JOURNAL OF Evolutionary Biology

doi: 10.1111/jeb.12844

Behavioural response to combined insecticide and temperature stress in natural populations of *Drosophila melanogaster*

A. FOURNIER-LEVEL*, A. NEUMANN-MONDLAK*, R. T. GOOD*, L. M. GREEN*, J. M. SCHMIDT*† & C. ROBIN*

*School of BioSciences, The University of Melbourne, Parkville, Vic., Australia
†Max Planck Institute for Evolutionary Anthropology, Leipzig, Germany

Keywords:

activity monitoring;
Cyp6g1;
DDT;
genetic variation;
G-matrix;
self-organizing map.

Abstract

Insecticide resistance evolves extremely rapidly, providing an illuminating model for the study of adaptation. With climate change reshaping species distribution, pest and disease vector control needs rethinking to include the effects of environmental variation and insect stress physiology. Here, we assessed how both long-term adaptation of populations to temperature and immediate temperature variation affect the genetic architecture of DDT insecticide response in *Drosophila melanogaster*. Mortality assays and behavioural assays based on continuous activity monitoring were used to assess the interaction between DDT and temperature on three field-derived populations from climate extremes (Raleigh for warm temperate, Tasmania for cold oceanic and Queensland for hot tropical). The Raleigh population showed the highest mortality to DDT, whereas the Queensland population, epicentre for derived alleles of the resistance gene *Cyp6g1*, showed the lowest. Interaction between insecticide and temperature strongly affected mortality, particularly for the Tasmanian population. Activity profiles analysed using self-organizing maps show that the insecticide promoted an early response, whereas elevated temperature promoted a later response. These distinctive early or later activity phases revealed similar responses to temperature and DDT dose alone but with more or less genetic variance depending on the population. This change in genetic variance among populations suggests that selection particularly depleted genetic variance for DDT response in the Queensland population. Finally, despite similar (co)variation between traits in benign conditions, the genetic responses across population differed under stressful conditions. This showed how stress-responsive genetic variation only reveals itself in specific conditions and thereby escapes potential trade-offs in benign environments.

Introduction

As climate change is shifting the distribution of pest and disease vectors, renewed efforts in pest management strategies are required. However, pest species have often evolved some degree of resistance when confronted to lethal dose of insecticides, proving the limits of current strategies. In times of major uncertainty about the climate and the emergence of new pests, understanding the interplay between resistance and adaptation to climate is a pressing question in evolutionary biology.

Lessons from the past offer valuable opportunity to improve strategies for tomorrow: over the past sixty years, massive amounts of the insecticide DDT (1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane) were applied worldwide to control pests and disease vectors. This widespread and nonspecific selection pressure has driven the emergence and diffusion of resistance alleles, making insecticide exposure the best example of how insect populations adapt to stressful environments

Correspondence: Alexandre Fournier-level, School of BioSciences, The University of Melbourne, Parkville, Vic. 3010, Australia.
Tel.: +61 383 447 258; fax: +61 383 445 139;
email: afournier@unimelb.edu.au

(Crow, 1966; Merrell, 1994). Despite being banned in most countries, DDT is still in use in some parts of the world and remains a persistent pollutant in natural ecosystems because of its environmental stability, acute toxicity and low water solubility (World Health Organization, 1989; Eskenazi *et al.*, 2009). DDT thus provides an unmatched opportunity to investigate how a systematic and well defined selective pressure with global, albeit uneven distribution, has shaped insects' natural populations. Moreover, as geographically dispersed populations of insects have come in contact with DDT, we can ask to which extent adaptation to climate alters the genetic architecture of insecticide resistance.

The fruit fly *Drosophila melanogaster* has a cosmopolitan distribution, adapting to diverse climates from tropical to temperate, and has colonized human-modified environments, therefore being exposed to high amounts of insecticides. With respect to climate adaptation, the distribution of genetic variation in *D. melanogaster* has been extensively studied along the North American and East Australian coasts (Weeks *et al.*, 2002; Turner *et al.*, 2008; Paaby *et al.*, 2010; Fabian *et al.*, 2012; Reinhardt *et al.*, 2014; Adrion *et al.*, 2015). Despite independent colonization and demographic histories, some degree of parallel evolution across these continents was observed for the loci believed to increase thermotolerance, with allele frequencies changing with latitude (Rako *et al.*, 2007; van Heerwaarden & Sgrò, 2011). This strong genetic differentiation demonstrates the importance of thermal adaptation and allows sampling populations specifically preadapted to different temperature regimes. In addition to genetic adaptation, a population may respond to environmental stress through phenotypic plasticity, that is the expression of an altered phenotype for a given genotype across different environments (Merilä & Hendry, 2014). In *Drosophila simulans*, high plasticity in thermal tolerance was shown to bear a significant fitness cost and to be under significant genetic constraint (Bubliy *et al.*, 2013); however, it is unclear whether plasticity is a ubiquitous solution to a changing environment. A powerful way to disentangle adaptation from plasticity is to investigate populations originating from diverse regions where they have adapted to particular climates and to assess them in multiple environments. The adaptive hypothesis can thus be assessed by testing populations of different origin in a common environment and the plasticity hypothesis by testing a specific population across multiple environments.

Resistance to DDT was observed as early as 1947 (Brown, 1958), and attributed to the metabolism of DDT into nontoxic DDE (Perry, 1960). In *D. melanogaster*, the cytochrome P450-encoding gene *Cyp6g1* contributes to DDT resistance in many populations (Daborn *et al.*, 2002). Long-standing exposure to DDT has triggered the gradual evolution of the *Cyp6g1* locus from the ancestral and susceptible *M* haplotype to highly resistant *AA*, *BA* and *BP* haplotypes that have emerged via gene duplication and multiple transposable element insertions (*Accord*, *HMS-Beagle* and *P*-elements), increasing gene expression (Schmidt *et al.*, 2010). Interestingly, the resistant haplotypes have not reached global fixation and the *Accord* retrotransposon was found to follow a pattern of latitudinal differentiation in the USA and in Australia (Catania *et al.*, 2004; Turner *et al.*, 2008). Temperature variation is therefore a potential explanation for this heterogeneous distribution of resistance alleles. More generally, temperature has frequently shown to modulate insecticide mortality, with a surprisingly inconsistent effect across taxa and insecticides (Amarasekare & Edelson, 2004; Muturi *et al.*, 2011; Janssens *et al.*, 2014). However, this modulating effect has never been investigated for multiple populations of a single species.

Here, we investigate the response of three populations of *D. melanogaster* sampled from different climatic regions, to gradients of DDT and temperature. The sample represents populations from a hot tropical climate in North Queensland (Australia), from a warm temperate climate in Raleigh, North Carolina (USA) and a cool oceanic climate in Tasmania (Australia). We first measured the effect of temperature on DDT mortality to test the extent of plasticity within and across populations of different climatic origins. Then, we used a more sensitive assay that monitored the activity of flies continuously over the 24 h of the exposure to specific DDT and temperature combinations. Substantial changes in activity regime were observed over time, leading to the identification of two temporally distinct phases of response over the course of the stress exposure. These phases were further analysed as function-valued traits to estimate the genetic component of the variance for all DDT and temperature combinations in a unified model. Finally, the values of the functions for activity metrics and mortality under specific regimes of temperature and DDT dose were used to determine the level of genetic constraint within population using genetic variance/covariance matrices (*G*-matrices) analysis. Altogether, our study reported extensive interaction between DDT and temperature and showed how increasing tolerance to DDT across populations was correlated with reducing genetic variance for activity traits. Despite similar trait variation in benign conditions, the response to stress was heterogeneous across populations, probably due to differences in the intensity of selection for stress resistance each population has faced.

Materials and methods

Drosophila melanogaster source populations, breeding and maintenance

To study the behavioural effect of DDT combined with temperature stress, three naturally sourced populations

were used: a set of inbred lines from the *Drosophila* Reference Genetic Panel (DGRP; Mackay *et al.*, 2012; Table S1) originally sourced from Raleigh (North Carolina, USA); a cool-climate population from Hobart (Tasmania, Australia); and a hot-climate population from Innisfail (Queensland, Australia). To be able to compare the Australian field-caught populations (which had been maintained for 12 generations as mass bred populations) to the highly inbred DGRP, these were inbred by single-pair brother–sister mating for 10 generations to increase homozygosity. All flies were bred in constant 25 °C in 250-mL plastic bottles under permanent light to remove the influence of circadian rhythm. Insecticide susceptibility and the activity of insecticide degrading enzymes exhibit circadian rhythms (Hooven *et al.*, 2009; Beaver *et al.*, 2010). However, as circadian rhythm varies between lines (Low *et al.*, 2012; and personal observations of DGRP lines) and as it is credible that it is also affected by temperature – a key variable of our experimentation (Hamby *et al.*, 2013) – we decided to minimize its influence by maintaining flies in constant light (Hooven *et al.*, 2009). The culture media was based on Bloomington corn media. The founder populations represented an initial 192 genotypes for the Raleigh (DGRP) population, 30 for the Tasmania and 30 for the Queensland population, respectively. Unless stated otherwise, all assays were performed using 10 nonvirgin female flies aged between 5 and 12 days with at least four replicates for each dose/temperature/genotype combination. Flies were left 24 h to recover in holding vials after being sorted on a CO_2 gas plate.

Population sampling and selection of a reference middle-DDT dose

To compare the populations with minimal sampling bias, reference subsets of 12 genotypes were chosen for each population. These subsets included the genotypes having a maximally different response to DDT within each population. This was based on two phenotypes measured in the same experiment: proportion of flies knocked down after 3 h of exposure to DDT (3hKD) and proportion of mortality after 24 h (24hDT). Assays were performed using 10-mL glass vials coated with a solution of DDT diluted in 200 µL acetone, stoppered with a cotton ball soaked in 5% sucrose and placed at constant 25 °C.

Due to dramatic differences in DDT dose–response, a reference middle-DDT dose was first determined for each population to approximate the lethal dose for 50% of the population (LD_{50}). This was measured in a 24-h-and-25 °C assay with each population tested as bulk of 50 randomly chosen females for three different doses (30, 120 and 480 µg per 10-mL vial). For the Raleigh and Tasmania populations, we identified 60 and 120 µg of DDT as the round doses the closest to the LD_{50}, respectively. For the Queensland population, due to extremely high resistance, no mortality above 40% was observed when exposed to 480 µg, which led us to use 480 µg of DDT as low dose in subsequent assays. For the Raleigh, Tasmania and Queensland populations, we measured 3hKD and 24hDT when exposed to 60, 120 and 480 µg of DDT, respectively. At these doses, variance in 24hDT was equal among populations (Bartlett's K-squared = 0.72567, $P = 0.6957$). However, variance in 3hKD was greater in the initial Queensland population (Bartlett's K-squared = 24.951, $P = 3.819e\text{-}06$), but no difference in 3hKD was seen between the Raleigh and Tasmania populations (Bartlett's K-squared = 1.0983, $P = 0.2946$). Finally, 12 genotypes maximizing variability for the two traits were selected for each population (Fig. S1).

Molecular characterization of neutral RFLP markers and the *Cyp6g1* haplotype

PCR based RFLP assays were performed to characterize the level of neutral molecular diversity present in the samples. Genomic DNA was extracted from ~10 adult flies following a CTAB Phenol-Chloroform extraction protocol. Thirteen different sets of primers corresponding to markers *2L1*, *2L3*, *2L5*, *2R2*, *2R4*, *2R6*, *3L1*, *3L3*, *3L5*, *3R1*, *3R3*, *X3* and *X5* from Mackay *et al.* (2012) were used to amplify DNA fragments, restriction-digested and visualized by agarose gel electrophoresis. In addition, *Cyp6g1*, shown to be a major DDT resistance gene in *Drosophila* (Daborn *et al.*, 2002), was genotyped. *Cyp6g1* encodes a P450 oxidase whose expression level has been associated with variation in resistance (Daborn *et al.*, 2002; Festucci-Buselli *et al.*, 2005; Chung *et al.*, 2007). The *Cyp6g1* gene exhibits copy number variation and the resistance haplotypes are marked by various transposable elements. The ancestral susceptible haplotype has a single-gene copy and has no insertion (referred to as the *M* or '*minus*' haplotype), and then, the successive insertion of the *Accord* LTR element, the *HMS-Beagle* retrotransposon and a partial P-element steadily increased the *Cyp6g1* expression level and gave rise to the '*Accord-Accord' (AA)*, *'Beagle-Accord' (BA)* and *'Beagle-P-element' (BP)* haplotypes, all of which have at least one copy of *Cyp6g1* (Schmidt *et al.*, 2010). To specifically test the effect of the *Cyp6g1* haplotype on the activity response to DDT, the presence of *Accord*/*Beagle*/*P-element* was genotyped in the Tasmanian and Queensland population based on the three PCR assays of (Schmidt *et al.*, 2010; primers HI, LI, JK) and retrieved from the DGRP database for the Raleigh population. The amplicon typing matched those described in Schmidt *et al.* (2010) with the exception of a novel haplotype found once in the Tasmanian and once in the Queensland population. This novel haplotype had at least two copies of *Cyp6g1* (primers JK) and an *HMS-Beagle* insertion upstream of

at least one of these copies (LI); however, the HI primers which bridge the *Accord* transposable element insertion sites failed to produce an amplicon, perhaps because of primer binding site polymorphism or because of further indel events. We therefore refer to this new haplotype as the *BX* haplotype.

Activity monitoring assays under changing temperature and DDT concentration

Fly activity was measured using the *Drosophila* Activity Monitoring system (DAM6, Trikinetics, Waltham, MA, USA). The DAM6 system consists of a Plexiglass support where tubes carrying flies are inserted so that infrared beams record any crossing of the tube over a given time as a proxy for activity. Each 10-mL vial containing 10 females and a specific dose of DDT was inserted in the DAM6, and the flies were allowed to recover from manipulation stress for an hour after which the number of crossings was recorded every two minutes over 23 h. Assays were performed at three different temperatures (20 °C, 25 °C and 30 °C) and four population-specific doses approximately corresponding to control, half the population LD_{50} (low dose), LD_{50} (medium dose, but see previous sections for the Queensland population) and twice the LD_{50} (high dose). These doses corresponded to 0, 30, 60 and 120 µg of DDT in 10-mL vials for the Raleigh population; 0, 60, 120 and 240 µg of DDT for the Tasmania population; and 0, 480, 960 and 1920 µg of DDT for the Queensland population. For any combination of temperature and genotype, all different doses were run side by side and replicated twice in one assay and twice at a later date to avoid potential batch effects. After 24 h, the tubes were pulled out of the DAM6 unit and mortality was scored (Fig. 1).

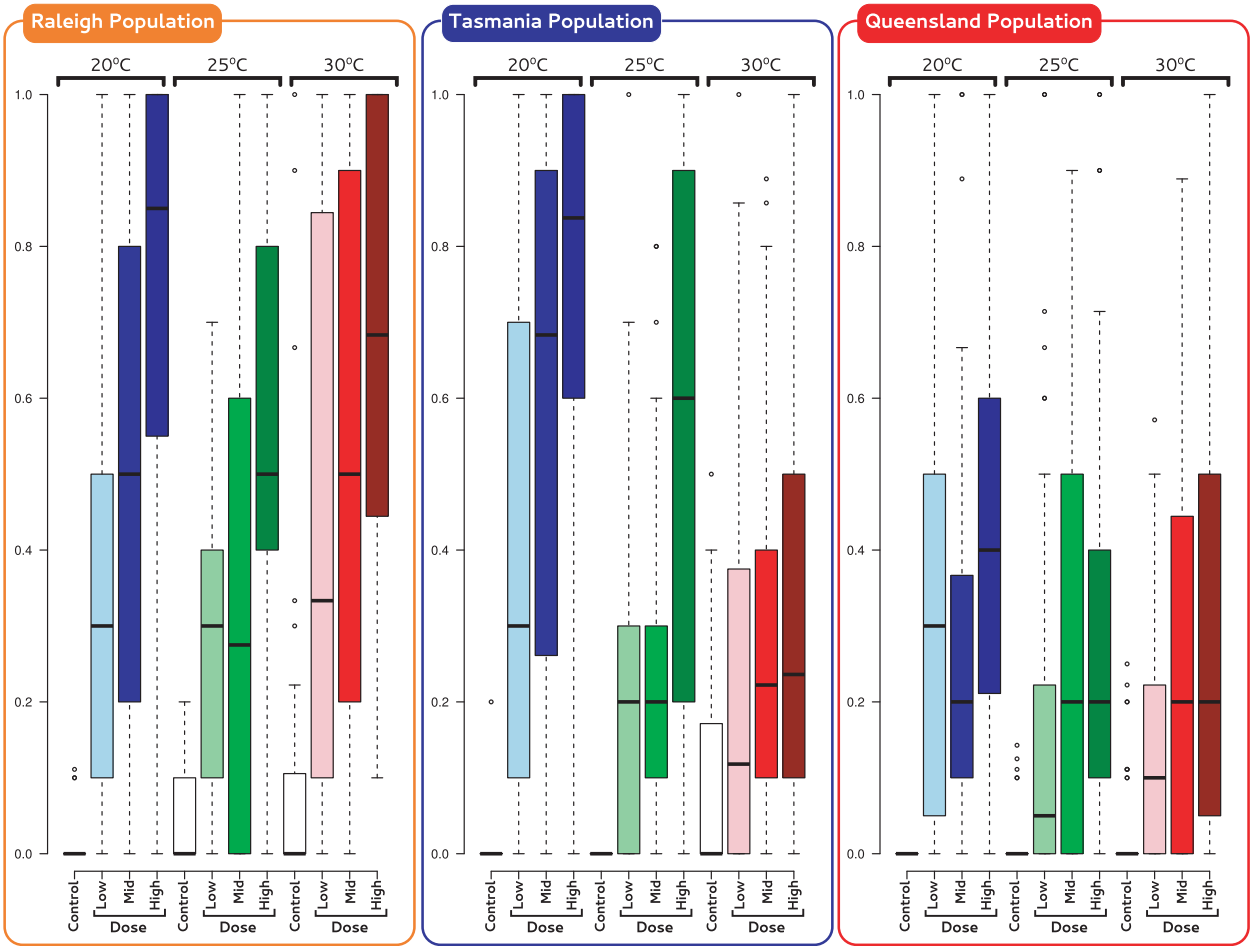

Fig. 1 Mortality to DDT for a gradient of temperature for 12 genotypes of each of the three populations. A specific DDT dose range was used for each population, expressed in µg per 10-mL vial bearing 10 females. For Raleigh: Control = 0 µg, Low = 30 µg, Mid = 60 µg, High = 120 µg; for Tasmania: Control = 0 µg, Low = 60 µg, Mid = 120 µg, High = 240 µg; and for Queensland: Control = 0 µg, Low = 480 µg, Mid = 960 µg, High = 1920 µg. The dose range for each population was chosen so mortality was similar across population for the mid dose at 25 °C (ANOVA, $P > 0.3$).

Self-organizing map analysis

Log-transformed activity counts over time were analysed using Kohonen's self-organizing map method (SOM; Kohonen, 2001) as implemented in the R\kohonen package (Wehrens & Buydens, 2007). Activity over time profiles were projected over a 6 × 7 hexagonal nontoroidal map (the opposite edges of the map are not connected) with a run length of 10 000, defining 42 units, each representing a consensus profile between activity readings sharing a similar pattern. This SOM size was determined by minimizing the distance between each map unit and the observed activity profiles mapped to this unit (the smaller the better), penalized by the overall size of the map. After map units were defined through SOM as presented in Fig. 2a, the units were clustered into groups. These groups were defined through hierarchical clustering weighted by the count (frequency of observing a specific behaviour type; Fig. 2b) and the mortality associated with the profiles supporting each unit (Fig. 2c, d), using the 'flat' and most frequently observed profile of the experiment as reference (Group 1).

The effects of the population of origin, temperature and DDT dose nested in the population effect and *Cyp6g1* promoter type on the belonging to a behavioural group were tested through multinomial logistic regression using the multinom() function of the R\nnet package in the following model:

$$\log\left(\frac{Pr(Y_i = 2)}{Pr(Y_i = 1)}\right) = \alpha_2 \text{Pop} + \beta_2 Cyp6g1 + \gamma_2 \text{Temp} * \text{Pop} + \delta_2 \text{Dose} * \text{Pop}$$

$$\cdots$$

$$\log\left(\frac{Pr(Y_i = \text{k})}{Pr(Y_i = 1)}\right) = \alpha_k \text{Pop} + \beta_k Cyp6g1 + \gamma_k \text{Temp} * \text{Pop} + \delta_k \text{Dose} * \text{Pop}$$

for the k groups, where α, β, γ and δ are the predictors of the log-odds ratio of behaving like Group {2,...,k} instead of reference Group 1. The significance of the logistic regression coefficients was assessed using *z*-tests.

Modelling of early and later activity and mortality

Twenty-three-hour activity profiles were divided into two phases: first an early activity phase followed by a later activity phase. This is justified because mortality

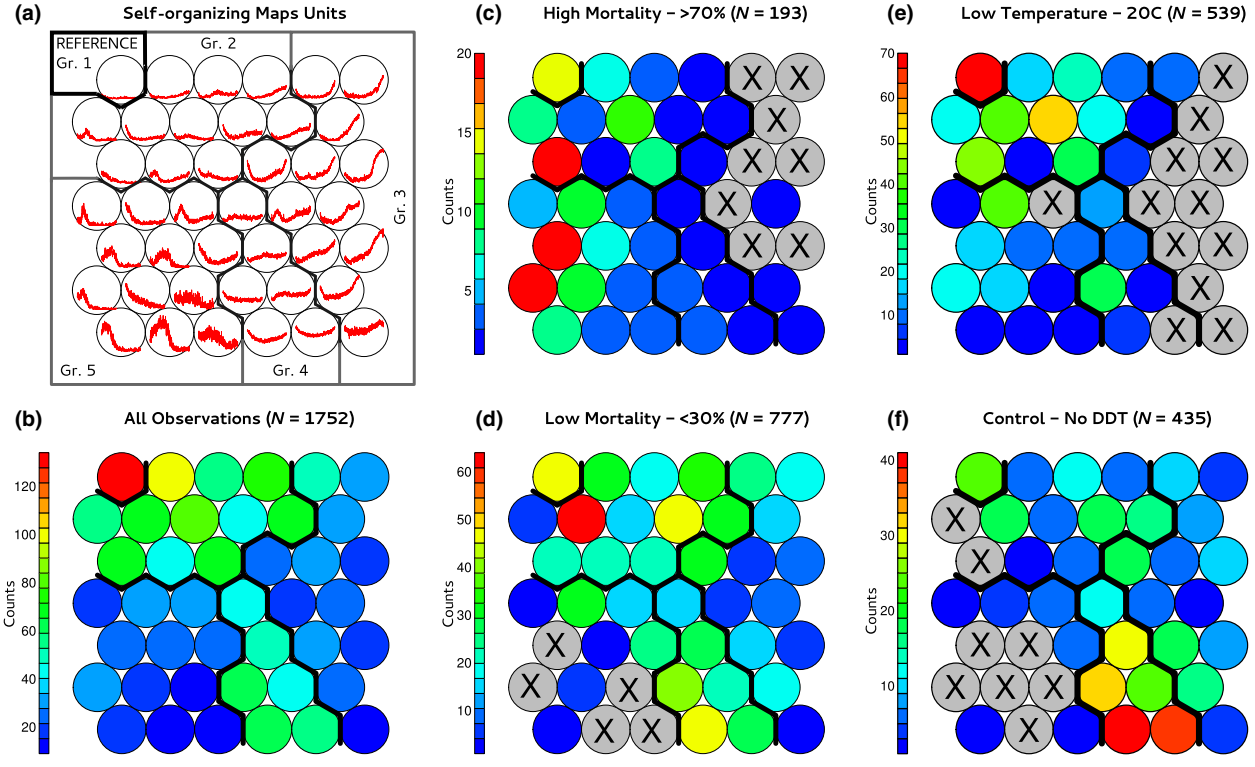

Fig. 2 Self-organizing map of the activity profiles over 23 h. Activity is measured as the number of cross of an infrared beam sitting in the middle of the 10-mL vial per individual fly (10 females per vial) on the DAM6 system (Trikinetics, MA, USA). a) The 42 units of the maps were defined to best represent the profiles in the 1752 observations. The groups (Gr.) correspond to clusters of similar profiles based on hierarchical clustering weighted by the count and mortality observed (see Materials and Methods). b–f) Count plots reporting the number times a given unit was observed in the data for specific sets of conditions. *N*: number of observation used in each analysis. X: units not observed in the specific set of conditions.

was significantly greater in Group 5, corresponding to a profile with intense early activity which sharply decreased afterwards. Early and later activity levels were computed as the sum of the activity counts from the beginning of the experiment to the breakpoint time and from the breakpoint time to the end of the experiment, respectively. The breakpoint time between the two phases was determined as the time for which the early activity was maximally correlated with mortality and the early and later activity minimally correlated using Pearson's moment. Finally, to analyse the overall response to temperature and DDT in the different populations and to determine how much variation was present within population, the early activity, later activity and mortality were analysed independently for each population using Bayesian general linear mixed models implemented in the R\MCMCglmm package (Hadfield, 2010) as:

$$\begin{aligned} Y_{ij} = {} & \alpha\text{Temp} + \beta\text{Dose} + \gamma\text{Temp}^2 + \delta\text{Dose}^2 \\ & + \eta\text{Temp} * \text{Dose} + \kappa_i\text{Geno} + \lambda_i\text{Geno} * \text{Temp} \\ & + \xi_i\text{Geno} * \text{Dose} + \varepsilon_{ij} \end{aligned}$$

where α, β, γ, δ and η are the predictors of the fixed effects of temperature and dose; κ_i, λ_i and ζ_i are the predictors of the random genotype effects; and ε_{ij} is the residual term. The MCMC sampling was run 25 000 times with a burn-in period of 5000 iteration and a thinning interval of 20. The models were subsequently adjusted by removing nonsignificant fixed effects or random effect based on deviance information criteria (DIC) to optimize the model fit.

Measuring genetic constraint in the genetic variance/covariance structure across populations

Matrices of variance/covariance (*G*-matrices) between traits for each population were extracted from the solution of the adjusted mixed-linear models described previously for five subsets of experimental conditions, centred on the population mean, and each subset of traits was compared across populations using the indices and tests suggested in Charmantier *et al.* (2014). The five subsets of traits corresponded to (i) all traits in all temperature and DDT dose conditions, (ii) traits for all temperature in the absence of DDT (control), (iii) traits for all temperature in the presence of DDT (dose > 0), (iv) traits for the 25 °C temperature for all DDT conditions (control and all DDT doses) and (v) traits for the low- and high-temperature conditions (20 °C and 30 °C) for all DDT conditions. We first computed the sum of all eigenvalues as an index of the total genetic variance available and the maximum variance along the first eigenvector relative to all eigenvalues (Kirkpatrick, 2009). Modified Mantel test was then used to test proportionality between *G*-matrices, and random skewers test was used to test similarity in response to perturbation of the *G*-matrices based on the R functions provided in Roff *et al.* (2012). Finally, the properties of the common subspace between two *G*-matrices and the angle between the first eigenvector of these *G*-matrices were measured using Krzanowski common subspace test (Krzanowski, 1979) as implemented in the MCMCglmm\Krzanowski.test() function. Significance levels for all tests described here were assessed by comparing the observed test statistic to the null distribution obtained through randomization (randomly reallocating individuals to one of the two populations compared). Random draws were performed by permuting 1000 times the lines of the two G-matrices compared in a given test. For the random skewers test, 100 skewers were drawn from a uniform U(−1,1) distribution. To obtain robust estimates, the solution of the MCMCglmm models were computed five times independently and for each set of solutions, the 1000-randomization procedure was run five times, this ensured stable sampling of *P*-values for all the tests described previously.

Results

Molecular diversity in sampled populations

To represent contrasted climatic origins, three populations were sampled: a warm-temperate reference population in Raleigh (NC, USA), a hot-tropical population in Innisfail (Queensland, Australia) and a cool-oceanic population in Hobart (Tasmania, Australia). For each population, 12 inbred lines were selected from larger initial populations to jointly maximize the diversity in response to DDT for 3-hour knockdown and 24-hour mortality phenotypes (Fig. S1). We first tested that the sampling scheme lead to an even representation of neutral genetic diversity across populations using 13 RFLP markers scattered over the genome. All 13 makers were polymorphic in each of the three population samples (Table S1) with a Nei diversity index (Nei, 1978) computed from these markers of 0.35 for the Raleigh population, 0.41 for the Tasmania population and 0.41 for the Queensland population. These similar Nei index values suggested no dramatic difference in levels of neutral molecular diversity between populations, allowing further cross-population comparison.

The haplotype structure of the *Cyp6g1* resistance locus was also examined with molecular markers and showed within and among population diversity (Table S1). The Raleigh population was the only population bearing the DDT susceptible ancestral *M* haplotype (frequency = 0.1), with most individuals having the moderately resistant *Accord-Accord* (*AA*, frequency = 0.7) and *Beagle-Accord* (*BA*, frequency = 0.2) haplotypes. The Tasmanian population consisted in mostly *AA* haplotype (frequency = 0.8) with one *BA*

and one *Beagle-uncharacterized allele* (*BX*) haplotype (frequency = 0.1 and 0.1, respectively). Finally, *BA* haplotype was less frequent in the Queensland population (frequency = 0.5) than in the other population (*t*-test, $P < 0.01$) with a single *BX* haplotype (frequency = 0.08), whereas the highly resistant *Beagle-P-element* haplotype (*BP,* frequency = 0.42) was more frequent than in the other populations (*t*-test, $P < 0.01$).

Temperature-modulated mortality to DDT

The mortality of lines exposed to DDT was very different across populations (Fig. 1). The Raleigh population was the most susceptible (LD_{50} for all three temperatures = 52 μg), the Tasmanian was intermediary (LD_{50} = 301 μg) and the Queensland was the most resistant (the extrapolated LD_{50} = 405 924 μg). Given this dramatic difference in DDT sensitivity, we selected a dose for each population such that mortality across populations was similar at the reference temperature of 25 °C (60 μg per 10-mL vial for the Raleigh population; 120 μg for the Tasmania population; and 960 μg for the Queensland population; ANOVA, $F_{2,89} = 1.216$, $P = 0.301$). These doses were assigned to the middle doses where each population had a separate series of three doses such that low dose = middle dose/2 and high dose = 2× middle dose. Despite some discrepancies in the Raleigh/25 °C and the Queensland/20 °C assays, increasing DDT dose increased mortality (GLM, $F_{3,1304} = 148.79$, $P < 0.001$) among the other seven population-by-temperature combinations.

Once the DDT dose gradient was determined, we tested whether the mortality to DDT was modulated by the temperature. Indeed, temperature variation (20 °C, 25 °C or 30 °C) significantly affected mortality (GLM, $F_{2,1305} = 39.23$, $P < 0.01$). The populations responded differently to the temperature gradient; the Tasmania population showed high plasticity with decreasing mortality at higher temperatures, the Queensland population was only moderately effected by temperature, whereas the Raleigh population showed lower mortality at 25 °C (Fig. 1). *t*-Tests on the linear models across all populations show that 20 °C and 30 °C led to higher mortality and thus could be considered more stressful conditions ($t_{20\ °C\ vs.\ 25\ °C} = -5.78$, $P < 0.001$ and $t_{25\ °C\ vs.\ 30\ °C} = 1.38$, $P < 0.001$). Altogether, the extensive variation in mortality rate across experimental conditions reflected how temperature variation modulated the level of mortality to a gradient of DDT dose, promoting different levels of stress response within and across populations.

Activity patterns associated with exposure to DDT and temperature variation

To sensitively examine the stress response over the course of the exposure to DDT and temperature, the activity of flies was monitored in real time over 23 h. The activity profiles ranged from continuously low activity to dramatic activity change over time. To identify discrete patterns in these data, activity profiles were classified using a self-organizing map (SOM; Kohonen, 2001). The 1732 activity readings (36 lines from three populations, three doses plus control, three temperatures and four to six replicates) were classified into 42 activity profile types arrayed within a SOM (Fig. 2a). The most common activity profile corresponded to monotonous and low activity in the top-left corner of the map and was considered as the reference in further analyses. The map mainly distinguished two features of the activity profiles: the profiles with an early activity peak on the bottom-left corner and with a later increase in activity in the top-right corner. In addition, the top-left corner corresponded to low activity for both early and late phases of the assay period, in opposition to the bottom-right corner having high activity for both early and late phases. The 42 activity profiles were further clustered into five groups (see Materials and Methods), resulting in: Group 1 including solely the reference activity profile; Group 2 including activity profiles deviating only slightly from the Group 1; Group 3 including profiles with later activity peak and overall low mortality; Group 4 including profiles with both high early and later activity; and Group 5 including profiles with early activity peak and high mortality. Details of the count for each population and condition are reported in Fig. S2a-c.

The temperature, DDT dose and population of origin had a significant influence in determining the activity profile of flies (multinomial logistic regression; Table 1). Increase in temperature particularly triggered a Group 3 type of activity response for all three populations (e.g. from Table 1, we can see that for every additional Celsius degree, the Raleigh population exhibits exp (0.60) = 1.82 more chance to show a Group 3 than a Group 1 activity profile); Group 4 was less affected by temperature. In contrast, an increase in DDT dose generally increased the likeliness of a Group 5 response [in the Raleigh population: increasing the DDT dose from low to middle or from middle to high increases the chance of observing a Group 5 activity profile by exp (0.94) = 2.56] but was highly unlikely to promote Group 3 or 4 responses. The exception to this was the Tasmania population, which was more likely to exhibit a Group 5 response irrespective of the temperature or DDT dose. In addition, the presence of *Cyp6g1 BA* ($n = 283$ observations with this haplotype in the whole data set) or *BP* ($n = 392$) resistant haplotypes reduced the likeliness of observing a Group 5 response, strengthening the proposition that Group 5 reflects a DDT susceptible profile. The estimates for the *BX* and *M* haplotypes effects have to be interpreted with caution due to their low frequency in the data.

Table 1 Log of the odds for the logistic regression explaining the presence in particular behavioural groups (1 is the reference).

	Population			*Cyp6g1* promoter type				Temperature			Dose within Population		
Group	RAL†	TAS	QLD	CypBA	CypBX	CypBP	CypM	RAL	TAS	QLD	RAL	TAS	QLD
2	−3.16*	7.10**	-1.83^{NS}	−1.47**	1.22^{NS}	−1.29*	10.54**	0.22*	-0.06^{NS}	0.33**	0.25^{NS}	-0.23^{NS}	0.45*
3	13.30**	3.86^{NS}	0.56^{NS}	−1.36**	1.68^{NS}	−1.63*	10.12**	0.60**	0.44**	0.62**	-0.46^{NS}	−1.24**	-0.09^{NS}
4	−5.69**	6.12*	1.47^{NS}	−2.45**	2.55*	-2.08^{NS}	10.34**	0.34**	0.12*	0.31**	−0.57*	−1.57**	−0.50*
5	−3.97*	7.62**	-0.42^{NS}	−3.44**	0.42^{NS}	−3.99**	12.15**	0.15*	-0.09^{NS}	0.32**	0.94**	-0.07^{NS}	0.88**

The level of significance was assessed using z-tests; NS, nonsignificant.
*$P < 0.05$.
**$P < 0.001$.
†The column reports the combined effect of the intercept of the model and the specific effect of the Raleigh population.

The exploratory 42 SOM profiles were further analysed as function-valued trait so that the DDT and temperature responses could be modelled over a continuous gradient. Two quantitative traits were derived to describe the key changes in activity regime over the course of the stress exposure. As the most distinct phases revealed by the SOM analysis of the activity profiles was the presence of a distinctive early and later phase, the transition between the two phases was determined to occur at a breakpoint time of 10 h 40 min (see Materials and Methods and Fig. S3). The early activity and later activity were defined as two continuous traits by summing the activity counts over the time each phase lasted, and those traits were subsequently analysed in a quantitative genetics framework.

Within and across population response to temperature and DDT

To determine the extent of genetic variation involved in the insecticide response within populations, the sum of early and later phase activity as well as mortality observed after 24 h were analysed using mixed-linear models (Table 2). The solutions of the models are presented in Fig. 3, with the fixed effects of the abiotic factors (temperature and DDT dose) presented as population-specific response surfaces and the superimposed random effect of within-population genetic variation presented as heatmaps. Altogether, these mixed-linear models explained a very substantial proportion of phenotype variation with global R^2 of 0.53 for early activity, 0.69 for later activity and 0.72 for mortality. Among all fixed effects (temperature^2, temperature, dose^2, dose and temperature-by-dose), the interaction between temperature and DDT was the only fixed effect significant for all traits (early activity, later activity and mortality) in all three populations. Despite quantitative differences in the magnitude of the fixed effects, for each trait, the shape of the response at the whole population level (not including the genetic differences within population) was fairly consistent across populations.

In all models, all three random effects (genotype, genotype-by-temperature and genotype-by-dose) improved the models fit based on Deviance Information Criterion (DIC). For all traits and populations, genetic effects had the highest variance when exposed to high temperature and to low-DDT dose. However, the levels of within-population genetic variance differed dramatically across populations. The Raleigh population showed the overall highest genetic variance with less than 32% of the total phenotypic variance left as residual (for early activity; Table 2). Furthermore, the variance of the genotype-by-temperature effect for early activity and mortality was greater than the one of the genotype-by-dose effect, showing stronger temperature related genetic plasticity. Tasmania showed an intermediate level of overall genetic variation (Fig. 3) but with stronger temperature plasticity (variance of the genotype-by-temperature effect always greater than that of the genotype-by-dose effect; Table 2). The Queensland population showed altogether much less genetic variance with between 51 and 84% of residual variance. Furthermore, in contrast to the other populations, the variance of the genotype-by-dose effect was greater than any other genetic variance, perhaps reflecting the fact that this is the only population sample with the highly resistant BP haplotypes.

Genetic constraint in the response to temperature and DDT gradient

The level of genetic correlation among traits based on the solution from the mixed models differed substantially across the different conditions. Increasing the temperature significantly increased the correlation among traits, suggesting increased constraint (Fig. 4). In the contrary, increasing the insecticide dose reduced this correlation, suggesting that DDT stress induced more diverse trait combinations among genotypes. To determine whether the genetic constraint in trait variation within population was different between benign and stressful conditions (i.e. temperature extremes and presence of DDT), trait genetic variance and covariance matrices (*G*-matrices) for particular temperature and DDT dose conditions were extracted from the mixed-linear models. Each population's *G*-matrices were defined to specifically contrast the traits response to

Table 2 Posterior mean variance and regression coefficient estimates and *P*-values for the mixed-linear model explaining the behavioural traits in each population. Empty cells correspond to nonsignificant effects not included in the final model.

		Raleigh population			Tasmania population			Queensland population		
	DIC	5715.11			7582.04			8150.04		
		σ^2	$\sigma^2/\sigma_p{}^2$		σ^2	$\sigma^2/\sigma_p{}^2$		σ^2	$\sigma^2/\sigma_p{}^2$	
Early activity										
Random effects (variance)	Geno	10024.13	0.32		6215.99	0.38		142.02	0.01	
	Geno × Temp	8952.71	0.28		2234.24	0.14		1334.13	0.05	
	Geno × Dose	2536.16	0.08		1683.27	0.10		2486.08	0.10	
	residual	10208.60	0.32		6413.34	0.39		20383.22	0.84	
		post.mean	*z*-Score	*P* (> *z*)	post.mean	*z*-Score	*P* (> *z*)	post.mean	*z*-Score	*P* (> *z*)
Fixed Effects (regression coefficient)	Intercept	−1888.70	−1.92	0.043*	−1497.31	−3.27	5E-04***	−1545.34	−3.39	5E-04***
	Temp	163.83	2.04	0.035*	138.67	3.71	5E-04***	136.11	3.66	5E-04***
	Temp2	−3.06	−1.91	0.046*	−2.64	−3.54	5E-04***	−2.61	−3.52	5E-04***
	Dose	2.77	4.08	5E-04***	*NI*			*NI*		
	Dose2	*NI*			3.47E-03	5.03	5E-04***	7.75E-05	4.59	5E-04***
	Temp × Dose	−0.11	−4.34	5E-04***	−5.07E-02	−7.56	5E-04***	−5.32E-03	−4.02	5E-04***
	DIC	5932.31			7982.64			7955.99		
		σ^2	$\sigma^2/\sigma_p{}^2$		σ^2	$\sigma^2/\sigma_p{}^2$		σ^2	$\sigma^2/\sigma_p{}^2$	
Later activity										
Random effects (variance)	Geno	16626.08	0.41		328.57	0.01		234.23	0.01	
	Geno × Temp	3639.45	0.09		14760.92	0.50		642.88	0.03	
	Geno × Dose	3428.87	0.09		2779.67	0.09		3826.70	0.19	
	Residual	16369.39	0.41		11862.24	0.40		14941.43	0.76	
		post.mean	*z*-Score	*P* (> *z*)	post.mean	*z*-Score	*P* (> *z*)	post.mean	*z*-Score	*P* (> *z*)
Fixed effects (regression coefficient)	Intercept	−3546.22	−4.90	5E-04***	352.69	12.90	5E-04***	−360.86	−7.87	5E-04***
	Temp	286.86	4.82	5E-04***	*NI*			27.06	14.26	5E-04***
	Temp2	−5.07	−4.26	5E-04***	*NI*			*NI*		
	Dose	*NI*			−1.93	−4.04	5E-04***	*NI*		
	Dose2	2.08E-02	4.44	5E-04***	8.29E-03	5.98	5E-04***	1.20E-04	7.19	5E-04***
	Temp × Dose	-0.16	−7.18	5E-04***	−4.57E-02	−3.78	5E-04***	−1.23E-02	−9.42	5E-04***

Table 2 (*Continued*)

		DIC	−130.76			−137.27			−236.89		
			σ^2	σ^2/σ_p^2		σ^2	σ^2/σ_p^2		σ^2	σ^2/σ_p^2	
Mortality											
Random effects (variance)	Geno		6.96E-03	0.07		2.14E-02	0.26		1.04E-02	0.16	
	Geno × Temp		4.15E-02	0.42		1.10E-02	0.13		9.49E-03	0.15	
	Geno × Dose		1.83E-02	0.19		1.08E-02	0.13		1.17E-02	0.18	
	residual		3.20E-02	0.32		3.95E-02	0.48		3.29E-02	0.51	
			post.mean	z-Score	*P* (> *z*)	post.mean	z-Score	*P* (> *z*)	post.mean	z-Score	*P* (> *z*)
Fixed effects (regression coefficient)	Intercept		5.18E-02	0.85	0.389	5.36E-02	0.92	0.363	8.57E-02	2.01	0.046*
	Temp		*NI*			*NI*			*NI*		
	Temp2		*NI*			*NI*			*NI*		
	Dose		1.33E-02	5.80	5E-04***	8.64E-03	9.75	5E-04***	3.21E-04	4.49	5E-04***
	Dose2		−4.29E-05	−3.01	0.005**	−8.46E-06	−3.12	0.001***	*NI*		
	Temp × Dose		−1.32E-04	−2.45	0.01*	−1.82E-04	−8.74	5E-04***	−6.49E-06	−2.51	0.014*

*$P(> z) < 0.05$; ***$P(> z) < 0.001$; NI: not included.

DDT (control vs. Dose) and to temperature stresses (25 °C vs. stressful temperatures).

The eigenanalysis of these matrices showed the Raleigh population as bearing the greatest amount of genetic variation for all subsets of conditions followed by the Tasmania and finally the Queensland population (Table 3). Furthermore, the variation along the first eigenvector of each *G*-matrix or effective dimension, corresponding to a measure of genetic variation most readily available to selection, represented at least 49% of the total genetic variation in any population for any set of conditions (effective dimension $\max\lambda/\Sigma\lambda < 2$).

The tests performed on *G*-matrices for each subset of condition showed differences in matrices structure across populations (Table 4). Both the Mantel (correlation) and random skewers tests (response to perturbation) showed Queensland as the most differentiated population, whereas Raleigh and Tasmania populations were more similar. Strikingly, the *G*-matrices in the most stressful set of conditions (with DDT or for temperatures of 20 °C or 30 °C) were systematically less similar than in more benign conditions (no DDT or 25 °C). This suggests that stress tends to promote different reactions for populations that have different resistance levels.

We next tested the presence of a common subspace between matrices which indicates that the pair of populations compared can evolve the same trait values. The *G*-matrices measured in the absence of DDT overlapped the most, whereas those measured in the presence of DDT had a reduced common subspace (a greater *P* (< rand.) in Table 4 indicates that the observed value is smaller than in the randomized data and thus the overlap is smaller than expected by chance). The analysis of the angle between the first eigenvectors of these *G*-matrices shows the same general pattern as for the volume of the common subspace. For the tested range of condition, these results showed that the presence or absence of DDT led to strong differences in how traits respond within population but far less so temperature. When comparing populations, the *G*-matrices for the Raleigh and the Queensland populations always showed the greatest angle, further emphasizing the difference in the nature of the genetic variation available to respond to environmental stress in these two populations. Overall, these results show the populations tested reacted differently under stressful conditions induced by DDT or temperature, even with similar trait covariation structure in benign conditions.

Discussion

The Physiological response to DDT and temperature across populations

This study shows that insect activity measures through *Drosophila* Activity Monitors captures highly heritable

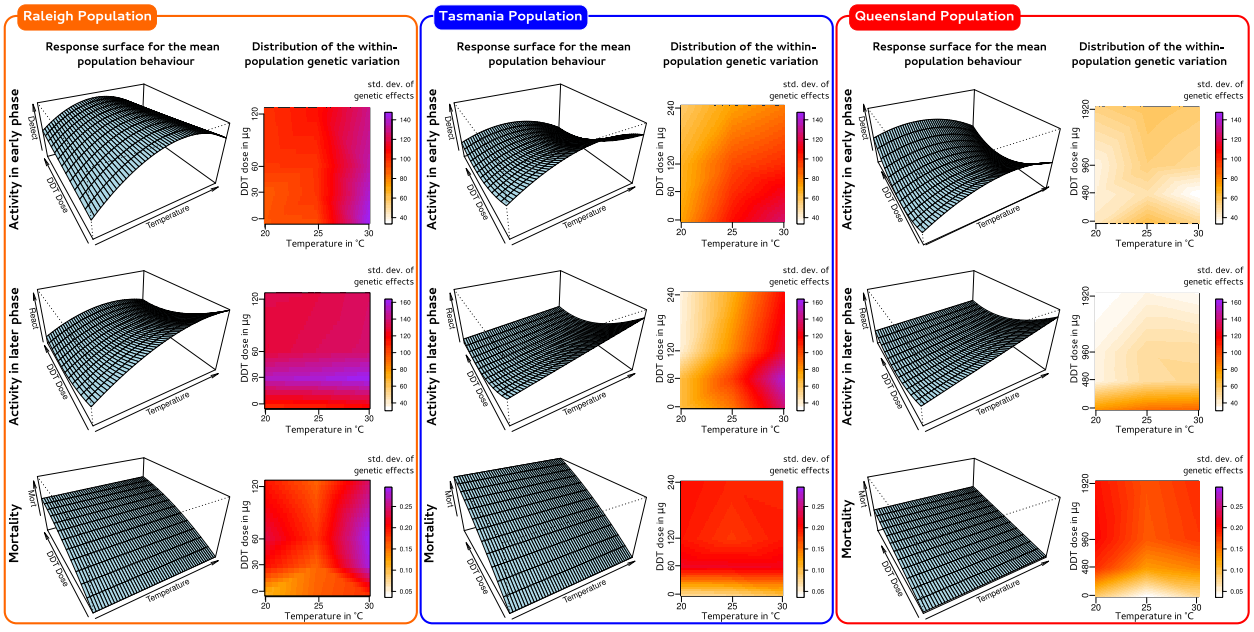

Fig. 3 Response landscape for the models reported in Table 2 for the three populations. The 3-dimensional surfaces represent the variation for the gradient of fixed effects (DDT dose and temperature) and the heatmaps, the within-population genetic variation as random effects (genotype, genotype-by-DDT dose and genotype-by-temperature). For each combination of trait and population, the *x*- and *y*-axis of the surface and heatmap are represented on the same scale. The *z*-axis of the surface for each trait is identical across populations.

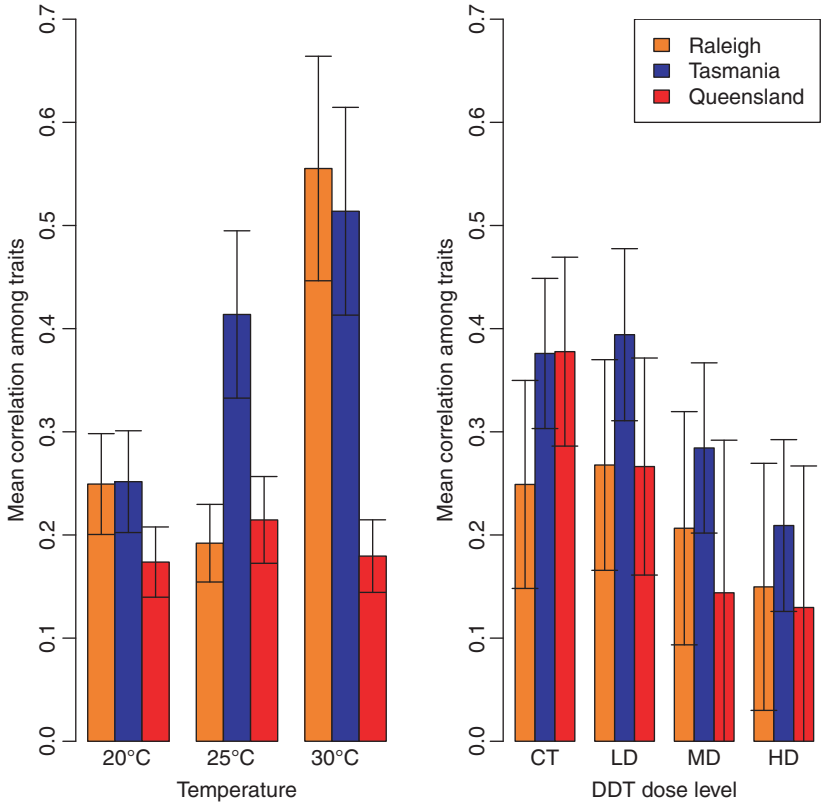

Fig. 4 Mean genetic correlation between traits (early activity, later activity and mortality) along the temperature gradient (left panel) and the DDT dose gradient (right panel). The correlation was computed using Pearson's moment correlation using the solution of the mixed-linear model for each genotype. For each temperature, all traits measured for all DDT dose at this specific temperature were combined; for each DDT dose, all traits for all temperatures at this specific dose were combined. CT: control; LD: low dose; MD: middle dose; HD: high dose.

Table 3 Sum of the eigenvalues and ratio of the maximum eigenvalues to the sum of all eigenvalues. *P* (< rand.) indicates the probability of the observed data to have a value inferior to those obtained through randomization and is used as empirical *P*-values. All significance levels were calculated using five sets of MCMC solutions times five replicates of 1000 randomizations.

			P (< rand.) for pairwise comparison			*P* (< rand.) for pairwise comparison	
Subsets of conditions	Population	$\Sigma\lambda$	vs. RAL	vs. TAS	$\max\lambda/\Sigma\lambda$	vs. RAL	vs. TAS
All conditions	RAL	429 826			1.32		
	TAS	215 065	0.113		1.35	0.513	
	QLD	65 959	0.001***	0.003***	1.95	0.871	0.899
Control No DDT	RAL	92 442			1.27		
	TAS	63 762	0.220		1.20	0.339	
	QLD	31 305	0.021*	0.080	1.09	0.220	0.255
DDT dose > 0	RAL	337 383			1.26		
	TAS	151 304	0.104		1.28	0.520	
	QLD	34 655	0.001***	0.001***	1.77	0.917	0.803
25 °C	RAL	192 736			1.22		
	TAS	65 968	0.069		1.23	0.484	
	QLD	24 934	0.008*	0.028~	2.02	0.987*	0.966~
20 °C/30 °C	RAL	223 584			1.41		
	TAS	149 097	0.198		1.32	0.367	
	QLD	41 230	0.001***	0.002***	1.97	0.761	0.931

~*P*(< rand.) < 0.05; **P*(< rand.) < 0.01; ***P*(< rand.) < 0.005.

Table 4 Mantel, random skewers and Krzanowski subspaces tests for proportionality and identity between G-matrices. *P* (< rand.) indicates the probability of the observed data to have a value inferior to those obtained through randomization and is used as empirical *P*-values. All significance levels were calculated using five sets of MCMC solutions times five replicates of 1000 randomizations.

		Mantel	*P* (< rand.)	Random skewers	*P* (< rand.)	Sum of subspace	*P* (< rand.)	Angle of subspace	*P* (< rand.)
All conditions	RAL vs. TAS	0.83	0.366	0.77	0.347	3.33	0.459	4.823	0.325
	RAL vs. QLD	0.39	0.962~	0.46	0.961~	2.71	0.964~	10.161	0.231
	TAS vs. QLD	0.48	0.998***	0.51	0.996***	3.28	0.787	4.375	0.735
Control No DDT	RAL vs. TAS	0.89	0.449	0.84	0.423	4.16	0.593	5.45E-07	0.806
	RAL vs. QLD	0.66	0.907	0.71	0.862	4.52	0.056	1.21E-06	0.786
	TAS vs. QLD	0.73	0.977*	0.73	0.963~	4.39	0.161	8.36E-07	0.682
DDT dose > 0	RAL vs. TAS	0.84	0.402	0.80	0.410	3.43	0.795	2.369	0.324
	RAL vs. QLD	0.42	0.951~	0.50	0.944	2.82	0.842	10.388	0.016*
	TAS vs. QLD	0.41	0.998***	0.51	0.998***	2.36	1.000***	2.610	0.415
25 °C	RAL vs. TAS	0.95	0.081	0.92	0.053	4.33	0.347	0.011	0.665
	RAL vs. QLD	0.55	0.627	0.63	0.642	3.52	0.839	0.014	0.757
	TAS vs. QLD	0.63	0.949	0.68	0.927	3.57	0.616	0.009	0.855
20 °C/30 °C	RAL vs. TAS	0.84	0.563	0.80	0.451	4.17	0.137	0.353	0.834
	RAL vs. QLD	0.38	0.977*	0.44	0.987*	3.69	0.642	3.830	0.254
	TAS vs. QLD	0.47	0.998***	0.52	0.994***	3.67	0.482	1.552	0.650

~*P*(< rand.) < 0.05 or > 0.95; **P*(< rand.) < 0.025 or > 0.975; ***P*(< rand.) < 0.01 or > 0.99.

traits that are sensitive enough to provide novel insights into insecticide biology (Denecke *et al.*, 2015). Both DDT and temperature stress trigger increased activity. However, the response to DDT exposure leads to an early reaction, whereas temperature leads to a later one. This is coherent with the mode of action of DDT, which causes nervous system misfiring, whereas the response to temperature may first involve activity reduction before triggering escape past a time threshold (Hoffmann & Parsons, 1997; Dillon *et al.*, 2009; Ma & Ma, 2012).

Interestingly, we detected a significant interaction between the DDT dose and the temperature for all traits analysed in all three populations (Table 2). Such a modulating effect of the temperature on insecticide action has been reported for five of seven insecticides tested in the larvae of the grasshopper *Melanoplus differentialis* (Thomas) (Amarasekare & Edelson, 2004) and for an organophosphate insecticide in the damselfly *Ischnura elegans* (van der Linden) (Janssens *et al.*, 2014). A temperature effect is also illustrated by the reduced efficiency of malathion on the mosquitos *Culex restuans*

(Theobald) and *Aedes albopictus* (Skuse) at 20 °C compared to 25 °C and 30 °C (Muturi *et al.*, 2011). These mosquito results contrasts with our *Drosophila* results to the extent that mortality was highest at 20 °C in our study. This discrepancy may be due to the respective mode of action of DDT and malathion or the way the different species have evolved to overcome them. Malathion is an insecticide blocking acetylcholine esterase activity, and resistance in field populations is often associated with target-site resistance. Conversely, DDT acts on sodium channels and resistance in *D. melanogaster* is mostly mediated by P450 detoxification and so higher temperature may increase the insect metabolic rate and thus the detoxification efficiency. Altogether, our finding joins the body of literature reporting that the insect response to insecticides depends on temperature and reinforces the possibility that different genetic architectures may arise from insecticide pressures in different environments.

Heterogeneous selection for resistance across populations

The three populations examined in this study exhibited substantially different resistance levels; from the more sensitive Raleigh population to the highly resistant Queensland population. They differ in the degree of interaction between DDT and temperature; with the Tasmania population being the most plastic. They also differ with respect to genetic variance for the traits examined; with the Queensland flies exhibiting less genetic variance for all of them. Although the molecular variation present in the natural populations of origin might have been different, this is striking considering the initial population from which the Queensland population was sampled exhibited high variation for the 3-h knockdown phenotype (see Materials and Methods). Given that the Queensland flies in this study, like those sampled in a prior study by Schmidt *et al.* (2010), have a higher frequency of the DDT resistance alleles at the *Cyp6g1* locus, it is our contention that all these population differences can be attributed to selection for insecticide resistance depleting variation at resistance loci and possibly on other correlated traits. Given the equivalent levels of neutral standing variation present in all three populations, a bottleneck or founder effect of the resistant genotype alone (Piiroinen *et al.*, 2013) is unlikely to have generated the pattern of genetic variation observed. So we propose that there has been an indirect shift in the genetic architecture of traits such as has been reported in the case of the resistance of the moth *Choristoneura rosaceana* (Harris) to various classes of insecticides wherein multiple life history traits were affected (Carriere & Roff, 1995).

The Queensland population shows extreme levels of resistance and limited temperature plasticity, and also has a much higher frequency of the most resistant *Cyp6g1* haplotypes. In the activity assays reported here, all the lines bearing the most derived *Cyp6g1* alleles (i.e. *BP* and *BA* haplotypes) show predominantly 'flat' activity profiles (Gr. 1 and 2 in Fig. 2a) which differ from more susceptible fly lines that exhibited an early burst of activity (Gr. 5 in Fig. 2a; Table 1). Whereas this study reaffirms that large effect alleles at the *Cyp6g1* locus contribute to DDT resistance, it also implies that other loci contribute to DDT resistance and the associated temperature plasticity. Specifically, the Tasmania and Raleigh populations have similar *Cyp6g1* allele frequencies (here and in other studies: Catania *et al.*, 2004; Schlenke & Begun, 2004) yet the Tasmania population exhibits twice the resistance of the Raleigh population and is much more plastic. In addition, as our study specifically used female bred under constant light, we only investigated a subset of the potential factors known to modulate the genetic basis of insecticide response (Mackay, 2001; Beaver *et al.*, 2010; Hamby *et al.*, 2013). In this context, our report is probably still underestimating the true extent of plasticity in relation to stress response in natural population of *D. melanogaster*.

Strong genetic constraint in the behavioural response to DDT

Our results showed no dramatic difference in the way the three populations responded to the temperature gradient alone (Fig. S2a-c, Control column). All populations, no matter the level of exposure to DDT, altered their activity in response to temperature in a similar fashion: from mostly low-activity profiles at 20 °C (Gr. 1) to continuously high profiles at 25 °C (Gr. 4) and finally exhibiting stress at 30 °C with an escape behaviour through increased later activity (Gr. 3). There is thus no ubiquitously altered response to temperature induced by DDT resistance. However, when traits were clustered together based on the conditions they were measured in, differences in genetic correlations between traits emerged (Tables 3 and 4). Strikingly, these differences were only observed in the stressful set of conditions, in coherence with the hypothesis that enhanced level of additive genetic variation is favoured by environmental stress (Hoffmann & Parsons, 1997). Insecticide bioassays are usually conducted under standard environmental conditions and insecticide resistance has relatively rarely been associated with a fitness cost. However, we report strong levels of genotype-by-environment interaction, with activity traits showing a stronger environmental influence of both temperature and DDT dose and mortality being more influenced by genetic factors (Table 2). Under this type of genetic determinism, insecticide resistance may not have an immediate fitness consequence under benign conditions but could in return become more pronounced under stressful conditions. Genotype-by-environment interaction could therefore represent a potential mecha-

nism for mitigating the deleterious effects (i.e. cost) of insecticide resistance as cost would not be ubiquitous but only be revealed under specific stress. This may also suggest that in spite of being found in various taxa and insecticide classes (Kliot & Ghanim, 2012), identifying trade-offs requires testing a broad range of conditions and may have been underestimated in the literature.

The *G*-matrices analysis suggested a common genetic structure for traits across populations (Table 3) but the stress response did not necessarily sit in the same space (Krzanowski test; Table 4). In theory, if selection was to act solely on standing variation, the dimension of the common subspace between the *G*-matrices of the populations compared should have a volume roughly equivalent to the one of the smallest *G*-matrix considered (Aguirre *et al.*, 2014). In the analysis reported, significantly nonoverlapping spaces were found between the most and least resistant population when exposed to DDT. This result can be interpreted by considering that mutations of strong effect are capable of changing the space in which the genetic variation lies in a population (Jones *et al.*, 2003). Such a mutational shift is expected in the context of resistance mutation and would lead to defining a new space for evolution of traits. Therefore, the *Cyp6g1-BP* haplotype specific to the Queensland population potentially reflects such a mutational shift. In this scenario, the dramatic effect of *BP* is constraining the variation for activity traits through a conditionally pleiotropic effect, only revealed in stressful conditions.

Acknowledgments

We would like to thank J. Schirriffs and A.A. Hoffmann for sharing flies collected in Tasmania and Queensland. We are also grateful to P. Batterham for sharing laboratory space and T. Perry for their help in setting up the *Drosophila* Activity Monitoring system. This work was funded by the Human Frontier in Science Long Term Fellowship LT000907/2012-L and the Early Career Research Grant from the Faculty of Science of the University of Melbourne to AFL. The authors declare no conflicting interest.

References

Adrion, J.R., Hahn, M.W. & Cooper, B.S. 2015. Revisiting classic clines in *Drosophila melanogaster* in the age of genomics. *Trends Genet.* **31**: 434–444.

Aguirre, J.D., Hine, E., McGuigan, K. & Blows, M.W. 2014. Comparing G: multivariate analysis of genetic variation in multiple populations. *Heredity (Edinb.)* **112**: 21–29. Nature Publishing Group.

Amarasekare, K.G. & Edelson, J.V. 2004. Effect of temperature on efficacy of insecticides to differential grasshopper (Orthoptera: Acrididae). *J. Econ. Entomol.* **97**: 1595–1602.

Beaver, L.M., Hooven, L.A., Butcher, S.M., Krishnan, N., Sherman, K.A., Chow, E.S.-Y. *et al.* 2010. Circadian clock regulates response to pesticides in *Drosophila* via conserved Pdp1 pathway. *Toxicol. Sci.* **115**: 513–520.

Brown, A.W. 1958. The insecticide-resistance problem: a review of developments in 1956 and 1957. *Bull. World Health Organ.* **18**: 309–321.

Bubliy, O.A., Kristensen, T.N. & Loeschcke, V. 2013. Stress-induced plastic responses in *Drosophila simulans* following exposure to combinations of temperature and humidity levels. *J. Exp. Biol.* **216**: 4601–4607.

Carriere, Y. & Roff, D.A. 1995. Change in genetic architecture resulting from the evolution of insecticide resistance : a theoretical and empirical analysis. *Heredity (Edinb.)* **75**: 618–629.

Catania, F., Kauer, M.O., Daborn, P.J., Yen, J.L., Ffrench-Constant, R.H. & Schlotterer, C. 2004. World-wide survey of an accord insertion and its association with DDT resistance in *Drosophila melanogaster*. *Mol. Ecol.* **13**: 2491–2504.

Charmantier, A., Garant, D. & Kruuk, L.E.B. 2014. *Quantitative Genetics in the Wild*. Oxford University Press, Oxford.

Chung, H., Bogwitz, M.R., McCart, C., Andrianopoulos, A., Ffrench-Constant, R.H., Batterham, P. *et al.* 2007. Cis-regulatory elements in the Accord retrotransposon result in tissue-specific expression of the *Drosophila melanogaster* insecticide resistance gene *Cyp6g1*. *Genetics* **175**: 1071–1077.

Crow, J.F. 1966. Evolution of resistance in hosts and pests. In: *Scientific Aspects of Pest Control* (National Academy of Science), pp. 263–275. National Research Council Publication No. 1402, Washington, D.C.

Daborn, P.J., Yen, J.L., Bogwitz, M.R., Le Goff, G., Feil, E., Jeffers, S. *et al.* 2002. A single p450 allele associated with insecticide resistance in *Drosophila*. *Science* **297**: 2253–2256.

Denecke, S., Nowell, C.J., Fournier-Level, A., Perry, T. & Batterham, P. 2015. The wiggle index: an open source bioassay to assess sub-lethal insecticide response in *Drosophila melanogaster*. *PLoS One* **10**: e0145051. Public Library of Science.

Dillon, M.E., Wang, G., Garrity, P.A. & Huey, R.B. 2009. Review: thermal preference in *Drosophila*. *J. Therm. Biol* **34**: 109–119.

Eskenazi, B., Chevrier, J., Rosas, L.G., Anderson, H.A., Bornman, M.S., Bouwman, H. *et al.* 2009. The Pine River statement: human health consequences of DDT use. *Environ. Health Perspect.* **117**: 1359–1367.

Fabian, D.K., Kapun, M., Nolte, V., Kofler, R., Schmidt, P.S., Schlötterer, C. *et al.* 2012. Genome-wide patterns of latitudinal differentiation among populations of *Drosophila melanogaster* from North America. *Mol. Ecol.* **21**: 4748–4769.

Festucci-Buselli, R.A., Carvalho-Dias, A.S., de Oliveira-Andrade, M., Caixeta-Nunes, C., Li, H.-M., Stuart, J.J. *et al.* 2005. Expression of *Cyp6g1* and Cyp12d1 in DDT resistant and susceptible strains of *Drosophila melanogaster*. *Insect Mol. Biol.* **14**: 69–77.

Hadfield, J.D. 2010. MCMC methods for multi-response generalized linear mixed models: the MCMCglmm R package. *J. Stat. Softw.* **33**: 1–22.

Hamby, K.A., Kwok, R.S., Zalom, F.G. & Chiu, J.C. 2013. Integrating circadian activity and gene expression profiles to predict chronotoxicity of *Drosophila suzukii* response to insecticides. *PLoS One* **8**: e68472. Public Library of Science.

van Heerwaarden, B. & Sgrò, C.M. 2011. The effect of developmental temperature on the genetic architecture underlying size and thermal clines in *Drosophila melanogaster* and *D. simulans* from the east coast of Australia. *Evolution* **65**: 1048–1067.

Hoffmann, A.A. & Parsons, P.A. 1997. Consistent heritability changes under poor growth conditions. *Trends Ecol. Evol.* **12**: 460–461.

Hooven, L.A., Sherman, K.A., Butcher, S. & Giebultowicz, J.M. 2009. Does the clock make the poison? Circadian variation in response to pesticides. *PLoS One* **4**: e6469. Public Library of Science.

Janssens, L., Dinh Van, K. & Stoks, R. 2014. Extreme temperatures in the adult stage shape delayed effects of larval pesticide stress: a comparison between latitudes. *Aquat. Toxicol.* **148**: 74–82.

Jones, A.G., Arnold, S.J. & Bürger, R. 2003. Stability of the G-matrix in a population experiencing pleiotropic mutation, stabilizing selection, and genetic drift. *Evolution* **57**: 1747–1760.

Kirkpatrick, M. 2009. Patterns of quantitative genetic variation in multiple dimensions. *Genetica* **136**: 271–284.

Kliot, A. & Ghanim, M. 2012. Fitness costs associated with insecticide resistance. *Pest Manag. Sci.* **68**: 1431–1437.

Kohonen, T. 2001. *Self-Organizing Maps*, 3rd edn. Springer, Berlin/Heidelberg, Berlin, Heidelberg.

Krzanowski, W.J. 1979. Between-groups comparison of principal components. *J. Am. Stat. Assoc.* **74**: 703–707.

Low, K.H., Chen, W.-F., Yildirim, E. & Edery, I. 2012. Natural variation in the *Drosophila melanogaster* clock gene period modulates splicing of its 3′-terminal intron and mid-day siesta. *PLoS One* **7**: e49536. Public Library of Science.

Ma, G. & Ma, C.-S. 2012. Effect of acclimation on heat-escape temperatures of two aphid species: Implications for estimating behavioral response of insects to climate warming. *J. Insect Physiol.* **58**: 303–309.

Mackay, T.F.C. 2001. Quantitative trait loci in *Drosophila*. *Nat. Rev. Genet.* **2**: 11–20.

Mackay, T.F.C., Richards, S., Stone, E.A., Barbadilla, A., Ayroles, J.F., Zhu, D. *et al.* 2012. The *Drosophila melanogaster* genetic reference panel. *Nature* **482**: 173–178. Nature Publishing Group, a division of Macmillan Publishers Limited. All Rights Reserved.

Merilä, J. & Hendry, A.P. 2014. Climate change, adaptation, and phenotypic plasticity: the problem and the evidence. *Evol. Appl.* **7**: 1–14.

Merrell, D.J. 1994. *The Adaptive Seascape: The Mechanism of Evolution*. U of Minnesota Press, Minneapolis.

Muturi, E.J., Lampman, R., Costanzo, K. & Alto, B.W. 2011. Effect of temperature and insecticide stress on life-history traits of *Culex restuans* and *Aedes albopictus* (Diptera: Culicidae). *J. Med. Entomol.* **48**: 243–250.

Nei, M. 1978. Estimation of average heterozygosity and genetic distance from a small number of individuals. *Genetics* **89**: 583–590.

Paaby, A.B., Blacket, M.J., Hoffmann, A.A. & Schmidt, P.S. 2010. Identification of a candidate adaptive polymorphism for *Drosophila* life history by parallel independent clines on two continents. *Mol. Ecol.* **19**: 760–774.

Perry, A.S. 1960. Metabolism of insecticides by various insect species. *J. Agric. Food Chem.* **8**: 266–272. American Chemical Society.

Piiroinen, S., Lindström, L., Lyytinen, A., Mappes, J., Chen, Y.H., Izzo, V. *et al.* 2013. Pre-invasion history and demography shape the genetic variation in the insecticide resistance-related acetylcholinesterase 2 gene in the invasive Colorado potato beetle. *BMC Evol. Biol.* **13**: 13.

Rako, L., Blacket, M.J., McKechnie, S.W. & Hoffmann, A.A. 2007. Candidate genes and thermal phenotypes: identifying ecologically important genetic variation for thermotolerance in the Australian *Drosophila melanogaster* cline. *Mol. Ecol.* **16**: 2948–2957.

Reinhardt, J.A., Kolaczkowski, B., Jones, C.D., Begun, D.J. & Kern, A.D. 2014. Parallel geographic variation in *Drosophila melanogaster*. *Genetics* **197**: 361–373.

Roff, D.A., Prokkola, J.M., Krams, I. & Rantala, M.J. 2012. There is more than one way to skin a G matrix. *J. Evol. Biol.* **25**: 1113–1126.

Schlenke, T.A. & Begun, D.J. 2004. Strong selective sweep associated with a transposon insertion in *Drosophila simulans*. *Proc. Natl. Acad. Sci. USA* **101**: 1626–1631.

Schmidt, J.M., Good, R.T., Appleton, B., Sherrard, J., Raymant, G.C., Bogwitz, M.R. *et al.* 2010. Copy number variation and transposable elements feature in recent, ongoing adaptation at the *Cyp6g1* locus. *PLoS Genet.* **6**: e1000998.

Turner, T.L., Levine, M.T., Eckert, M.L. & Begun, D.J. 2008. Genomic analysis of adaptive differentiation in *Drosophila melanogaster*. *Genetics* **179**: 455–473.

Weeks, A.R., McKechnie, S.W. & Hoffmann, A.A. 2002. Dissecting adaptive clinal variation: markers, inversions and size/stress associations in *Drosophila melanogaster* from a central field population. *Ecol. Lett.* **5**: 756–763.

Wehrens, R. & Buydens, L.M.C. 2007. Self- and super-organizing maps in R: the kohonen package. *J. Stat. Softw.* **21**: 1–19.

World Health Organization. 1989. *DDT and Its Derivatives: Environmental Aspects*, Environmental Health Criteria monograph No. 83, Geneva.

Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1 Scatterplot of the proportion of knockdown after 3 h and the proportion of mortality after 24 h for the three populations (mean of three replicates). Doses are expressed in μg of DDT per 10-mL vial. The solid dots represent all the genotypes tested and the coloured triangles, the 12 genotypes chosen within each population to maximize the diversity for both traits.

Figure S2 (a–c) Count plots reporting the percentage of times a given unit was observed in the data for each of the three populations in each set of conditions.

Figure S3 Correlation coefficients between each pair of quantitative traits (Early Activity, Later Activity and Mortality) for a range of putative breakpoint in time. The vertical red line represents the optimal breakpoint, jointly minimizing the correlation between activity traits and maximizing their correlation with mortality.

Table S1 Population genotype marker.

Data deposited at Dryad: doi: 10.5061/dryad.7f368

Received 2 December 2015; revised 3 February 2016; accepted 5 February 2016