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RESEARCH ARTICLE

Changes Across Development Influence Visible and Cryptic Natural Variation of *Drosophila melanogaster* Olfactory Response

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Abstract Relative to an equivalent source of variation that do not present a hidden state, cryptic genetic variation is likely to be an effective source for possible adaptations at times of atypical environmental conditions. In addition to environmental perturbations, it has also been proposed that genetic disturbances can generate release of cryptic genetic variation. The genetic basis and physiology of olfactory response in *Drosophila melanogaster* is being studied profusely, but almost no analysis has addressed the question if populations harbor cryptic genetic variation for this trait that only manifests when populations experiences a typical or novel conditions. We quantified olfactory responses to benzaldehyde in both larval and adult lifecycle stages among samples of chromosome two substitution lines extracted from different natural populations of Argentina and substituted into a common inbred background. We also evaluated whether an effect of genetic background change, occurred during chromosome substitution, affect larval and adult olfactory response in terms of release of cryptic genetic variation. Results indicate the presence of genetic variation among chromosome substitution lines in both lifecycle stages analyzed. The comparative analyses between chromosome 2 substitution lines and isofemale lines used to generate the chromosome 2 substitution lines shown that only adults exhibited decanalizing process for olfactory response to benzaldehyde in natural populations of *D. melanogaster*, i.e., release of hidden genetic variation. We propose that this release of hidden genetic variation in adult flies is a consequence of the shift in genetic background context that happens in chromosome 2 substitution lines, that implies the disruption of natural epistatic interactions and generation of novel ones. All in all, we have found that changes across *D. melanogaster* development influence visible and cryptic natural variation of olfactory behavior. In this sense, changes in the genetic background can affect gene-by-gene interactions (epistasis) generating different or even novel phenotypes as consequence of phenotypic outcome of cryptic genetic variation.

Keywords Cryptic genetic variation · Natural genetic variation · Olfactory response · *Drosophila melanogaster*

Introduction

Quantitative natural variation in phenotypes is the result of multifactorial genetic causes (Chandler et al. 2013; Mackay 2014; Stapley et al. 2010) which is also modified during development and affected by environmental changes (Mensch et al. 2008; Pigliucci 2010; Travisano and Shaw 2012; Carreira et al. 2013). The astonishing amount of phenotypic variation for each trait gives place to a myriad of potential possibilities in terms of the generation, maintenance and elimination of variation. In this scenario, canalization acts as a genetic buffer for this large amount of phenotypic variation and plays a role in the stabilization of phenotypes (Gibson and Dworkin 2004; Gibson and Wagner 2000; Flatt 2005; Le Cunff and Pakdaman 2012). Canalization intends to describe the robustness of phenotypes to perturbation (Gibson and Wagner 2000, Dworkin 2005a) so that a genotype's phenotype remains relatively

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invariant when individuals of a particular genotype are exposed to different environments (environmental canalization) or when individuals sharing the same single or multilocus genotype differ in their genetic background (genetic canalization). Canalization results in the accumulation of phenotypically cryptic genetic variation, which can be released after a "decanalizing" event. Thus, canalized genotypes maintain a cryptic potential for expressing particular phenotypes, which are only uncovered under particular decanalizing environmental or genetic conditions (Chandler et al. 2013; Gibson and Dworkin 2004; Le Rouzic and Carlborg 2008; Polaczyk et al. 1998). Thus, according to the model proposed by these authors, accumulation of mutations at conditionally neutral loci for a sufficiently long time can lead to the accumulation of cryptic variation even in the absence of canalization. All in all, given the different aspects involved in the evolution of phenotypes, identifying the underlying genetic complexity involved in variation of complex traits could be of interest in ecology, genetics, developmental biology and evolutionary biology.

Olfactory response is a complex trait that allows organisms to interact with the external world in several ways, e.g.: evaluation of environmental quality through chemical cues and, for insects, in localization of oviposition sites. Studies performed using *Drosophila melanogaster* demonstrated that olfactory response is a complex quantitative trait, determined by ensembles of multiple segregating genes that encompasses context-dependent interactions (Anholt et al. 2003; Anholt and Mackay 2004; Lavagnino et al. 2013; Sambandan et al. 2006; Swarup et al. 2013). This implies that the relationship between the genome and the olfactory phenotype is not static, but the genetic networks that orchestrate the behavioral phenotype are dynamic and plastic (Anholt and Mackay 2015; Zhou et al. 2012). In this sense it has been shown that the topology of genetic networks can be altered by both environmental factors and changes in the genetic background (Anholt and Mackay 2015; Zhou et al. 2012). Particularly, different studies (Swarup et al. 2012; Wang et al. 2012; Yamamoto et al. 2009) demonstrated that differences in genetic backgrounds modify epistasis (gene by gene interactions) enabling changes in phenotypic expression and consequently in phenotypic variation.

As a holometabolous insect, *D. melanogaster* adult and larva stages are anatomically and behaviorally much different although, the basic organization of the larval olfactory circuit is surprisingly similar to its adult counterpart but is numerically much simpler (Ramaekers et al. 2005; Vosshall and Stocker 2007). Certainly the genetic underpinnings that enable larval and adult olfactory response to external stimulus are distinct but present a partial overlap, indicating that different life stages share some genetic factors but others are stage-exclusive (Gerber and Stocker 2007; Lavagnino et al. 2013; Vosshall and Stocker 2007; Zhou et al. 2009). There has been an increased effort to investigate the extent and nature of naturally occurring genetic and phenotypic variation for olfactory response (Fanara et al. 2002; Lavagnino et al. 2008; Mackay et al. 1996; Satorre et al. 2014; Swarup et al. 2012, 2013); however, it is surprising that almost no studies analyzed variation in genetic architecture of olfactory response among natural populations with the exception of a study of Lavagnino et al. (2008) that showed the existence of a vast amount of phenotypic and genetic variation in different populations in both larval and adult olfactory response. Furthermore, these authors also found that this variation was not evenly distributed between populations and stages; since estimates of evolvability (Houle 1992) were higher for larvae than for adults in all populations analyzed.

In the present paper, we study phenotypic and genetic variation in larvae and adults olfactory response to benzaldehyde using isogenic chromosome 2 substitution lines derived from natural populations of *D. melanogaster* to further dissect the genetic architecture of this trait. We select chromosome 2 since the others chromosome (X and chromosome 3) have been studied previously (Mackay et al. 1996). Besides, Swarup et al. (2013) detected that 43 % of the total single nucleotide polymorphisms that contribute to natural variation in olfactory perception are located in chromosome 2 suggesting the active role of this chromosome in the genetic architecture of olfactory response. Our results reveals that chromosome 2 harbor natural genetic variation involved in OB for both lifecycle stages of *D. melanogaster*. Considering that isogenic chromosome 2 substitution lines were derived from the same populations utilized by Lavagnino et al. (2008) in their study performed with wild-derived lines (isofemale lines), we also evaluated whether an effect of genetic background change, occurred during chromosome substitution, affect larval and adult olfactory response in terms of release of cryptic genetic variation. The analysis showed that only adult olfactory response exhibited cryptic genetic variation as consequence of the change in genetic background context, suggesting a new aspect in which the genetic architecture for olfactory response, an essential compound of olfactory behavior, differs between larvae and adults of *D. melanogaster*.

Materials and Methods

Drosophila Stocks

Flies were collected by net sweeping over fermented banana baits at seven locations along a latitudinal gradient

in Western Argentina. Populations were named for the nearby city where sampling took place. Collection locations, host plant prevalence, latitude, longitude, altitude and climatological data (http://www.smn.gov.ar/) for each population are presented in Table 1. Chromosome 2 substitution lines were set up from single wild-caught females (isofemale line) from each population. After ten generations of full-sib mating of each isofemale line on cornmeal–molasses–agar medium under standard conditions of 25 ± 1 °C, 70 % humidity and a 12-h light: 12-h dark cycle, a single chromosome 2 was extracted from each line and substituted into the genetic background of an isogenic *Canton-S* B strain (IsoB afterwards) by standard techniques using balancer chromosomes (Fig. 1). Briefly, to construct chromosome 2 substitution lines, males of each line was crossed to *w*; *Cy*/IsoB; *Sb*/IsoB females [crossing 1 (C1)]. A single *w/Y*; *Cy*/+*2*; *Sb*/+*3* male from the progeny of each cross was crossed to *w*; *Cy*/*Sp*; IsoB females (C2). Next, *w/Y*; *Cy*/+*2*; *Sb*/IsoB males were crossed to *w*; *Cy*/*Sp*; IsoB females (C3) to remove *Sb* balancer chromosome. *Cy* balancer chromosome was eliminated by crossing females and males of genotype *w*; *Cy*/+*2*; IsoB (C4). Flies with genotype *w*; +*2*; IsoB were intercrossed at C5 to maintain an isogenic chromosome 2 substitution line. By means of this protocol we generated isogenic chromosome 2 substitution lines for one wild derived chromosome in an otherwise isogenic background common to all lines. The number of chromosome 2 substitution lines obtained for each population is mentioned in Table 1. These lines were maintained via full-sib mating under the same conditions described above.

Behavioral Assays

Olfactory response was measured for both adults and larvae using benzaldehyde (Merck Schuchardt OHG, Hohenbrunn, Germany) as a standard odorant. To measure olfactory response in adult flies, we used the assay described by Anholt et al. (1996). Flies were collected 3–5 days after eclosion, 24 h before the essay, using light CO_2 as anesthetics and were stored in single-sex groups of five individuals in 2.5 × 9-cm plastic vials containing standard lab media. Each single-sex group was placed in a test vial without food 2 h before the initiation of the adult behavioral essay to stimulate the activity of flies. All test vials were marked with two lines, 3 and 6 cm from the bottom. A cotton swab dipped in 0.3 % (v/v) benzaldehyde was inserted in each vial so that the tip of the cotton swab lined up against the 6 cm mark. The vial was placed horizontally during the assay with both sides of the vial closed with white colored surfaces to avoid the effect of negative geotaxis and phototaxis respectively. Flies were allowed to recover for 15 s from the disturbance during the insertion of the cotton swab. Then ten counts of the number of flies in the bottom compartment of the vial, demarcated by the 3-cm line, were taken at 5 s intervals starting with the15-s time point. The adult response index (ARI) was calculated for each sex as the number of flies in the bottom sector of the vial, averaged over the ten measurements, and varies between 0 (total attraction) and 5 (total repulsion) (Anholt et al. 1996). To quantify larval olfactory responses, we employed the assay of Aceves-Piña and Quinn (1979), modified by Cobb et al. (1992). Briefly, adult females were allowed to lay eggs for 8 h on Petri dishes filled with agar medium and yeast paste. Larvae were allowed to develop on these Petri dishes for 36 h, when they were washed from the yeast paste and the behavioral test was started. Between 10 and 30 larvae were placed at the centre of a 10 cm Petri dish filled with 10 ml of 2.5 % agar. A 5-µl drop of 1 % (v/v) benzaldehyde (Ayyub et al. 1990; Cobb et al. 1992; Ganguly et al. 2003; Oppliger et al. 2000) and a 5 µl drop of distilled water were placed on filter paper discs on opposite ends of the Petri dish. To prevent diffusion of odorant through the agar and to eliminate larval gustatory responses, the filter paper discs containing the odorant or water were placed on inverted lids cut off 1.5 ml microcentrifuge tubes. The number of individuals within a 30 mm radius from each filter disc and the larvae that remain between both 30 mm radii were counted 5 min after the introduction of the larvae. Olfactory responses

Table 1 Information of natural hosts, geographical coordinates and climatic data of the seven populations of *Drosophila melanogaster* analyzed

Population	Host	Latitude (south)	Longitude (west)	Altitude (m)	Mean anual temperature (°C)	N
Guemes	Unknown	24°41′	65°03′	695	20.2	9
San Blas	Unknown	28°25′	67°06′	1061	17.2	7
Chilecito	Grape	29°10′	67°28′	1043	20	8
Barreal	Apple	31°32′	69°27′	1910	12.1	5
Uspallata	Apple, Quince	32°35′	69°22′	1915	12.2	6
Lavalle	Grape, Quince	32°50′	68°28′	647	17.1	6
Neuquén	Apple	38°57′	68°04′	260	14.5	15

N number of isogenic chromosome 2 substitution lines analyzed

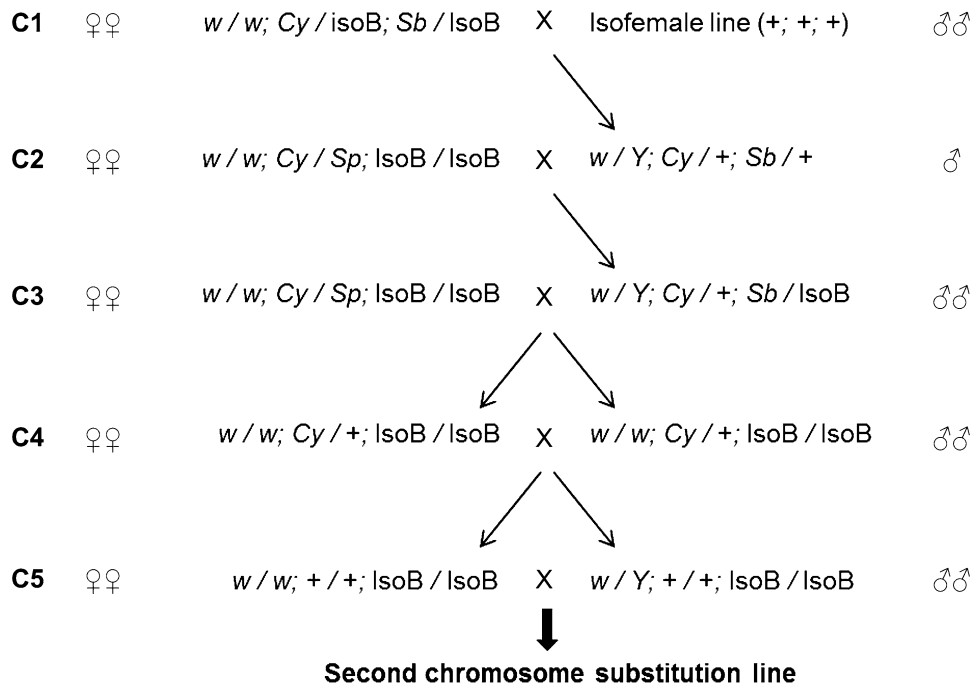

Fig. 1 Description of the crosses to generate chromosome 2 substitution lines into an isogenic *Canton-S* B genetic background (IsoB) of *Drosophila melanogaster*. Chromosome 2 substitution lines were recognized by the phenotypic marker white eyes (*w*). Chromosome balancers utilized were recognized by dominant phenotypic markers *Curly* (*Cy*) and *Stubble* (*Sb*) located at second and third chromosome, respectively. Natural chromosomes are represented as *plus*

tend to decline after 5 min, presumably as a result of saturation of the vapor phase (Kaiser and Cobb 2008; Rodrigues 1980). A larval response index (LRI) was calculated for each dish as: $LRI = [(n_{odorant} - n_{control})/n_{total}] \times 100$, where n designates the number of larvae and the subscripts indicate the sides of the Petri dish containing odorant, water (control) and the entire dish, respectively. This index varies between −100 (total repulsion) and +100 (total attraction), wherein a $LRI = 0$ indicates indifferent behavior. Larvae respond to odorants in the same way when in groups as when tested individually; thus, there is no alteration of LRI due to the presence of the other individuals (Kaiser and Cobb 2008; Monte et al. 1989). All behavioral tests were performed between 14:00 and 16:00 h under controlled temperature (25 ± 1 °C), light (5.4 ± 0.2 × 10−5 lx) and humidity (42 ± 5 %). Replicate measurements (5–7) were made for each line tested, distributed in different batches in which 10–15 lines were simultaneously assessed.

Statistical Analyses

We used an analysis of variance (ANOVA) to evaluate the sources of ARI variance in adult olfactory response of isogenic chromosome 2 substitution lines of all the populations analyzed according to the three-way nested mixed ANOVA model: $Y = \mu + P + S + L(P) + P \times S + L(P) \times S + E$, where μ is the overall mean, P and S are the fixed effects of population and sex, respectively, L(P) stands for the random effect of Line nested in Population and E is the error or the among replicate variance. We also performed two-way ANOVAs for each population separately to investigate if there are genetic differences within each population based on the model: $y = \mu + L + S + L \times S + E$. In these ANOVAs, a significant L effect is an indication of genetic variation within population, while a significant L × S interaction is an estimate of the genotype by sex interaction (GSI) which may be interpreted as genetic variation in sexual dimorphism. Significant GSI can arise from: (1) differences in the among-line variance in males and females (change in magnitude); and/or (2) deviations from unity of the cross-sex genetic correlation ($r_{GSI} < 1$; changes in rank order). We analyzed the contribution of these two sources of variation using the equation derived by Robertson (1959): $V_{GSI} = [(\sigma_M - \sigma_F)^2 + 2\ \sigma_{MF}\ (1 - r_{GSI})]/2$, where V_{GSI} is the GSI variance component, r_{GSI} is the cross-environment genetic correlation and, σ_M and σ_F are the square roots of the among-line variance components for males (M) and females (F). The first term of the equation corresponds to differences in among-line variance whereas the second corresponds to deviations from the perfect correlation between sexes ($r_{GSI} < 1$). The cross-environment genetic correlation ($r_{GSI} < 1$) is the genetic correlation of measurements in males and females and here reflects the degree to which the same genes control the phenotypic value in the two sexes. r_{GSI} was estimated as: $r_{GSI} = COV_{MF}/\sigma_M\sigma_F$, where COV_{MF} is the covariance of olfactory response to benzaldehyde between males and females.

We also performed an analysis of variance (ANOVA) to evaluate the sources of LRI variance of isogenic chromosome 2 substitution lines among populations (P) as well as the contribution of genetic variation within population through the factor L(P) following the mixed model: $Y = \mu + P + L(P) + E$. Additional ANOVAs were performed for each population separately according to the model: $Y = \mu + L + E$ to estimate the genetic component of phenotypic variance of larval olfactory response.

Estimation of Quantitative Genetics Parameters

We estimated quantitative genetic parameters in isogenic chromosome 2 substitution lines derived from each natural population and for adult and larvae, separately. Since all isogenic chromosome 2 substitution lines are inbred, the variance component among lines (σ^2_L) is an estimate of $2FV_G$, and the S × L variance component ($\sigma^2_{S \times L}$) is an estimate of $2F(1/2V_G)$, assuming a strictly additive model. For fully inbred lines, $F = 1$, so the genetic variance (V_G) of avoidance score was estimated for adult as $1/2\sigma^2_L + \sigma^2_{S \times L}$ and simply as $1/2\sigma^2_L$ for larvae olfactory response considering that the LRI measurement was realized without differentiate sexes (Falconer and Mackay 1996; Lynch and Walsh 1998). The estimate of environmental variance (V_E) used was the error variance, and the phenotypic variance (V_P) of avoidance score was estimated, as usual, from $V_G + V_E$. The heritability of avoidance score (h^2) was calculated as V_G/V_P. V_E, V_P and h^2 was also estimated for each lifecycle separately. To facilitate comparisons between larvae and adult, and among populations for each stage, we calculated the genetic coefficients of variation (CV_G) using the equation: $CV_G = 100(V_G)^{1/2}/X$, where X is the LRI or ARI population mean. This procedure is based on the notion that trait means, rather than variances, are more appropriate for standardizing genetic variance when the objective is to compare among traits and/or populations (Houle 1992).

Analysis of Cryptic Genetic Variation

To elucidate if adult and/or larva olfactory response to benzaldehyde release cryptic genetic variation as consequence of changes in the genetic background, we compared isogenic chromosome substitution lines (*Canton-S* B isogenic genetic background) with isofemale lines (wild-derived genetic background) used to generate the isogenic chromosome substitution lines (see Fig. 1) and that were previously scored for olfactory response (Lavagnino et al. 2008). In a subsets of 17 and 22lines in larva and adults respectively, for both chromosome substitution lines and isofemales lines we tested whether there is canalization, i.e. release of cryptic genetic variation, both for larvae and adult olfactory response following the analysis sensu Gibson and Wagner (2000) and also sensu Dworkin (2005a, b). Gibson and Wagner (2000) propose that it is possible to detect relaxed canalization, i.e. release of cryptic genetic variation, of a trait by comparing mean phenotypic values and phenotypic variance of the trait between lines in "normal", or wild type, and perturbed situations consequence of environmental or genetic factors. When differences between the two types of lines are found in both phenotypic variance and mean phenotype it is considered that the trait under study relaxed canalization, i.e. release of cryptic genetic variation. But, if differences are only found in mean phenotype and there is no change in phenotypic variance then the trait is considered that remains canalized in spite of the perturbation. Following this analysis scheme we compared mean phenotypic values and phenotypic variance between isofemale lines (wild-type) and chromosome 2 substitution lines (perturbed). Phenotypic variance differences were tested by means of variance homogeneity tests and differences between mean olfactory response by mean of a *t* test; in the cases when there was no homogeneity of variances between the two samples a *t* test for heterogeneous variances was performed. We also performed an analysis of canalization sensu Dworkin (2005a, b) by means of an analysis of variance (ANOVA) to evaluate the effect of the differences between genetic background (treatment effect) in adult olfactory response using ARI as variable according to the three-way nested mixed ANOVA model: $y = \mu + L + S + T + L \times S + L \times T + S \times T + L \times S \times T + E$, where μ is the overall mean, S and T are the fixed effects of sex and treatment, respectively, L stands for the random effect of Line and E is the error or the among replicate variance. We also performed two-way ANOVAs for each sex separately based on the model: $y = \mu + L + T + L \times T + E$. For larval olfactory response we utilized LRI as variable but, in this case, the sex factor was not considered. In all ANOVAs achieved a significant L × T interaction is interpreted as an indication of possible release of cryptic genetic variation due to the change of genetic background (perturbation). This indication should be further confirmed. Thus, if L × T interaction was significant we proceed to calculate the genetic correlation across treatments $r_{GTI} = COV_{wt-subs}/\sigma_{wt}\sigma_{subs}$ where $COV_{wt-subs}$ is the covariance of olfactory response to benzaldehyde between isofemale lines (wt) and chromosome substitution lines (subs), and CV_G as an estimate of genetic variability for different types of lines (treatments). The criteria followed indicated that if L × T interaction was significant, the r_{GTI} across treatments should be closer to 1 than to 0 and an increased genetic variability should be observed in the isogenic substitution genetic background (perturbed) lines (CV_G subs > CV_G wt) consequence of a

release of cryptic genetic variation, consistent with a decanalization of olfaction response phenotype. Finally, we calculated the contribution of the differences in among-line variance and the deviations from the perfect correlation ($r_{GTI} < 1$) between different genetic background (isofemale and isogenic chromosome substitution lines) to L × T interaction following the procedure described above.

Statistical analyses and estimates of variance components of random effects were performed using InfoStat (2008).

Results

We quantified 56 isogenic chromosome 2 substitution lines detecting substantial phenotypic variation in both larva and adult olfactory response to benzaldehyde (Fig. 2). With respect to population differentiation for larval olfactory response (Fig. 3a), the Chilecito population was the only population with a positive value for mean LRI (1.64) while chromosome 2 substitution lines derived from the Barreal population showed the highest mean avoidance score to benzaldehyde (LRI: −4.92). In the case of adult olfactory response, the lowest and highest score for ARI were measured in the Barreal (3.64) and Chilecito (4.05) populations, respectively (Fig. 3b). However, neither in larvae nor in adult olfactory response to benzaldehyde exhibited significant differences between populations (Table 2). We detected significant differences among chromosome 2 substitution lines in larvae and adults (Table 2) revealing that these populations harbor genetic variation located in chromosome 2 affecting olfactory response. It is possible that the absence of significant variation between populations is a consequence of high intra-population genetic

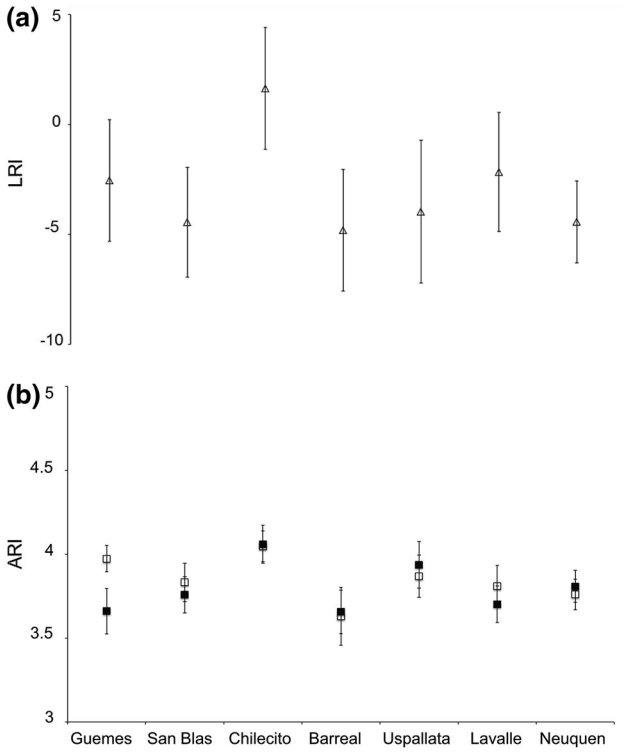

Fig. 3 Mean values of olfactory responses to benzaldehyde among chromosome 2 substitution lines of *Drosophila melanogaster* derived from different populations of Argentina for **a** larval response index (LRI, larvae were not sexed) and **b** adult response index (ARI) for female (*white bars*) and males (*black bars*). *Vertical bars* represent standard deviation

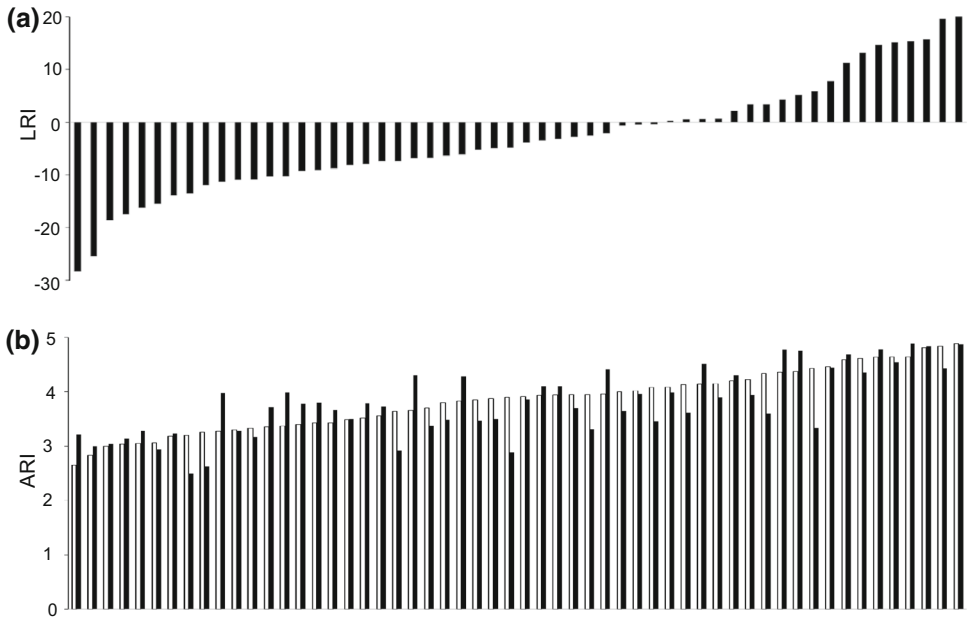

Fig. 2 Histogram of 56 chromosome 2 substitution lines means for olfactory responses to benzaldehyde for **a** larval response index (LRI) and **b** adult response index (ARI) for female (*white bars*) and males (*black bars*). *Line* means are ranked from smallest to largest response index

variation relative to inter-population genetic variation, since variation among lines account for 17 and 40 % of the total phenotypic variation in larvae and adults olfactory response, respectively. Interestingly, the analysis performed in adults revealed that the sex effect was non-significant (Table 2), suggesting an absence of sexual dimorphism for adult olfactory response that, as reveled by the non-significance of population by sex interaction, was consistent across populations (Table 2). Nevertheless, line by sex interaction, which is interpreted as GSI, exhibited a significant effect although this factor only explains the 3 % of total phenotypic variation.

We also studied the relative contribution of differences among chromosome 2 substitution lines to total phenotypic variation in each individual population in both stages of the life cycle to know whether the general pattern of genetic variation encountered presents differences between populations. The results of these ANOVAs for larval olfactory response (Table 3) revealed a significant line effect in the Guemes, San Blas, Chilecito, Lavalle and Neuquen populations wherein the contribution of the differences among chromosome 2 substitution lines in these populations to larval olfactory response varied between 16.7 and 26.6 %. These results indicate that chromosome 2 derived from these populations harbor natural genetic variation for larval olfactory response. Conversely, variation in chromosome 2 did not contribute to larval olfactory response phenotypic variation in the Barreal and Uspallata populations. In the case of adult olfactory response, our results showed significant differences among chromosome 2 in all populations except the San Blas population (Table 3), suggesting that the populations studied bear a substantial amount of genetic variation for this trait. Besides, the line by sex interaction term was significant in the San Blas and Guemes populations (Table 3), indicating sex-specific variation in adult olfactory response among chromosome 2 substitution lines from these populations. A significant line by sex interaction term may be due to deviations from the perfect genetic correlation across sexes (changes in the ranking order) and/or when variance among lines differs across sexes (changes in the magnitude). To further analyze the nature of line by sex interaction, we constructed reaction norms for olfactory responses to benzaldehyde of males and females for chromosome 2 substitution lines derived from the San Blas and Guemes populations (Fig. 4). The populations analyzed showed different patterns since in the San Blas population the 86 % of the line by sex interaction is explained by changes in the ranking order of chromosome 2 substitution lines across sexes, while in the Guemes population this term account for the 26.4 % of the line by sex interaction (Fig. 4).

Table 2 Analysis of variance for larval and adult olfactory responses to benzaldehyde

Source	Larvae			Adult		
	d.f.	MS	*P*	d.f.	MS	*P*
Population	6	165.03	0.975	6	2.31	0.755
Sex	–	–	–	1	0.35	0.433
Population by sex	–	–	–	6	0.42	0.608
Line (population)	49	846.62	0.000	49	4.14	0.000
Line (population) by sex	–	–	–	49	0.59	0.044
Error	355	338.38		626	0.43	

d.f. degrees of freedom, *MS* mean squares

Estimates of quantitative genetic parameters provided a supplementary view of patterns of variation in larva and adult olfactory response in each natural population (Table 4). Estimates for genetic variance (V_G) were low for adult olfactory response in all populations, in agreement with estimates for V_G for adult olfactory response of isofemale lines (Lavagnino et al. 2008). Larval olfactory response V_G showed higher values than in adult in all populations except in Barreal, where this quantitative genetic parameter was zero for larva but exhibited the highest value for adult. Heritability (h^2) estimates showed in all cases higher values for adult than for larvae olfactory response, probably as consequence of the lower environmental variance (V_E) recreated in larvae behavioral assays (Table 4).

Finally, in the analyses of decanalization, i.e. release of cryptic genetic variation; the analysis sensu Gibson and Wagner (2000) revealed a significant phenotypic mean change in both larvae and adult olfactory response index to benzaldehyde between isofemale lines (wild-type) and isogenic chromosome 2 substitution lines (genetic perturbed lines) although, significant increase in phenotypic variance for olfactory response index was detected only in adults of both sexes (Table 5; Fig. 5). In the case of the analysis sensu Dworkin (2005a, b) the ANOVAs results indicated that treatment (genetic background) factor and the line by treatment interaction exhibited significant differences only in adults (Table 6). The two-way ANOVAs performed for each sex showed the same outcome in males and females since there is a non-significant line by treatment by sex interaction (Table 6). In fact, significant results were observed for the treatment factor ($F_{1,21} = 37.97$ $P < 0.0001$; $F_{1,21} = 30.36$ $P < 0.0001$, female and male respectively) and for line by treatment interaction ($F_{1,21} = 6.52$ $P < 0.0001$; $F_{1,21} = 4.4$ $P < 0.0001$, female and male, respectively). The cross environment (background) genetic correlation (r_{GTI}) was 0.85 and 0.80 in male and female, respectively, while r_{GTI} measured in larva olfactory response index was 0 suggesting a possible change in genetic variance between wild type and perturbed sets of lines. Certainly For both sexes

Table 3 Phenotypic plasticity and relative contributions of sources of variation to the total phenotypic variation for larval and adult olfactory responses

	Guemes	San Blas	Chilecito	Barreal	Uspallata	Lavalle	Neuquen
Larvae							
Line	16.7*	18.5*	26.6**	0.2	7.1	21.2*	21.7***
Adult							
Line	45.6**	0	30.4**	53**	30.7**	38*	48.5***
Line × Sex	10.6*	18.4*	0.3	0	0	0.3	3.6

Numbers are percentages of total phenotypic variance

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$

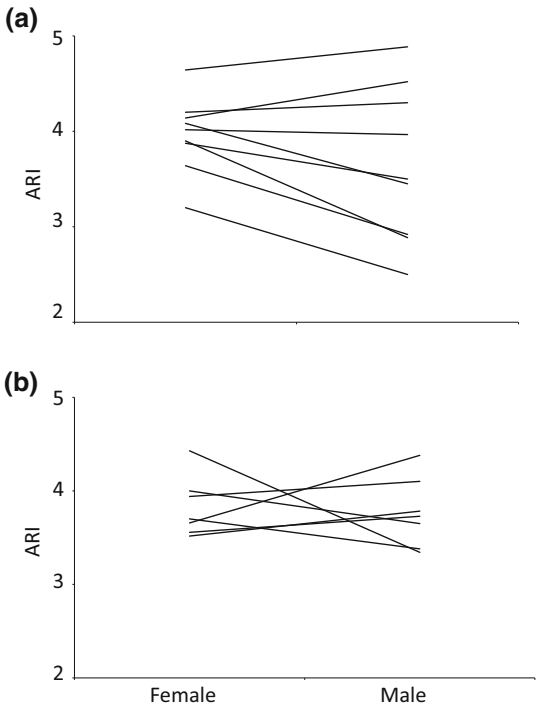

Fig. 4 Norms of reaction for adult response index (ARI) to benzaldehyde in the two sexes for chromosome 2 substitution lines of *Drosophila melanogaster* from the Guemes (**a**) and San Blas (**b**) populations

the significant L × T interaction is attributable to differences in among-lines variance in each genetic background since the change in magnitude account for 93 and 63 % in female and male respectively, of this significant interaction. In this sense, the plot of the reaction norm for olfactory response index in adults showed more within-line variation among isogenic chromosome 2 substitution lines than isofemale lines (Fig. 5), that may be interpreted as a release of cryptic genetic variation due to the perturbation or change in genetic background.

Finally, quantitative genetic parameters showed that perturbed lines showed a larger amount of genetic variation for adult olfactory response (CV_G subs = 4.82) than wild type lines (CV_G wt = 1.52), while the opposite pattern was found for larval olfactory response (CV_G subs = 35.75, CV_G wt = 524.92). All in all, the results from both analyses suggest that genetic perturbation produces a decanalization process; i.e. discloses cryptic genetic variation in adult olfactory response but not in larval olfactory response.

Discussion

We have analyzed the extent and nature of segregating quantitative genetic variation for larval and adult olfactory response of *D. melanogaster* among samples of chromosome 2 extracted from different natural populations and substituted into a common identical genetic background. Our survey of olfactory response variation revealed that chromosome 2 harbors an important portion of the natural variation for the trait in both life-cycle stages of *D. melanogaster*. The response to benzaldehyde in adult flies indicated that chromosome 2 substitution lines exhibited an absence of sexual dimorphism and scarce genetic variation in the magnitude of sex dimorphism. To our knowledge this is the first record of a negligible effect of sexual dimorphism in adult olfactory response, since previous studies showed that males had significant higher responses index (ARI) than females (Arya et al. 2015; Lavagnino et al. 2008; Mackay et al. 1996; Swarup et al. 2013). Certainly, Lavagnino et al. (2008) detected differences in olfactory response to benzaldehyde between sexes using isofemales lines in flies collected from the same populations used in our survey. On the other hand, Sambandan et al. (2006) showed that flies with the same isogenic genetic background than the one utilized in this study exhibited sexual dimorphism for adult olfactory response. Thus, chromosome 2 from these populations would not contribute significantly to sexual dimorphism of adult olfactory response and/or the interaction between wild-derived chromosome 2 and the isogenic genetic background determined the result observed.

We have shown that the genetic variation in larval olfactory response exceeds the variation quantified for adults among populations and, in line with this observation; evolvability estimates are consistently higher for larvae

Table 4 Estimates of quantitative genetic parameters of olfactory responsesin larvae and adults

	Guemes	San Blas	Chilecito	Barreal	Uspallata	Lavalle	Neuquen
Larvae							
V_G	38.11	34.36	54.97	0	15.73	42.66	41.23
CV_G	242.09	131.72	454.08	0	99.90	317.06	144.94
V_E	398.69	273.31	324.02	504.05	436.82	310.78	300.99
V_P	436.80	307.67	378.99	504.05	452.55	353.44	342.22
h^2	0.09	0.11	0.15	0	0.04	0.12	0.12
Adult							
V_G	0.24	0.12	0.10	0.24	0.14	0.14	0.22
CV_G	12.82	9.14	7.81	13.46	9.59	9.95	12.41
V_E	0.31	0.49	0.47	0.39	0.55	0.42	0.39
V_P	0.55	0.60	0.58	0.63	0.69	0.56	0.61
h^2	0.43	0.19	0.18	0.38	0.20	0.24	0.36

V_G genetic variance, CV_G genetic coefficient of variation, V_E environmental variance, V_P phenotypic variance, h^2 heritability

Table 5 Summary results of the analysis of canalization sensu Gibson and Wagner (2000) for larval and adult olfactory response index (RI) to benzaldehyde. Δ phenotypic variance: differences between olfactory response variances of lines of different types (σ^2 isofemale line (wild-type)—σ^2 chromosome 2 substitution line (perturbed)) and the *P* value of variance homogeneity tests are shown. Δ mean phenotype: differences between mean olfactory response of lines of different types (mean isofemale line (wild-type)—mean chromosome 2 substitution line (perturbed)) and the *P* value of a *t* test are show; in the case when there was no homogeneity of variances between the two samples a *t* test for heterogeneous variances was performed

	Larval olfactory RI	Adult olfactory RI♀	Adult olfactory RI♂
Δ phenotypic variance	−18.35 ($P = 0.7939$)	−0.25 ($P = 0.001$)	−0.32 ($P < 0.0001$)
Δ mean phenotype	6.01 ($P = 0.1465$)	0.65 ($P < 0.0001$)	0.81 ($P < 0.0001$)

than for adults. Low genetic variance for adult olfactory response was also found for different chromosome substitution lines extracted from a natural population of *D. melanogaster* (Mackay et al. 1996). Differences in genetic variation and evolvability between life stages were observed in lines where the entire genome is natural (Lavagnino et al. 2008). Despite that our results agree with the outcome observed in isofemale wild-derived lines, there are some differences in the pattern of genetic variation for olfactory response between both studies. In fact, Lavagnino et al. (2008) detected absence of genetic variation for adult olfactory response in the Chilecito and Neuquen populations and in both lifecycle stages in the Uspallata population. Conversely, in the present study we detected genetic variation in most of those cases when chromosome 2 substitution lines were involved except for larval olfactory response from Uspallata (Table 4). Interestingly, Lavagnino et al. (2008) and the present study analyzed similar numbers of lines indicating that the difference in genetic variation observed could not be attributable to sample size effect. Furthermore, as was previously stated, Mackay et al. (1996) estimated similar genetic variance and evolvability values for chromosome X and chromosome 3 substitution lines than our estimates for chromosome 2 substitution lines; although they analyzed a higher sample size (43 X and 35 chromosome 3 substitution lines). Thus, our sample size would not determine an underestimation of quantitative genetic parameters including genetic variance and evolvability.

Olfactory response is a complex trait orchestrated by ensembles of genes arranged in gene networks characterized by nonlinear interactions (i.e. epistasis) among gene products (Anholt et al. 2003; Anholt and Mackay 2015; Arya et al. 2015; Brown et al. 2013), wherein the genetic background where these gene networks function may be one of the factors that cause the generation of different phenotypic outcomes (Swarup et al. 2013). Swarup et al. (2012) demonstrated that the phenotypic expression of mutants affecting candidate genes involved in adult olfactory response depends on the genetic background. In fact, the effect of mutations reduces the response to benzaldehyde in the *Canton-S* background while in wild-type genetic backgrounds (isofemale lines) this effect was suppressed. Therefore, an alternative explanation of the differences in the estimates of genetic variation for olfactory response detected between isofemale lines (Lavagnino

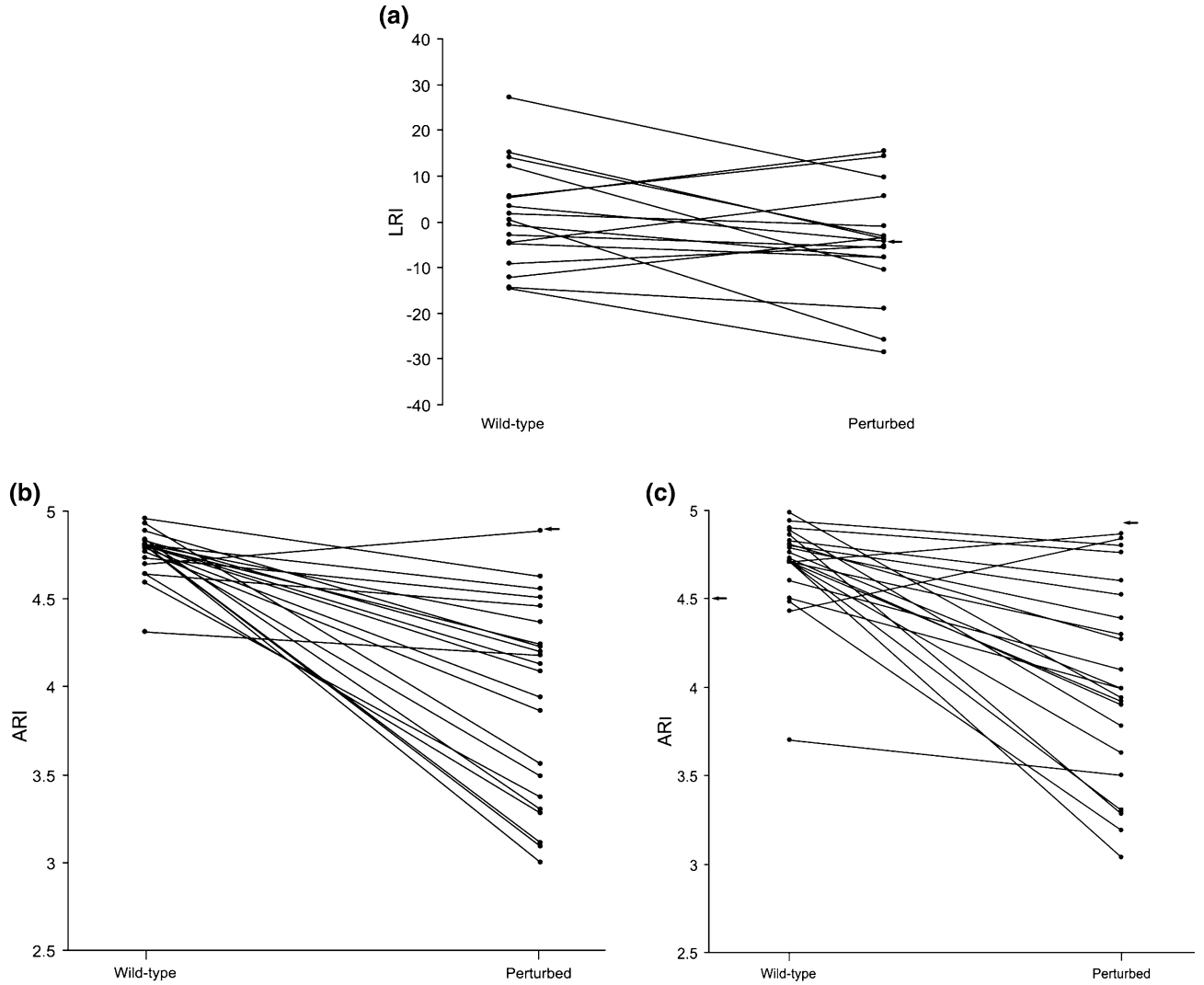

Fig. 5 Plot of the reaction norms of *line* means for larval (**a**, both sexes), adult female (**b**) and adult male (**c**) response index (RI) to benzaldehyde across treatments. Treatments refer to the same lines in different genomic contexts: wild-type (isofemale lines) or genetically perturbed (substitution lines). *Black arrows* indicate mean RI value of *Canton-S* B strain (IsoB) to benzaldehyde. This *line* provided genetic background for wild derived chromosome 2 substitutions (see 'Materials and Methods' section)

Table 6 Analysis of variance for *D. melanogaster* larval and adult olfactory response index to benzaldehyde of isofemale lines (wild-type) and chromosome 2 substitution lines for the analysis of canalization sensu Dworkin (2005a, b)

Source	Larvae			Adult		
	d.f.	MS	*P*	d.f.	MS	*P*
Line	16	1444.90	0.975	21	2.47	<0.0001
Sex	–	–	–	1	0.05	0.681
Treatment	1	2417.53	0.053	1	82.75	<0.0001
Line by sex	–	–	–	21	0.32	0.114
Line by treatment	16	493.51	0.105	21	2.21	<0.0001
Sex by treatment	–	–	–	1	4.69	0.042
Line by treatment by sex	–	–	–	21	0.21	0.58
Error	204	330.62		528	0.23	

d.f. degrees of freedom, *MS* mean square

et al. 2008) and chromosome 2 substitution lines (this study) could be attributable to genetic background effect. In general terms, this effect contemplates that the establishment of an isogenic background produces as outcome changes in variation patterns of quantitative trait genetic architecture through modifications in epistatic interactions (Carlborg et al. 2006; Chandler et al. 2013; Spencer et al. 2003; Swarup et al. 2012, 2013; Wright et al. 2006; Yamamoto et al. 2009). In this sense, we considered that chromosome 2 substitution lines are genetic perturbed lines due to the breakdown of natural epistatic interactions and dominance effects in each chromosome 2 as a consequence of the interaction of these wild derived chromosomes with the isogenic "foreign" background. Previous studies suggested that when epistatic networks related to a trait change, a decanalizing process could occur which is related to hidden quantitative genetic variation or cryptic genetic variation (Flatt 2005; Gibson and Wagner 2000; Gibson and Dworkin 2004; Mackay 2014; Paaby and Rockman 2014). Moreover, it has been proposed that decanalization and a related release of cryptic genetic variation could be detected by a comparison of perturbed lines either by changes in the environment or genetic context with wild-type lines (Dworkin 2005b, Gibson and Dworkin 2004; Gibson and Wagner 2000; Paaby and Rockman 2014). Our comparative analyses of genetically perturbed lines (chromosome 2 substitution lines) and wild derived lines (isofemale lines) shows results consistent with a scenario of descanalization and its associated release of cryptic genetic variation for adult but not for larval olfactory. Certainly, we can ask about the causes that determine that adult olfactory response harbors cryptic genetic variation in the populations analyzed while larval olfactory response does not. In principle, as we mentioned before, it has been strongly suggested that changes in the genetic background presumably imply disruption of natural epistatic interactions and generation of novel ones, which result in a decanalizing process that reveal the existence of hidden quantitative genetic variation; scenario that fits perfectly with our results for adult olfactory response in natural populations of *D. melanogaster*. In this sense, different studies (Swarup et al. 2012; Yamamoto et al. 2009) showed that epistasis appears to be a pervasive general feature of natural population and that there is variation in the magnitude of epistasis and phenotypic variation among the different genetic backgrounds. Actually, these studies revealed that effect of *P*-element insertions (mutational effect) were generally suppressing in wild-type genetic background compared to the effect observed in the same *Canton-S* B strain genetic background used in our study. Suppressing epistasis may be a general mechanism for conferring phenotypic robustness and hence canalization (Masel and Trotter 2010; Paaby and Rockman 2014; but see Hermisson and Wagner 2004). The olfactory system in larvae is the same as in adult flies but much simpler not only in terms of number of elements (neurons, organs) but for different aspects of genetic architecture complexity between stages (Gerber and Stocker 2007; Lavagnino et al. 2008, 2013; Vosshall and Stocker 2007). If we consider that adult olfactory response genetic architecture is orchestrated by more complex epistatic interacting networks in comparison to larval olfactory response, then a genetic perturbation or genetic background change affecting these epistatic interactions has a tangible effect in the more complex genetic architecture as adult olfactory response. All in all, our results contribute to consolidate the general idea that the genetic architecture of a trait is an important factor in its evolution; since it was shown that differences in genetic architecture of olfactory response between larval and adult stages influence decanalization and cryptic genetic variability release in *D. melanogaster* olfactory response. In other words, we have found that changes across *D. melanogaster* development influence visible and cryptic natural variation of olfactory response. Further studies will have to evaluate at a molecular level the genetic basis of phenotypic variation of larvae and adult olfactory response in flies collected in natural populations in order to determine how and to what extent this variation is affected by changes in the epistatic interacting gene networks.

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Compliance with Ethical Standards

Conflict of interest All authors declare that they have no conflict of interest.

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