Entomologia
Experimentalis et Applicata

DOI: 10.1111/eea.12192

Micro-geographical scale variation in *Drosophila melanogaster* larval olfactory behaviour is associated with host fruit heterogeneity

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Accepted: 17 March 2014

Key words: natural variation, phenotypic plasticity, micro-geographical distances, genotype by environment interaction, Diptera, Drosophilidae

Abstract

Organisms utilize environmental cues to deal with heterogeneous environments. In this sense, behaviours that mediate interactions between organisms and their environment are complex traits, especially sensitive to environmental conditions. In animals, olfaction is a critical sensory system that allows them to acquire chemical information from the environment. The genetic basis and physiological mechanisms of the olfactory system of *Drosophila melanogaster* Meigen (Diptera: Drosophilidae) are well known, but the effects of ecological factors on the olfactory system have received less attention. In this study, we analysed the effect of environmental heterogeneity (different host fruits) on variation in larval olfactory behaviour in a natural population of *D. melanogaster*. We generated half-sib lines of *D. melanogaster* derived from two nearby fruit plantations, *Vitis vinifera* L. (Vitaceae) ('grape') and *Prunus persica* L. (Rosaceae) ('peach'), and measured, using a simple behavioural assay, larval olfactory response to natural olfactory stimuli. Results indicate that patterns of variation for this trait depend on host fruit plantation where lines were collected. In fact, only lines derived from 'grape' showed phenotypic plasticity for larval olfaction, whereas a genotype*environment interaction was detected solely in lines derived from 'peach'. Therefore, our results demonstrate the existence of genetic differences in *D. melanogaster* larval olfactory behaviour at a micro-geographical scale and also reveal that the trait studied presents a dynamic genetic architecture which is strongly influenced by the environment.

Introduction

The analysis of complex traits is challenging considering the intricate web of interactions between genotype and phenotype ('genotype-phenotype map'; Houle et al., 2010) that should be elucidated, preferably in various environments, in order to infer how organisms respond to heterogeneous environments (Pigliucci & Preston, 2004; Dixon et al., 2009). Thus, understanding the effect of environmental factors involved in variation of complex traits is of major interest in ecology, genetics, and evolutionary biology, as environmental variation plays an important role in the generation of evolutionary novelties (West-Eberhard, 2003; Gilbert & Epel, 2009; Moczek et al., 2011), genomic scale changes (Kondrashov, 2012), and during ecological speciation (Rundle & Nosil, 2005). Moreover, several studies have addressed the relevance of environmental variation (context-dependent effect) in relation to patterns of trait variation within and between populations (Wayne et al., 2005; Fanara et al., 2006; Folguera et al., 2008; Mensch et al., 2010; Del Pino et al., 2012). In this context, considering that natural populations must deal with different and changing environments, a set of strategies to cope with environmental diversity has been proposed (Meyers & Bull, 2002).

A mechanism that has been frequently suggested in the context of strategies to cope with the 'problem' of environmental diversity is phenotypic plasticity (Schlichting & Pigliucci, 1998; Whitman & Ananthakrishnan, 2009). Phenotypic plasticity refers to the property of a genotype to express different phenotypes in different environments

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(Pigliucci, 2005; Fordyce, 2006). The plot of mean phenotypic values of a genotype across a range of environments is a way of visualizing its environmental sensitivity, or reaction norm. However, not all genotypes respond in the same way to the same source of environmental heterogeneity, indicating the existence of genetic variation for phenotypic plasticity. Such variation in the reaction norms among genotypes is called genotype*environment interaction (Conner & Hartl, 2004; Mackay & Anholt, 2007). Phenotypic plasticity and genotype*environment interaction are complex matters and their study involves the assessment of the underlying genetic variation, liability to environmental conditions, and trait association with fitness, as well as the relationship with other aspects of the phenotype (Schlichting & Pigliucci, 1998; Conner & Hartl, 2004). Furthermore, it has been proposed that genetic variation can be maintained by genotype*environment interaction in natural populations (Via & Lande, 1987; Gillespie & Turelli, 1989; Fernandez Iriarte & Hasson, 2000; Ungerer et al., 2003; Fanara et al., 2006).

In order to better understand the evolution of complex traits in natural populations, it is important to address the role of environmental variation. The effect of environmental heterogeneity can be contrasting at short distances even within the ranges of dispersion of animal species. When this occurs, it can be considered as micro-geographical variation. Several studies showed the relevance of micro-geographical environmental variation relative to biological variation within and between natural populations (Dobzhansky, 1939; McKenzie & Parsons, 1974; Barker et al., 1986; Alonso-Moraga et al., 1988; McPheron et al., 1988; Sokolowski & Carton, 1989; Karan et al., 1999; Haerty et al., 2003; Wayne et al., 2005).

Drosophila melanogaster Meigen (Diptera: Drosophilidae) is the quintessential insect model organism for genetic, physiological, developmental, and evolutionary research. However, ecological features of this species have been less investigated (but see, for example, Carson, 1971; Nunney, 1990; Rodriguez et al., 1992; Medina-Muñoz & Godoy-Herrera, 2005; Reaume & Sokolowski, 2006). Further research, both in situ in natural environments and in the laboratory, would help us understand diverse ecological aspects of *D. melanogaster* and, particularly, the relevance of micro-geographical environmental variation related to adaptive strategies and biological variation. Certainly, responses to the chemical environment play an important role in animal survival, as chemical cues influence several key behaviours such as adult and larval feeding. When the olfactory system performs accurately, it detects and discriminates odour cues and integrates relevant information into the nervous system, thus enabling the elicitation of suitable behavioural responses. Larval olfactory behaviour (LOB) is an adaptive trait (Asahina et al., 2008), determined by ensembles of multiple segregating genes that are sensitive to the environment (Kreher et al., 2005; Gerber & Stocker, 2007; Lavagnino et al., 2013). Most studies of larval olfaction aimed at its genetic and molecular basis as well as its physiological mechanisms (Kreher et al., 2005; Gerber & Stocker, 2007; Lavagnino et al., 2013); recently phenotypic and/or genetic variation in natural populations were characterized (Mackay et al., 1996; Lavagnino et al., 2008; Lavagnino & Fanara, 2011; Richgels & Rollmann, 2012; Swarup et al., 2013). Despite the wealth of knowledge about the olfactory response to individual chemical stimuli in larvae (Kreher et al., 2005; Lavagnino et al., 2008, 2013; Khurana & Siddiqi, 2013), the effect of the whole fruit as a stimulus remains almost unexplored—notable exceptions are Ruebenbauer et al. (2008) for adult olfaction and Lavagnino & Fanara (2011) for larval olfaction.

Here, we present a study of *D. melanogaster* LOB using larvae derived from a natural population that exhibits environmental variation (change of host fruit presence) at a micro-geographical scale. LOB was analysed in response to complex olfactory stimuli that are present in the natural environment, i.e., rotten fruits that act as hosts, to assess whether (1) larvae derived from a natural population show phenotypic and genetic variation in a micro-geographical scale associated with different host fruits; (2) LOB differs in its response to different complex olfactory stimuli present in natural environments, and (3) LOB displays a genotype*environment interaction which varies at a micro-geographical scale associated with different host fruits. Our results indicate that host fruit variation at micro-geographical scale partly explains *D. melanogaster* LOB variation encountered in a natural population.

Materials and methods

Collection site

Fly collection was carried out in March 2010 in a natural population located near the town of Lavalle (Province of Mendoza, Argentina; altitude 647 m), by gathering rotten fruits in plantations of two plant species that are used by *D. melanogaster* as breeding and feeding sites: *Vitis vinifera* L. (Vitaceae) ('grape': 32°42′17.1″S, 68°37′35.9″W) and *Prunus persica* L. (Rosaceae) ('peach': 32°42′35.5″S, 68°39′44.4″W). The plantations are 3.1 km apart. The fruits were isolated in containers and taken to the laboratory. During 15 days, emerged adult flies from each container were identified by species and sex. This protocol ensures that all emerged flies are offspring of flies that laid eggs in nature. Previous studies have demonstrated that adult *D. melanogaster* flies have a dispersion capacity that

exceeds the 3.1-km distance between the two plantations (Coyne et al., 1982; Coyne & Milstead, 1987). Because there were no geographical barriers to gene flow visible in the area between plantations (JJ Fanara, pers. obs.), we consider that differences in host fruit between our collecting sites are micro-geographical.

Drosophila melanogaster lines

Lines were generated by crossing a single male emerging from one type of host fruit with various virgin females emerging from the same type of fruit. Each crossing lasted 24 h, after that time, females were kept alone in vials containing laboratory rearing medium (agar with maize meal, molasses, and sugar). Thus, all lines originating from one host fruit plantation are half-sib lines. Lines were maintained by full-sib mating on laboratory medium under standard conditions (25 ± 1 °C, 70% r.h., and L12:D12 photoperiod) and never exposed to host fruits until the initiation of behavioural assays (see below). Ten lines derived from the 'grape' plantation and nine from 'peach', all proven to be viable and fertile, were used to quantify LOB.

Behavioural assays

The behavioural assays used to quantify LOB in response to whole-fruit stimuli are the same as previously described in Lavagnino & Fanara (2011). Briefly, adult females from each line were allowed to lay eggs for 8 h on an agar medium with commercial yeast paste. Before the onset of the behavioural essays (36 h after eclosion) larvae were washed from the yeast paste with distilled water (H_2O_d). Between 30 and 50 larvae were placed in the centre of a 10-cm Petri dish containing 10 ml of 2.5% agar. Filter paper discs with 300 µl of the stimulus preparation consisting of whole rotten fruit ('grape' and 'peach') diluted in H_2O_d (6 g ml^{-1}) and 300 µl of H_2O_d were placed on opposite ends of the Petri dish. To recreate the natural situation, prior to conducting the assays, whole-fruit preparations were left to decompose for 8 days at 25 ± 1 °C and 70% r.h. To prevent diffusion of odorants through the agar and to eliminate larval gustatory responses, the filter paper discs containing stimuli were placed on inverted lids cut off from 15-ml Falcon tubes. Lid sidewalls were covered with black tape to avoid phototaxis. Seven minutes after their introduction, the larvae within a 30-mm radius from each filter disc and the larvae that remained between both 30-mm radii were counted. Olfactory responses tend to decline after 7 min, presumably as a result of saturation of the vapour phase (Rodrigues, 1980; Kaiser & Cobb, 2008).

A larval response index (LRI) was calculated for each Petri dish as $[(n_{fruit} - n_{water})/n_{total}] \times 100$, where 'n' designates the number of larvae and the subscripts indicate the sides of the Petri dish containing the whole-fruit stimuli (fruit) or H_2O_d (water), respectively. This index varies between −100 (total repulsion) and +100 (total attraction). LRI = 0 indicates indifferent behaviour. Larvae respond to odorants the same when in groups as when tested individually (Monte et al., 1989; Kaiser & Cobb, 2008); thus, there is no alteration of LRI due to the presence of the other individuals. All behavioural assays were performed between 14:00 and 16:00 hours, at 25 ± 1 °C, 42 ± 5% r.h., and $5.4 \pm 0.2 \times 10^5$ lux light intensity. Seven replicates in response to each of the whole-fruit stimuli used were performed for each line. The whole set of replicates was randomized into different assay sessions which consisted of 40–50 replicates each. Finally, it was not necessary to turn the plates to avoid positional bias, because a control test with a random mix of larvae from all lines using H_2O_d as stimulus on both sides of the plate indicated the absence of side effects (matched-pair t-test: 'grape' larvae: $t = -1.44$, $P = 0.18$; 'peach' larvae: $t = -0.3$, $P = 0.77$; both $n = 10$).

Statistical analysis

We used analysis of variance (ANOVA) to evaluate the sources of LRI variance in LOB among host fruit plantations and stimuli according to the model: $Y = \mu + H + L(H) + S + H*S + L(H)*S + Er$, where H is a fixed effect of host fruit plantation, L is the random effect of line nested in H effect, S is the fixed effect of stimulus, and Er is the error variance. A significant H effect indicates the existence of micro-geographical variation in LOB for *D. melanogaster* individuals that have been raised in different host fruits plantations. A significant L(H) effect indicates intra-population genetic variation, i.e., genetic differences among lines for the trait analysed. If S is significant but not the interaction term H*S, then phenotypic plasticity exists for olfaction in larvae derived from both types of fruit. If the interaction term H*S is significant, Tukey's post-hoc comparisons are needed to elucidate which of the two sets of larvae, the ones derive from 'grape' or from 'peach', shows phenotypic plasticity for the trait analysed. Finally, significant L(H)*S interaction indicates genotype*environment interaction (genetic variation for phenotypic plasticity) for LOB.

To further understand the contribution of lines derived from each of the two plantations to genetic variation and to genotype*environment interaction, two ANOVAs dividing lines by plantation of origin were performed according to the model: $Y = \mu + L + S + L*S + Er$, where L is the random effect of line, S is the fixed effect of stimulus, and Er is the error variance. A significant L effect indicates genetic differences among lines for the trait analysed and a significant L*S interaction indicates

genotype*environment interaction for LOB. All statistical analyses were performed using InfoStat (2008).

Results

The values of LRI obtained from the behavioural assays were positive for all lines analysed in response to both 'grape' and 'peach' whole-fruit olfactory stimuli (Figure 1). Thus, lines derived from both plantations were attracted to both stimuli, one originating from its host fruit and the other from the alien fruit.

ANOVA indicated that the main effects of stimulus and host were not significant (Table 1), whereas the interaction of host*stimulus (H*S) was (Table 1, Figure 1). In fact, mean LRI values gathering lines from the same plantation in response to stimulus of the original host are lower those in response to the alien stimulus ('grape' derived lines: $LRI_{grape} = 22.16$, $LRI_{peach} = 32.74$; 'peach' derived lines: $LRI_{grape} = 28.30$, $LRI_{peach} = 24.80$), suggesting that the attraction towards the alien stimulus is stronger for larvae from both plantations. However, only larvae derived from 'grape' exhibited significant differences in LOB to stimuli, indicating stronger attraction to the alien stimulus ('peach') than to 'grape' (Tukey's test: $P<0.05$; Figure 1). This pattern indicates that larvae derived from the 'grape' plantation have phenotypic plasticity for larval olfactory response. The general ANOVA also revealed that 3% of total phenotypic variance is attributable to the significant contribution of the L(H) factor (Table 1). This result suggests that the natural population analysed harbours genetic variation for LOB when using whole-fruit preparations as olfactory stimuli.

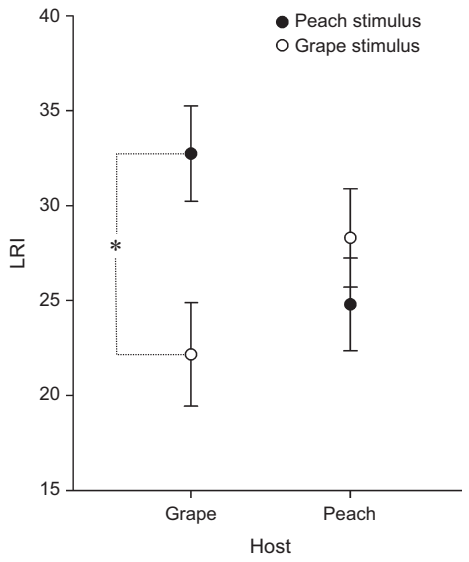

Figure 1 Olfactory response of *Drosophila melanogaster* larvae of half-sib lines derived from fruits of a grape plantation and a peach plantation, both near Lavalle, Argentina. Mean (± SE) larval response index (LRI) for all lines analysed in response to peach and grape whole-fruit stimuli. The asterisk indicates a significant difference between means (Tukey's test: $P<0.05$).

To elucidate whether the trends detected by the general ANOVA are similar between the two plantations, we assessed the relative contribution of differences among lines (genetic variance) and the Line*Stimulus (L*S) interaction (genotype*environment interaction) to total phenotypic variation separately for each fruit plantation. The analyses indicated that the contribution of the various factors to LOB variation differs between the two plantations (Table 2). Lines derived from 'grape' differed for the Stimulus factor, indicating that LOB exhibits phenotypic plasticity (Table 2, Figure 1). This is consistent with the outcome of the general ANOVA and the Tukey's post-hoc comparison as shown previously. Larvae derived from 'peach' harbour genetic variation, because a significant L*S term was found (Table 2). This genotype*environment interaction explains 18.3% of total phenotypic variance. A reaction norms plot for larval olfactory response to 'peach' and 'grape' whole-fruit stimuli for lines derived from 'peach' indicated that changes in rank order play a major role for the genotype*environment interaction (Figure 2).

Table 1 General analysis of variance for larval olfactory responses to grape and peach whole-fruit stimuli (Stimulus) in *Drosophila melanogaster* half-sib lines (Line) derived from 'grape' or 'peach' plantations (Host)

Source of variation	d.f.	MS	P
Host	1	53.72	0.79
Stimulus	1	1016.23	0.19
Host*stimulus	1	3287.35	0.005
Line (host)	17	709.66	0.041
Line (host)*stimulus	17	536.29	0.20
Error	228	413.64	

Table 2 Analysis of variance for larval olfactory response to grape and peach whole-fruit stimuli (Stimulus) in *Drosophila melanogaster* half-sib lines (Line) separated by origin (grape and peach plantation)

Source of variation	Grape			Peach		
	d.f.	MS	P	d.f.	MS	P
Line	9	832.23	0.08	8	571.78	0.13
Stimulus	1	3917.25	0.003	1	386.33	0.52
Line*stimulus	9	250.63	0.85	8	857.66	0.018
Error	120	469.03	–	108	352.09	–

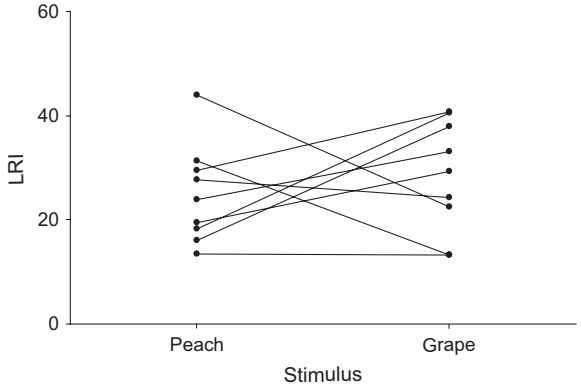

Figure 2 Reaction norms for larval response index (LRI) to grape and peach whole-fruit stimuli for *Drosophila melanogaster* half-sib lines derived from the peach plantation.

Discussion

Our behavioural assays demonstrated the existence of phenotypic and genetic variation for LOB in a natural population of *D. melanogaster* and, more importantly, that host fruit variation at a micro-geographical scale contributed to the LOB variation patterns. Specifically, the differences in phenotypic plasticity and genotype*environment interaction for *D. melanogaster* LOB in response to complex olfactory stimuli are associated with fruit heterogeneity in the natural environment. In a previous study, Lavagnino et al. (2008) observed both phenotypic and genetic variation for *D. melanogaster* larval and adult olfactory behaviour, using benzaldehyde as a standard odorant. In that investigation, lines were collected from various natural populations across Argentina (one of which is the one surveyed in the present study). Lavagnino et al. (2008) postulated that host fruit heterogeneity could contribute to variation patterns both within and between populations and to the difference between larval and adult variation in olfactory response. The current results provide evidence in favour of this hypothesis and indicate that host fruit heterogeneity plays an important role in larval olfactory response variation patterns.

Our results indicated the existence of larval behavioural responses depending on the host fruit plantation from where lines were derived. Behavioural responses to the two whole-fruit stimuli are predicted to differ because volatiles emanating from 'grapes' and 'peaches' are known to be different. The most intense odorants in 'grapes' are β-damascenone, hexanal, (*Z*)-3-hexen-1-ol, (*E*,*Z*)-2,6-nonadienal, and β-ionone (Fan et al., 2010), whereas in 'peach' the more frequent volatiles are trans-2-hexen-1-ol, hexyl formate, ethyl acetate, hexanal, and trans-2-hexen-1-al (Cheng et al., 2012); δ-decalactone is less abundant, but a major contributor to the overall aroma of 'peaches' (Horvat et al., 1990; Cheng et al., 2012). Moreover, this prediction of divergent LOB to different fruit odours is independent of the origin of larvae; however, this pattern was only found for 'grape'-derived larvae, indicating that there are considerable phenotypic differences for olfaction at short distances.

We have shown that lines derived from 'grape' and 'peach' plantations have genotype-specific characteristics related to larval olfaction, because 'grape'-derived genotypes expressed phenotypic plasticity, whereas 'peach'-derived genotypes revealed genotype*environmental interaction (i.e., genetic variation for phenotypic plasticity) for LOB. Therefore, the variational properties of genetic architecture of LOB (Hansen, 2006) depend on environmental heterogeneity displayed by 'grape' and 'peach' plantations (host fruits where individuals breed in nature). Considering that previous studies demonstrated that adult *D. melanogaster* flies have a dispersion capacity exceeding the 3.1-km distance that separates the plantations (Coyne et al., 1982; Coyne & Milstead, 1987) and the absence of visible geographical barriers to gene flow (JJ Fanara, pers. obs.), we assumed that gene flow between flies from the two plantations is not zero and, therefore, genotype-specific characteristics could be considered as intra-populational change in LOB genetic architecture occurring at micro-geographical distances. Distances similar to the one in the present study were considered as micro-geographical distances for *D. melanogaster* in previous studies in which genetic and phenotypic variation in different traits were quantified (Sokolowski & Carton, 1989; Karan et al., 1999). Studies using molecular markers could estimate the actual gene flow between the plantations.

Interestingly, larvae derived from the 'grape' plantation exhibited stronger attraction when the stimulus was 'peach' than when it was 'grape'. It has been proposed that larvae can learn about ecologically relevant traits (i.e., conditioned behaviour) and that such phenomena increase larval fitness (Thorpe, 1939; Quinn et al., 1974; Dukas, 1998; Davis, 2008). Retention of experiences could enable adults to locate oviposition sites with a positive impact on larval viability; for example, 'peaches' could form a longer-lasting and more nutritious resource than 'grapes'. However, there is no conclusive evidence that exposure to a conditioning stimulus in the larval stage induces a change in behaviour of adult *Drosophila* flies (Hershberger & Smith, 1967; Dukas, 2008). Besides, the lines used in our study were kept in vials containing laboratory rearing medium for more than 30 generations and were never exposed to the host fruits used as stimuli until the initiation of behavioural assays. We cannot rule out a scenario

of larval olfactorily conditioned behaviour in the natural population of *D. melanogaster* where 'peach' and 'grape' breeding sites coexist at short range, but our experimental design was not made to detect this. Then, how to explain that larvae derived from a given host fruit and reared in laboratory medium without any of the host fruits, display stronger olfactory responses to an 'alien' fruit stimulus than to its host fruit stimulus? On the one hand, a particular fruit may differ in its attractiveness to adults vs. larvae. Thus, oviposition site preference and adult olfaction may not be positively correlated with larval olfaction preference. Decoupling of larval and adult genetic architecture for olfactory behaviour has been demonstrated (Gerber & Stocker, 2007; Vosshall & Stocker, 2007; Zhou et al., 2009; Lavagnino et al., 2013), which would support the independence of adult olfaction preferences and larval responses. On the other hand, in the area where flies were collected 'grapes' are more abundant than 'peaches' (JJ Fanara, pers. obs.). If host preference is not strong, it is likely that flies utilize 'grapes' as breeding site more frequently than 'peaches'. Considering these circumstances, when *D. melanogaster* larvae are confronted with a 'novel' environmental cue (for instance, 'peach' as whole-fruit stimulus for larvae that developed in 'grape') an overexpression of olfactory phenotype could occur. Such a behavioural response is consistent with a scenario where a rapid location of feeding sites in new environments with novel resources is called for, for instance in cases of dispersion through human fruit commerce or environmental catastrophes that impose excessive movements to larvae.

As far as we know, this constitutes one of few studies in which intra-populational change of LOB genetic architecture variational properties at micro-geographical distances has been found. Future studies should incorporate analysis of intra-population heterogeneity in environmental factors, such as breeding host, to improve our understanding of the dynamics of phenotypic evolution.

Acknowledgements

This study was supported by grants from Universidad de Buenos Aires and CONICET. NJL is a recipient of a postdoctoral scholarship from CONICET (Argentina) and JJF is a member of Carrera del Investigador Científico of CONICET (Argentina).

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