ago3*), *piwi*, *zucchini* (*zuc*), *vasa* (*vas*), *thor*, *Methyltransferase 2* (*dnmt2*), *Suppressor of variegation 3-9* (*su(var)3-9*), *modifier of mdg4* (*mod(mdg4)*), *oskar* (*osk*), *Helicase at 25E* (*Hel25E*), and the *412* transposable element (TE). All these genes are expressed in ovaries. We tested three genes for use as normalization genes: *Ribosomal protein L32* (*rp49*), *18S rRNA* (*18S*) and *tubuline* (*tub*). Considering that *rp49* had the lowest coefficient of variation across samples, we normalized the whole dataset by *rp49* levels. Three biological replicates were obtained for each condition, and reactions were performed in triplicate. Standard curves were calculated using serial dilutions of cDNAs. An efficiency value between 1.8 and 2.0 was maintained. For each gene, standard curves were used to convert Ct values into absolute concentrations, which were subsequently divided

by *rp49* absolute concentrations in each sample. Primer sequences are given in supplementary Table S2.

Intra-line nucleotide diversity

To measure intra-line nucleotide diversity, we focused on one intron of the *Adh* gene. This gene is particularly well studied in *Drosophila* for diversity measures (McDonald and Kreitman 1991). Working on the intronic sequence gives us access to neutral diversity. We performed individual DNA extractions on five females per line using the NucleoSpin 96 Tissue Kit (Macherey Nagel, Düren, Germany) following the manufacturer's instructions. We PCR amplified a 453 bp region in the first intron of the *Adh* gene (FBgn0000055). See Table S2 for primer sequences. PCR products were directly sequenced using the Sanger procedure.

Statistical analyses

Estimates of the realized heritability for both traits were computed for each line by regression of the cumulative selection response (as a deviation from the control) on the cumulative selection differential based on the data from generation 0–10 (Falconer and Mackay 1996). Because the resulting selected lines are derived from inbred lines with different genetic backgrounds, we computed heritability estimates for each line separately.

All statistical analyses were performed using R. Data were analysed using mixed models with *line* as a random effect. Models were implemented in R using the lmer function of the lme4 package.

ST: At each generation during the 10 generations of selection, we recorded the survival time of the first three dead flies in each vial. We could not wait until longevity was recorded to choose individuals who gave birth to the next generation, so we decided to make measurements on the same number of flies for each line to limit biases. We assessed the significance of the selection effect by comparing the null model (survival_time ~ (1|line)) to the complete model (survival_time ~ generation

+ (1|line)) (likelihood ratio test, df = 1). Next, we analysed intra-line behaviours using linear models (survival_time ~ generation). The strength of the selection effect was estimated by the corresponding slope.

CCRT: We recorded chill coma recovery time from generation 1 to 10 for the selected lines. Contrary to our expectations, CCRT increased instead of decreasing. Therefore, we started to record CCRT in control lines from generation 6, and, for unknown reasons, we noticed that CCRT also increased in control lines. To test the existence of a response to selection, we only used data obtained from generations 6 to 10, assessing the significance of the treatment by generation interaction by comparing the complete model (recovery_time ~ treatment*generation + (generation|line)) to the model without an interaction term (recovery_time ~ treatment + generation + (generation|line)) (likelihood ratio test, df = 1). A negative interaction term for the selection treatment is expected if recovery time increases less in the selection condition compared to the control. We also analysed intra-line behaviours using linear models (recovery_time ~ treatment*generation). The effect of the treatment by generation interaction corresponds to the difference between the respective slopes for the selection and control treatments.

Gene expression levels: We assessed the significance of the selection effect by comparing the null model (expression ~ (1|line)) to the complete model (expression ~ treatment + (1|line)) (likelihood ratio test, df = 1). The corresponding p-values are provided on Figure 3. We determined per-line global patterns of variation between selection and control conditions using paired Wilcoxon tests on gene results.

FA and fitness: We assessed the significance of a global effect of the selection procedure by comparing the null model <FA or fitness> ~ (1|line) to the complete model <FA or fitness> ~ treatment + (1|line) (likelihood ratio test, df = 1). At the intra-line level, we compared the selection and control treatments using t tests for FA and Wilcoxon tests for fitness estimates, respectively.

Results

Response to selection

We used three inbred lines of *D. melanogaster* and one outbred line to test for selection response in two traits: reduced CCRT and starvation resistance (2,759 and 3,881 flies analysed, respectively). We used the outbred line as a positive control for response to selection since it is known that the traits of interest display a genetic basis (Falconer and Mackay 1996). Since inbred lines display non-genetic variability, whereas the outbred line both displays genetic and non-genetic variability, response to selection is expected to be stronger in the outbred line compared to the others.

Globally, we found weak, although significant, responses to our selection protocols. In the ST assay, survival time significantly increased over time (Chi2 = 15.5, p-value = 8.10^{-5}; generation effect = 0.44). In the CCRT assay, the generation by treatment interaction was significant (Chi2 = 30.5, p-value = 3.10^{-8}), with a negative effect (-2.5) due to selection, indicative of a relative shortening of recovery time along generations. However, in both selection experiments, we noticed a high inter-line variability, which we describe in more detail below.

As expected, the strongest response to selection for starvation resistance was observed for the outbred line (slope = 1.09, p-value = 2.10^{-4}) (Fig. 1, upper panel). Line 10.1 also responded to selection (slope = 0.63, p-value = 0.003). However, no significant response to selection could be detected in lines 6.6 (slope = -0.07, p-value = 0.75) and 15.4 (slope = 0.10, p-value = 0.48).

We observed that chill coma recovery time increased significantly less across generations in the selected lines compared to the control for the outbred line and 10.1 (slope difference = -2.89 (p-value = 1.10^{-6}), and -5.78 (p-value < 10^{-7}), respectively). The effect was not significant for line 15.4 (slope difference = 0.42 (p-value = 0.62)). Missing data in line 6.6 prevented us from comparing slopes regarding the same generations (Fig. 1, bottom panel). We also noticed a high variability of the response across and within generations. Such oscillations are frequently observed in experimental

selection protocols (Falconer and Mackay 1996).

Strikingly, we noticed an absolute decrease of chill coma recovery time only in the selected outbred line. For both traits (ST and CCRT), we noticed that the outbred line displayed a strong response to selection, as expected. Inbred line 10.1 also showed some response to both selection processes. In contrast, line 15.4 did not significantly respond to selection for either of the traits. Line 6.6 was insensitive to ST resistance selection. This illustrates the strong line effect in response to selection in inbred lines.

Realized heritability estimates

Broad heritability values of cumulative realized heritability (ΣR) for CCRT and ST were estimated per line (Table 1).

As expected, the outbred line presented the highest heritability estimates (0.20 for CCRT and 0.16 for ST). The heritability estimates for CCRT and ST were low and consistent with those estimated for most physiological or behavioural traits in outbred populations (Mousseau and Roff 1987; Roff 1997).

Morphological alterations and fitness estimates

We analysed 30 females per line in both selection experiments and recorded the number of flies with visible morphological alterations (including deformed scutellar bristles). We did not detect any increase in the number of aberrant phenotypes in selected lines compared to control lines (Fisher's exact tests; Table S3). We also recorded the number of scutellar bristles and considered as an aberrant phenotype any number different from four. We did not detect any increase in aberrant number of

scutellar bristles following the selection process, except in the case of line 6.6 for CCRT selection (Fisher's exact tests; Table S3).

We could not detect any effect of selection on FA using the complete data set (CCRT assay: Chi2 = 0.99, p-value = 0.319; ST assay: Chi2 = 1.12, p-value = 0.291). However, at the intra-line level, we detected a significant increase in FA of sternopleural bristle numbers for line 6.6 for both selection experiments (t tests; CCRT: p-value = 0.036; ST: p-value = 0.031) (Fig. 2).

Fitness was not altered after the selection experiments (CCRT assay: Chi2 = 1.18, p-value = 0.278; ST assay: Chi2 = 0.02, p-value = 0.890), except for a significant decrease in line 6.6 selected for reduced CCRT (Wilcoxon test, p-value = 0.03) and in line 15.4 selected for ST resistance (Wilcoxon test, p-value = 0.03) (Fig. 2; ST in upper panels and CCRT in bottom panels).

Expression level analysis

We quantified gene expression for a set of genes that could be involved in response to selection, genome stability, or both. Our set included genes associated with stress, epigenetics and the piRNA pathway. These genes were chosen because a previous experiment showed that their expression levels were modified after chill coma and starvation stresses on *D. melanogaster* and *D. simulans* species (unpublished data). Colinet et al. (2010) also showed an increase in the expression *Hsp27* and other *Hsp* genes after a cold shock. We found that the selection process had an effect on the expression levels of some genes: *thor* in the CCRT assay (p-value = 0.025), and, in the ST assay, *thor* (p-value = 0.007), *Hsp27* (p-value = 0.017), *modmdg4* (p-value = 0.029), and *ago3* (p-value = 0.016) (Fig. 3). Additionally, line 6.6 showed a significant global decrease in gene expression levels for cold treatment (Wilcoxon signed rank test, V = 135, p-value < 10^{-4}), while line 10.1 and the outbred line showed an overall upregulation (Wilcoxon signed rank tests, V = 16, p-value = 0.005, and V = 12, p-value = 0.002, respectively).

Intra-line genetic diversity

We sequenced a portion of the first intron of the *Adh* gene to assess genetic diversity within lines. All sequences retrieved from line 6.6 (16 sequences) and line 15.4 (15 sequences) were 100% identical. Among the sequences retrieved from line 10.1 (17 sequences), we found two variants that differed by 5 SNPs and a short indel. Among the sequences retrieved from the outbred line, only 8 were analysable due to a high proportion of heterozygotes, and these matched the same two variants as recorded in line 10.1. Such genetic diversity is expected in the outbred line.

Discussion

Response to selection is moderate in Drosophila inbred lines

In this study, we performed artificial selection experiments on inbred lines of *D. melanogaster* to affect resistance to chill exposure and nutrient restriction. In the absence of genetic variation, we expect selection to be inefficient, unless non-genetic variability can also be inherited. As far as we know, this is the first study of selection on traits performed on inbred lines of insects of a natural origin. Apart from this study, and using *D. melanogaster* transgenic lines, Ciabrelli et al. (2017) recently successfully performed divergent selection for eye colour determined by alternative epialleles, as defined by differential levels of H3K27me3 on a mini-white construct.

We observed responses to selection only in lines for which we cannot exclude genetic variation. This prevents us from concluding that we managed to select non-genetic variation. As expected, the response to selection was more intense in the case of the outbred line compared to any one of the inbred lines. Heritability estimates were low and consistent with those estimated for most physiological or behavioural traits in natural populations (Mousseau and Roff 1987; Scheiner and Callahan 1999; Scheiner et al. 2000; Gerken et al. 2016). Such low estimates were expected in inbred lines.

We established that the selection processes had an effect on gene expression for some genes involved in the stress response, epigenetics and TE control. It has already been shown that direct and

indirect responses to selection can affect several sets of genes with different pleiotropic effects (Mackay 2014). In the present study, we detected upregulation of *modmdg4* and *ago3* in the CCRT assay, downregulation of *thor* in both assays, and downregulation of *Hsp27* in the ST assay.

The response to selection is line dependent

We noticed variability across lines in the intensity of the response to selection, which we propose is partly related to the extent and nature of the epigenetic variability specific to each line. Such line variability was also observed regarding gene expression measures: the selection process induced changes in expression profiles; however, these changes were line dependent, as expected for independent inbred lines (England et al 2003).

In addition, we noticed a large variability of responses across and within generations (Fig. 2). Such oscillations are frequently observed in experimental selection protocols and are particularly observed in populations with low genetic load (Falconer and Mackay 1996), which is probably not the case of the inbred lines. Morgante et al. (2015) showed that a large phenotypic plasticity existed within lines from a natural population of *D. melanogaster*, which they called micro-environmental plasticity. Our data suggest that such within-line variability exists, which could be due to variability in the non-genetic component of phenotypic plasticity, but we cannot exclude residual genetic variability. Indeed, despite a large number of sib mating crosses, inbred lines probably carry residual polymorphism. Ciabrelli et al. (2017) performed deep-sequencing of the genomes of their inbred lines and reported hundreds of thousands of polymorphisms in each line. However, based on sequence analyses, they claim that these differences do not account for the observed phenotypic differences. Here, the data that we have on a limited genetic loci suggest that genetic variability may be somehow higher for line 10.1 compared to lines 6.6 and 15.4. Thus, we cannot exclude the possibility that the larger response to

selection observed in 10.1 compared to the other inbred lines is related to a higher level of residual genetic variability.

Implications for fitness and buffering mechanisms

Several studies have described stress-induced variation through natural and artificial selection (Badyaev 2005), but very few have suggested that artificial selection could be considered a stress (see Belyaev 1979; Belyaev et al. 1985; Trut 1999 for selection on tame behaviour in silver fox). In ongoing work in our group, we observed that selection experiments in *Drosophila* populations presented occurrences of aberrant phenotypes, significant FA index and alterations of canalized traits such as the number of scutellar bristles (B.F. Menezes et al., unpublished data). Therefore, artificial selection may lead to fitness decreases and impairments of buffering mechanisms. We may suspect alteration in the buffering mechanism following the selection protocol only in strains 6.6 and 15.4. In line 6.6, both selection procedures led to an increase in the aberrant numbers of scutellar bristles, an increase in fluctuating asymmetry (FA) levels, and a fitness decrease. The fitness of line 15.4 decreased following ST selection. No morphological alterations were detected for both 10.1 and the outbred line. One possible explanation is that the potential of response to selection, regardless of its mechanism, did not reach its plateau for both traits. Therefore, the environmental perturbations applied did not overcome the buffering mechanisms, while the cryptic genetic variation revealed by strain 6.6 indicates a possible rupture in some canalization processes (Dworkin 2005).

Indeed, we noticed that lines displaying the weakest response to selection were also those that had impaired buffering mechanisms and fitness decreases. This was mostly line 6.6 but also line 15.4 to a more limited extent. In contrast, line 10.1 responded to selection and showed no buffering impairment nor fitness decrease. In addition, line 6.6 displayed an overall decrease in the expression levels of most studied genes following the selection process, while line 10.1 mostly upregulated the studied genes.

Together, all these elements may indicate that line 6.6 is unable to trigger stress response pathways, which results in an inability to respond to selection and deleterious buffering impairments. These data should be considered as possible clues, since we did not measure the expression of genes, namely, stress response genes, after a stress stimulus.

Conclusion

We hypothesize that selection could act upon non-genetic inheritance (e.g., upon an epigenetic methylation pattern or a chromatin structure), which introduces a conceptual similarity between non-genetic and genetic inheritance. Non-genetic variability could arise randomly (e.g., as an epimutation) and subsequently be exposed to selection so that it follows similar dynamics to those of ordinary genetic variants. Many studies address questions about the prevalence of non-genetic effects in natural conditions and their inheritance mechanisms, such as epigenetics (Cubas et al. 1999; Vaughn et al. 2007; Bossdorf et al. 2008; Bossdorf and Zhang 2011; Zhang et al. 2013; Cortijo et al. 2014; Tricker 2015, Heard and Martienssen 2014, Ciabrelli et al. 2017). Contrary to what is known from plants, our results demonstrate that selection of non-genetic variation is not straightforward in *Drosophila.* One inbred line showed a response to starvation selection, but this line appears to have residual standing genetic variation. We observed a line-dependent weak response to selection, accompanied by some changes in gene expression and buffering mechanisms. For example, the expression levels of *ago3*, a gene involved in TE silencing through the piRNA pathway, was affected by the selection process. We speculate that these differences were maintained after the release of selection, which would indicate a transmission across generations. The transmission of epigenetic marks could explain the final phenotype after selection.

We note that, to accurately disentangle genetic from non-genetic compounds on the phenotypes of lines subjected to selection, we must be able to strictly control the genetic variance. As seen with our data, this approach is nearly impossible. In future experiments, a knowledge of the genomic sequences

before and after selection will be fundamental. With this work, we hope to provide some clues on the difficulties of clearly demonstrating the ability to select for non-genetic variability. The current advances in high throughput sequencing should help us to delve further into these questions.

Author contributions

- BFM, CV and MF – conception of ideas and methodology design;
- BFM, JSO, HM, NB and SM - data collection;
- BFM, MF and CV – data analysis;
- BFM, CV, JSO and MF – principal manuscript writers.

All authors contributed critically to the drafts and gave final approval for publication.

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Tables

Table 1: Realized heritability estimates. Σ R is the mean obtained from cumulative selection response. *p-value < 0.05, **p-value < 0.01, ***p-value < 0.001, NS non-significant.

Line	Trait	Σ R ± S.E.	
6.6	CCRT	0.069 ± 0.043	NS
	ST	0.034 ± 0.024	NS
10.1	CCRT	0.092 ± 0.018	**
	ST	0.132 ± 0.045	**
15.4	CCRT	0.079 ± 0.029	*
	ST	0.073 ± 0.009	NS
outbred	CCRT	0.199 ± 0.043	***
	ST	0.157 ± 0.021	***

Figures

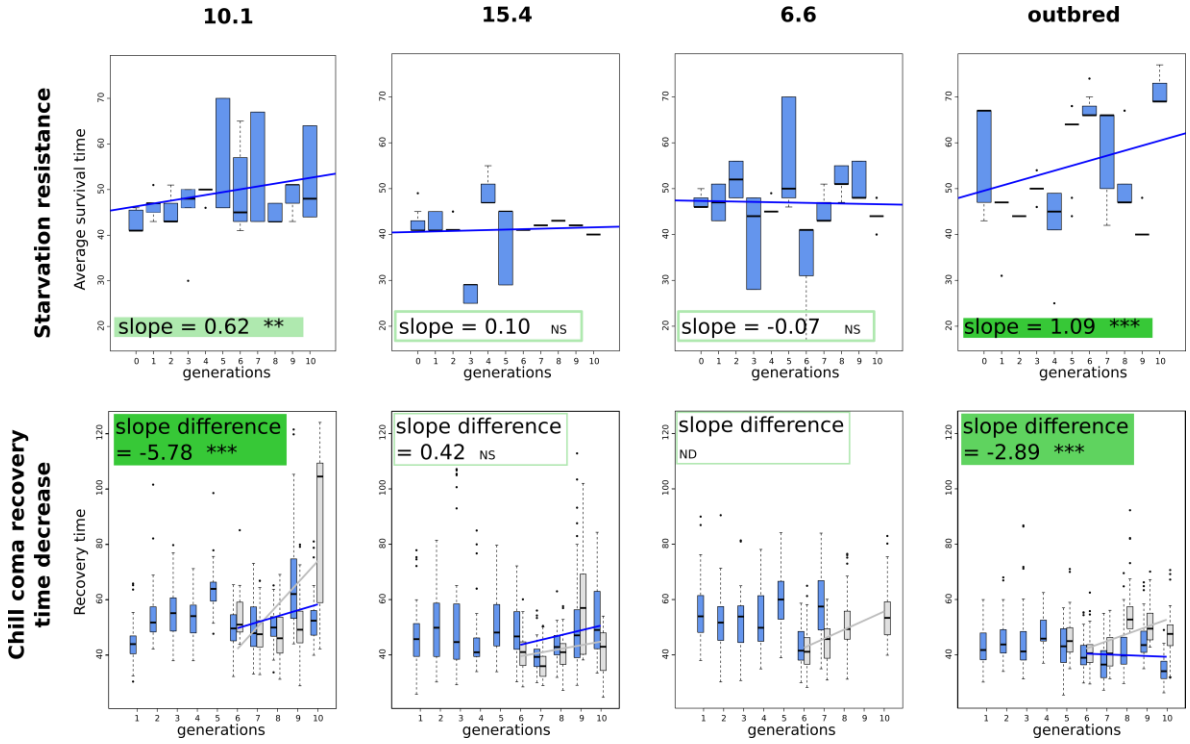

Figure 1: Phenotypic response to selection for ST (upper panel, average survival time in hours) and CCRT (bottom panel, recovery time in minutes). Blue: selected lines; grey: controls.

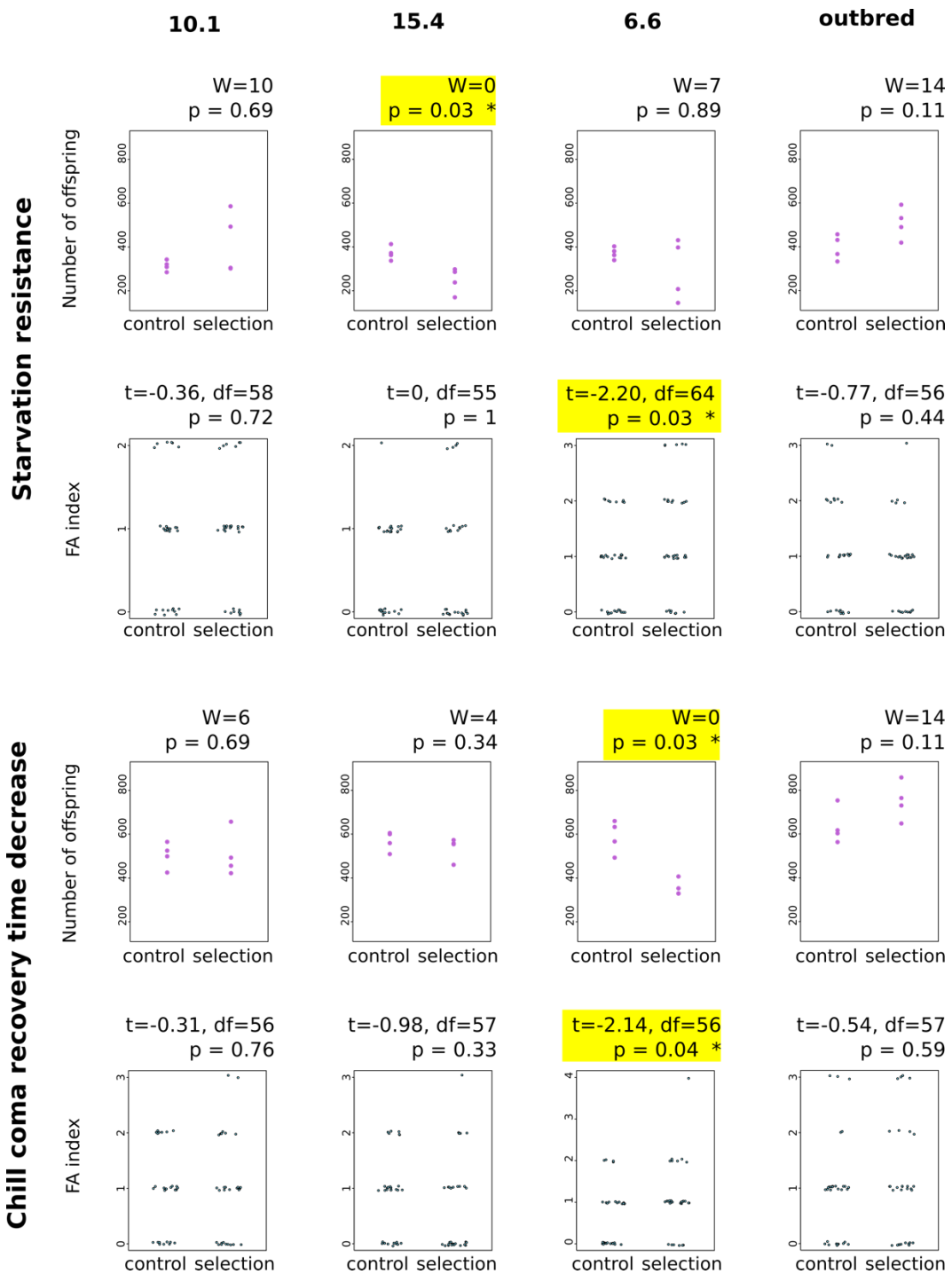

Figure 2: Fitness estimates and FA index for ST (upper panels) and CCRT (bottom panels).

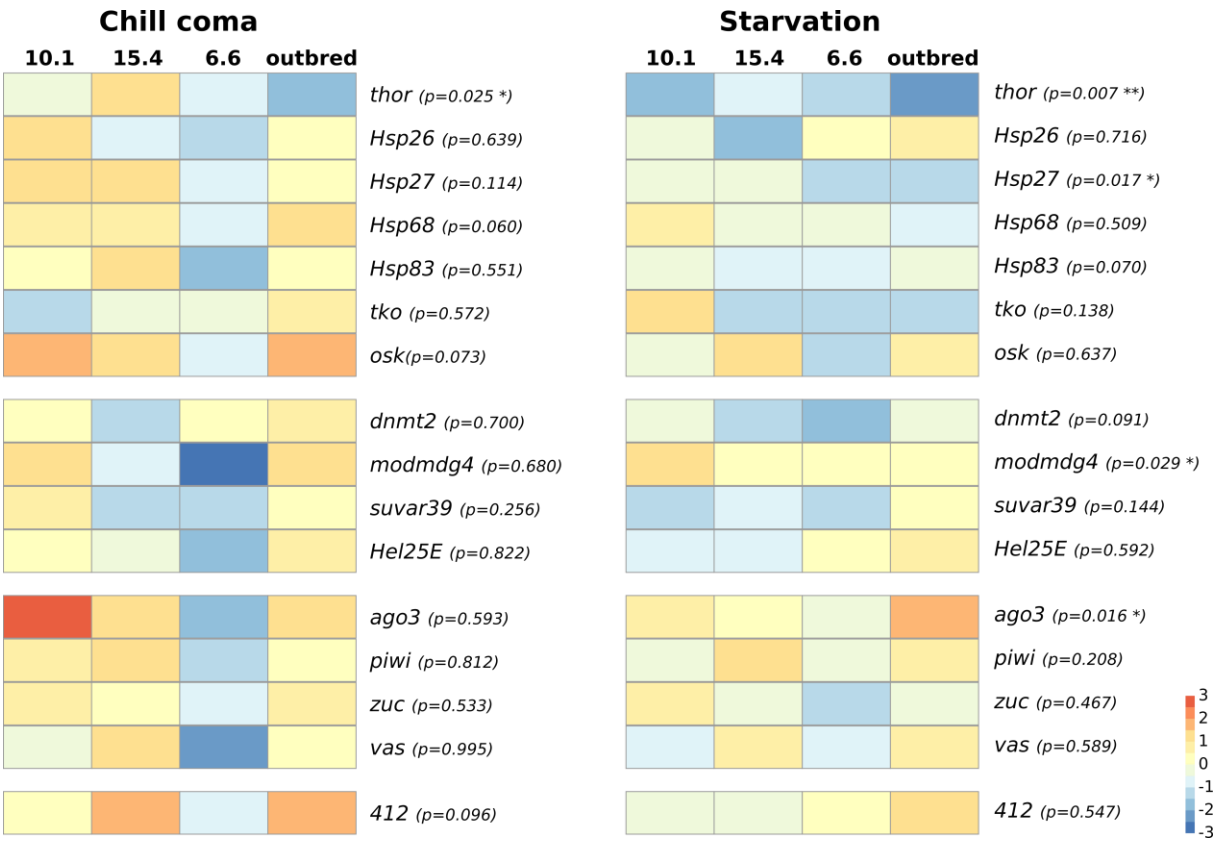

Figure 3: Relative expression levels of selected lines compared to controls (log2-fold changes). Genes were grouped based on their major functions (from top to bottom: response to stress, epigenetics, piRNA pathway and TE, respectively). P-values were obtained from significance tests of the selection effect (see Materials and Methods section).

File S1: Expression levels as estimated by RT-qPCR for all genes analysed for CCRT and ST.

CCRT

line	treatment	replicate	412	ago3	dnmt2	Hel25E	Hsp26	Hsp27	Hsp68	Hsp83	modmdg4	piwi	rp49	suvar39	thor	tko	vas	zuc	osk
10.1	selection	1	2,87E-09	1,93333E-06	7,85E-09	7,90333E-07	1,19667E-05	0,00000403	6,03333E-09	0,00000344	2,92667E-07	0,00000214	1,09667E-05	4,89667E-07	0,00000208	4,96E-08	0,00000182	0,00000209	4,54333E-07
10.1	selection	2	2,25667E-09	9,56667E-07	1,27967E-08	1,24967E-06	3,39667E-06	7,69333E-07	4,32E-09	0,00000203	6,50667E-07	5,66333E-07	6,95667E-06	0,00000135	1,20267E-07	8,71667E-08	7,165E-08	1,14667E-07	3,29333E-07
10.1	selection	3	2,355E-09	0,00000381	9,60333E-09	0,00000826	2,87667E-06	1,22167E-06	3,40667E-09	3,00333E-06	1,94333E-07	0,00000237	1,22667E-05	4,49667E-07	1,90333E-07	7,00333E-08	0,00000115	1,09433E-07	0,00000195
10.1	control	1	2,08333E-09	1,73967E-07	6,99E-09	0,000001715	2,13667E-06	0,00000804	2,93E-09	2,20333E-06	1,455E-07	3,68333E-07	9,84333E-06	6,21333E-07	2,445E-07	1,60333E-07	9,015E-08	1,03667E-07	0,00000194
10.1	control	2	2,2E-09	8,325E-08	7,19E-09	3,39667E-07	0,00000155	4,10333E-07	3,36E-09	3,82333E-06	7,37333E-08	1,13867E-07	0,00001 2	0,00000657	0,00000232	1,99333E-07	2,48333E-07	5,37667E-08	3,24667E-07
10.1	control	3	2,76333E-09	0,00000338	1,263E-08	6,82333E-07	4,74667E-06	1,15733E-06	2,85333E-09	2,14667E-06	3,47667E-07	4,03667E-07	0,00001 12	6,12333E-07	2,02667E-07	1,68667E-07	1,04033E-07	1,40667E-07	0,00000269
15.4	selection	1	1,93667E-08	5,86667E-07	1,79333E-08	1,08767E-06	6,55667E-06	0,00000175	1,61E-08	4,20667E-06	0,00000 1026	6,96667E-07	0,00001 42	6,36667E-07	0,00000251	2,44667E-07	1,65667E-07	2,92333E-07	1,09667E-06
15.4	selection	2	3,92E-08	4,05333E-07	1,04867E-08	0,00000142	0,00000438	1,85667E-06	1,86833E-08	6,00667E-06	0,00000056	0,00000352	1,32667E-05	4,28333E-07	3,97667E-07	0,00000336	0,00000801	1,86667E-07	0,00000843
15.4	selection	3	3,01E-08	0,00000481	1,23933E-08	0,00000167	0,00000796	2,28667E-06	8,64667E-09	4,75333E-06	6,44333E-07	0,00000 04	9,79667E-06	8,89333E-07	2,62667E-07	4,285E-07	4,18333E-07	2,12667E-07	5,42667E-07
15.4	control	1	2,98333E-09	9,78333E-08	1,665E-08	6,54667E-07	3,99667E-06	1,98333E-07	2,9E-09	5,58667E-07	3,28667E-07	0,00000 0076	2,90333E-06	5,80333E-07	3,16333E-07	1,32567E-08	3,945E-08	5,68333E-08	1,14333E-07
15.4	control	2	3,20333E-09	8,13333E-08	1,316E-08	3,91667E-07	0,00000232	2,05333E-07	3,83333E-09	1,01933E-06	0,00000292	4,48667E-08	6,33667E-06	6,10667E-07	8,18667E-08	1,86333E-07	1,28333E-07	5,08667E-08	0,00000125
15.4	control	3	2,06667E-09	2,99667E-08	4,92333E-09	0,00000576	3,14667E-06	2,87333E-07	1,89E-09	3,66333E-07	5,35667E-07	NA	3,64667E-06	0,00000345	3,25667E-08	1,35267E-07	6,67667E-08	4,75333E-08	6,85E-08
6.6	selection	1	1,91667E-08	1,94667E-06	3,66E-08	4,26333E-07	0,00000 71	2,30333E-06	6,89E-09	1,98667E-06	8,68333E-08	0,00000 0444	1,43333E-05	0,00000204	0,00000472	3,13667E-07	1,75667E-07	2,09667E-07	9,37667E-07
6.6	selection	2	4,80667E-09	4,94333E-07	2,32E-08	8,15333E-08	2,05667E-06	0,00000602	1,36667E-09	1,67333E-07	2,11E-08	1,94667E-07	8,46667E-06	1,71233E-07	1,49667E-07	9,36E-08	3,44333E-08	7,19E-08	2,90333E-07
6.6	selection	3	6,8E-09	7,49667E-07	3,51333E-08	2,50333E-07	0,00000287	9,35667E-07	4,04E-09	0,00000 1543	9,38667E-08	4,05667E-07	0,00000 86	3,85667E-07	0,00000258	2,16333E-07	1,02333E-07	1,68667E-07	5,92333E-07
6.6	control	1	8,91667E-09	0,00000284	4,19E-08	5,04333E-07	8,10333E-06	9,50333E-07	4,05667E-09	0,00000 1299	0,00000 0213	0,00000 051	1,01033E-05	0,00000572	0,00000645	1,81667E-07	1,21967E-07	1,46333E-07	6,63667E-07
6.6	control	2	1,44833E-08	0,00000 3235	1,88333E-08	6,43333E-07	0,00000602	0,00000187	7,98E-09	3,19667E-06	2,54333E-07	7,82333E-07	6,73667E-06	6,70667E-07	4,01667E-07	0,00000172	0,00000021	0,00000024	0,00000693
6.6	control	3	1,339E-08	0,00000 2415	2,54E-08	1,41133E-06	5,73667E-06	0,00000186	6,22667E-09	4,54333E-06	8,17333E-07	1,10433E-06	1,21033E-05	8,09333E-07	2,91333E-07	2,45667E-07	9,44667E-07	3,42667E-07	0,00000178
outbred	selection	1	1,48933E-07	1,00767E-06	4,68333E-08	2,40333E-06	7,07667E-06	2,28667E-06	2,35667E-08	6,92667E-06	3,31333E-06	8,90667E-07	1,26333E-05	7,91333E-07	3,05333E-07	0,00000609	0,00000716	0,00000318	0,00000757
outbred	selection	2	2,47333E-08	3,70667E-07	1,88333E-08	5,71667E-07	2,75333E-06	1,03267E-06	8,64667E-09	2,30667E-06	0,00000 0599	0,00000 0391	7,24333E-06	0,00000949	1,43667E-07	9,75333E-08	3,01667E-07	0,00000152	2,40333E-06
outbred	selection	3	3,21E-08	6,91667E-07	1,97667E-08	0,00000224	6,77667E-06	2,71667E-06	1,46933E-08	0,00000538	0,00000126	7,82333E-07	1,31333E-05	1,00567E-06	0,00000181	6,90333E-08	0,00000 1835	3,72333E-07	0,00000164
outbred	control	1	2,23667E-08	2,23333E-07	2,74333E-08	7,085E-07	0,00000 42	1,83667E-06	1,24333E-08	0,00000573	6,67667E-07	3,84667E-07	1,33333E-05	0,00000726	9,71667E-07	0,00000227	7,47667E-07	2,30333E-07	1,31667E-06
outbred	control	2	1,99667E-08	3,03667E-07	1,337E-08	0,00000 1381	4,07333E-06	1,98533E-06	2,95E-09	0,00000 3275	0,00000 0833	7,555E-07	0,00000 903	0,00000 1252	5,895E-07	1,15667E-07	1,04267E-06	0,00000208	9,57333E-07
outbred	control	3	1,15567E-08	3,46333E-07	1,69E-08	6,41667E-07	2,97333E-06	7,75667E-07	5,03333E-09	3,54333E-06	0,00000 0486	2,23333E-07	9,46667E-06	6,785E-07	0,00000 0526	8,15667E-08	0,00000 0322	9,10667E-08	7,33333E-07

ST

line	treatment	replicate	412	ago3	dnmt2	Hel25E	Hsp26	Hsp27	Hsp68	Hsp83	modmdg4	piwi	rp49	suvar39	thor	tko	vas	zuc	osk
10.1	selection	1	3,095E-10	5,885E-07	8,28E-09	0,00000 0488	0,00000 769	4,085E-07	7,905E-09	0,00000 1335	0,00000 1122	3,555E-07	0,00000 462	0,00000 0169	5,755E-08	4,175E-07	1,8685E-07	0,00000 0165	4,565E-07
10.1	selection	2	6,465E-10	4,045E-07	1,0125E-08	0,00000 0589	0,00000 51	0,00000 105	8,38E-09	0,00000 4855	1,0225E-06	2,835E-07	3,16217E-06	1,615E-07	6,245E-08	0,00000 0462	3,575E-07	0,00000 0289	0,00000 1008
15.4	selection	1	1,04E-08	0,00000 0776	8,445E-09	9,385E-07	0,00000501	9,565E-07	1,3625E-08	0,00000588	0,00000 1385	0,00000 0599	0,00000749	0,00000 0431	1,7105E-07	2,105E-07	3,995E-07	3,285E-07	0,00000 1235
15.4	selection	2	0,00000 0018	7,035E-07	8,525E-09	0,00000124	0,00000 3335	8,895E-07	1,3885E-08	0,00000414	0,00000 1553	1,1565E-06	0,00000 6085	NA	9,895E-08	0,00000 0161	0,00000 2395	0,00000 0238	0,00000 333
6.6	selection	1	1,79E-09	0,00000 0256	2,535E-08	0,00000 0863	0,00001 077	0,00000 0855	6,78E-09	0,00000385	0,00000 0976	0,00000 033	0,00000 7285	0,00000 0164	1,0335E-07	3,075E-07	2,085E-07	0,00000 0145	0,00000 0593
6.6	selection	2	1,75E-09	4,295E-07	6,04E-09	0,00000 0504	0,00000 441	5,245E-07	5,285E-09	0,00000244	0,00000 0793	0,00000 0337	0,00000 2905	2,4455E-07	8,76E-08	0,00000 0198	8,79E-08	0,00000 0197	0,00000 0444
outbred	selection	1	9,685E-09	0,00000 1435	1,92E-08	7,995E-07	0,00001 3965	0,00000 0958	4,69E-09	0,00000 51	0,00000 194	0,00000 0807	0,00000 528	0,00000 1045	5,83E-08	2,985E-07	6,695E-07	3,015E-07	0,00000 1135
outbred	selection	2	1,86E-08	1,0735E-06	1,945E-08	0,00000 17	0,00002 335	8,855E-07	1,531E-08	0,00000405	0,00000 1147	0,00000 0338	0,00000401	0,00000 0605	1,0245E-07	0,00000 0166	1,2185E-06	0,00000 0255	0,00000 1135
10.1	control	1	3,48E-10	0,00000 032	2,445E-08	5,685E-07	0,00000777	0,00000112	5,63E-09	0,00000336	0,00000 0232	3,62333E-07	0,00000456	0,00000 0263	2,04333E-07	2,08667E-07	9,01667E-08	0,00000 0164	9,59667E-07
10.1	control	2	1,0115E-09	0,00000 0306	1,0225E-08	0,00000 1065	0,00000797	1,38333E-06	6,48E-09	7,25667E-06	0,00000 078	4,73667E-07	0,00000336	5,36667E-07	0,00000 0308	3,13667E-07	0,00000 1085	0,00000 0167	1,37133E-06
10.1	control	3	6,11333E-10	2,78667E-07	4,86E-09	1,25333E-06	0,00000834	7,45333E-07	5,16667E-09	6,11667E-06	7,12333E-07	4,42333E-07	0,00000687	9,53667E-07	0,00000 0157	2,59667E-07	2,59333E-07	2,07333E-07	5,50333E-07
15.4	control	1	2,76667E-08	9,16667E-07	1,41E-08	0,00000302	1,23333E-05	1,11333E-06	1,53E-08	0,00001036	0,00000 08	6,12333E-07	1,16233E-05	0,00000573	2,07333E-07	4,05333E-07	0,00000 0679	0,00000346	1,03733E-06
15.4	control	2	1,307E-08	3,53667E-07	1,47667E-08	1,21933E-06	0,00000872	9,76667E-07	1,66333E-08	0,00000393	9,04333E-07	2,55333E-07	3,88333E-06	0,00000691	1,241E-07	0,00000 0377	6,29333E-07	2,19333E-07	5,22667E-07
15.4	control	3	1,36333E-08	0,00000313	2,14933E-08	1,38133E-06	0,00000769	8,53667E-07	1,02367E-08	5,63333E-06	0,00000 0869	2,005E-07	0,00000404	3,57667E-07	0,00000242	3,41333E-07	5,72667E-07	1,97333E-07	0,00000805
6.6	control	1	6,86667E-10	0,00000023	2,54667E-08	4,22333E-07	0,00000 2645	0,00000123	4,69333E-09	0,00000311	0,00000 035	3,36667E-07	1,87333E-06	4,61333E-07	1,83333E-07	3,44667E-07	0,00000 0153	0,00000 03	1,0185E-06
6.6	control	2	2,25667E-09	8,41333E-07	4,37E-08	5,94333E-07	7,89333E-06	2,03333E-06	8,70333E-09	0,00000523	0,00000 0775	5,35333E-07	0,00000649	6,22667E-07	0,00000213	4,77667E-07	0,00000 0399	4,74667E-07	9,92333E-07
6.6	control	3	1,83333E-09	0,00000611	3,40333E-08	4,08333E-07	7,03667E-06	1,95333E-06	9,17E-09	0,00000 54	1,04833E-06	4,74333E-07	0,00000 82	8,32667E-07	1,64667E-07	7,21667E-07	9,775E-08	0,00000 0336	0,00000 1173
outbred	control	1	6,25333E-09	2,76667E-07	2,36333E-08	1,17867E-06	1,19467E-05	0,00000184	1,12233E-08	0,00000691	1,15667E-06	2,47667E-07	5,39333E-06	3,735E-07	1,49767E-07	0,00000 0405	8,84333E-07	0,00000 022	0,00000 0622
outbred	control	2	6,1E-09	0,00000048	2,06E-08	7,23667E-07	1,23267E-05	0,00000176	1,26667E-08	0,00000331	1,18067E-06	2,695E-07	2,98333E-06	7,72667E-07	3,40333E-07	4,66333E-07	5,07667E-07	0,00000 0313	4,36333E-07
outbred	control	3	5,97667E-09	0,00000 0197	7,025E-09	5,11333E-07	0,00000678	2,47667E-06	1,72333E-08	5,51333E-06	6,225E-07	2,20333E-07	0,00000396	5,215E-07	0,00000 0314	4,78667E-07	4,02333E-07	3,24667E-07	8,61667E-07

Table S1: Morphological alterations scored at generation 20. Aberrant phenotypes labelled as "+" were the most representative; aberrant phenotypes labelled as "1" were observed just once.

Aberrant phenotypes		
Phenocopies	**Body deformations**	**Additional traits**
[+]*Taxi*-like	[+]Smashed wings	[1]Double veins
[+]*Ebony*-like	Thick veins and wing blisters	Wing spots
Vestigial-like	Crooked body (head, thorax, legs)	[+]Body spots
Apterous-like	[+]Malformed tergites	Body "warts"
Curly-like	[+]Scutellar bristles	

Table S2: primer sequences used for PCR and qPCR.

Genes / TEs	*Forward*	*Reverse*
rp49	CGG ATC GAT ATG CTA AGC TGT	GCG CTT GTT CGA TCC GTA
piwi	GG ACA GCA GAA CAT CGT GTT GTT TC	GAC CCA TTC CAC TAG CTG C
ago3	GCA AAC TCC CCA CAA ATA TT	GGC AAT GGG TTT TCT AGA TG
su(var)3-9	AGG AAC TTG CAG AAG CAG GA	CTC ACA TGT CGC ACC AGT CT
Hsp26	GTG GAC GAC TCC ATC TTG GT	TCC TTG GAC TTG TCC TCG AC
Hsp27	AAA GAT GGC TTC CAG GTG TG	CCC TTG GGC AGG GTA TAC TT
Hsp68	GGC ACT CAA GGA CGC TAA AAT G	CTG AAC CTT GGG AAT ACG AGT G
Hsp83	ATG CCA GAA GAA GCA GAG ACC	ACT CAT AGC GGA TCT TGT CCA G
tko	CAA CCT GCA AGA GCA CAA CA	AGC AAT GCA TTT GAA CAA CG
zuc	TTT GGC GGA TTC AAT AAA GC	TTG TGC ATC AAG TTC GTG GT
dnmt2	TAC ACC CAT TAC ACC GAG GG	CTT CCC GTG GCG TGA AAT AG
vas	TGT CTG ACG ACT GGG ATG ATG	ATT TCC TCC TTG GTA GCC GC
thor	CAG ATG CCC GAG GTG TAC TC	CAT GAA AGC CCG CTC GTA GA
mod(mdg4)	CCG CAA GAT GTT CAC TCA GA	GAT TCC GCG GTG CTA ATA AA
Hel25E	GAT GAG GAG CAG ACC GAG AC	ACT TGG CCT GAC AGA GGA TG
412	TTG ATG GGC AAA AGA TCC AT	TTG CTG GAA TTG TCG TTT CA
oskar	CGA CAA CGT GAC GGA TTT CCT	GGA GGT GAC CGT TCT TCA GG
adh	AGC AAA AGG GCA CAC AAT TGA	CGG CAC ACA CGG TTT GTT T

Table S3: Aberrant phenotypes (A) and scutellar bristle numbers (B). Aberrant: morphological alterations and phenocopies different from wild-type such as phenotype (A); scutellar bristle number different from four (B).

Aberrant phenotypes							
A) **Line**		**CCRT**			**ST**		
		aberrant	Non aberrant	p-value	aberrant	Non aberrant	p-value
10.1	Selected	3	27	0.61	1	29	1
	Control	1	29		0	30	
15.4	Selected	1	29	1	0	30	0.49
	Control	0	30		2	28	
6.6	Selected	5	25	1	1	34	1
	Control	4	26		2	33	
outbred	Selected	9	21	0.10	2	28	1
	Control	3	27		1	29	
Scutellar bristle number							
B) **Line**		**CCRT**			**ST**		
		aberrant	Non aberrant	p-value	aberrant	Non aberrant	p-value
10.1	Selected	0	30	0.24	3	27	1
	Control	3	27		2	28	
15.4	Selected	1	29	1	0	30	0.49
	Control	0	30		2	28	
6.6	Selected	19	11	0.004	10	25	0.61
	Control	7	23		13	22	
outbred	Selected	3	27	0.10*	8	22	0.18
	Control	9	21		3	27	