

## Effect of laboratory acclimation on the variation of reproduction-related characters in *Drosophila melanogaster*

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### SUMMARY

The natural variation of sex-specific characters between populations can favor their behavioral isolation, eventually leading to the formation of new species. Marked variations for male courtship, mating and the production of sex pheromones – three complex characters potentially inducing sexual isolation – were found between *Drosophila melanogaster* populations of various origins acclimated for many generations in research laboratories. However, the natural variation of these three characters between natural populations and their evolution after long-term acclimation in the laboratory remains unknown. We measured many traits involved in these characters in six stocks initiated with distinct populations sampled in a restricted geographic area. Several sex-specific traits varied between stocks freshly brought back to the laboratory. After 100 generations spent in the laboratory without any experimental selection, traits varied in a strain-dependent manner. This variation was not related to a reduction of their variance except for copulation duration. This indicates that reproduction-related characters can diverge between neighboring *D. melanogaster* populations, and differently adapt to stable laboratory conditions.

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Key words: *Drosophila*, courtship, laboratory acclimation, mating, natural variation, pheromone.

### INTRODUCTION

*Drosophila melanogaster* is a very favorable model species to study genes potentially involved in sensory communication related to courtship and mating (Hall, 1994; Hing and Carlson, 1996; Yamamoto et al., 1997; Yamamoto and Nakano, 1999; Greenspan and Ferveur, 2000; Hall, 2002; Moehring and Mackay, 2004). Given its short generation duration, it also allows the effect of experimental selection and that of laboratory acclimation on natural characters to be studied (Coyne and Orr, 2004).

Natural variability of cuticular hydrocarbons, of fitness characters or of olfactory responses in lines has been examined in lines either (1) collected in the field and freshly brought back to the laboratory, or (2) acclimated for many generations in the laboratory (Wu et al., 1995; Ferveur et al., 1996; Korol et al., 2000; Foley et al., 2007; Lavagnino et al., 2008; Yukilevitch and True, 2008). However, no long-lasting survey was performed to see how such sex-specific characters in wild-derived *D. melanogaster* stocks freshly collected in nature could evolve after a long period of acclimation to laboratory conditions (Charmantier and Garant, 2005; Hutter et al., 2008). The few *Drosophila* studies that have dealt with variation of sex-specific characters in natural populations before and after laboratory acclimation were carried out on *Drosophila serrata*, *Drosophila birchii*, *Drosophila pseudoobscura*, *Drosophila mojavensis* and several other species living in the Sonoran Desert (Toolson and Kuper-Simbron, 1989; Magiafoglou and Hoffmann, 2003; Toolson et al., 1990; Stennett and Etges, 1997; Higgie et al., 2000; Etges and Jackson, 2001; Magiafoglou and Hoffmann, 2003). The effects of selection and drift were also studied on acoustic features of the male song between *Drosophila virilis* strains (Huttunen et al., 2008). If a very stable genetic polymorphism pattern was noted over several decades

especially for *Drosophila* species living in desert environments [*Drosophila nigrospiraculata*, *Drosophila pachea*, *Drosophila mettleri* (Pfeiler and Markow, 2001)], the cuticular hydrocarbon profiles of *D. pseudoobscura* and *D. serrata* rapidly changed after few generations in the laboratory (Toolson and Kuper-Simbron, 1989; Higgie et al., 2000). However, neither study has simultaneously measured the long-term effect of laboratory acclimation on sexual behavior and on cuticular pheromones.

To answer this question, we collected *D. melanogaster* flies in geographically close places (mostly in north-east Burgundy vineyards) during the autumn of 2002, and we established six wild-derived stocks. In these stocks, we measured different aspects of (1) male courtship and (2) mating behavior in homotypic pairs, (3) male and female locomotor activity – a general non-sexual behavior, and (4) cuticular hydrocarbon production in mature flies of both sexes. After a five-year period of acclimation in the laboratory (representing about 100 generations), and without any experimental selection, we measured the same phenotypes. Therefore, the principal goal of this study was to determine the general effect of long-term lab acclimation on the natural variability of sex-related characters in *D. melanogaster* flies.

### MATERIALS AND METHODS

#### Fly collection and maintenance

Wild-type flies (*Drosophila melanogaster* Meigen) were collected late October 2002 either on rotting stocks of grapes from Burgundy vineyards [Chambertin (CHB), Marsannay (MAY), Mercurey (MER) and Morey-Hautes-Côtes (MHC)] or from compost in Antigny-la-Ville (ANT) and Dijon (DIJ) (see Fig. 1 for collection sites). On the following day, adult flies were aspirated (without

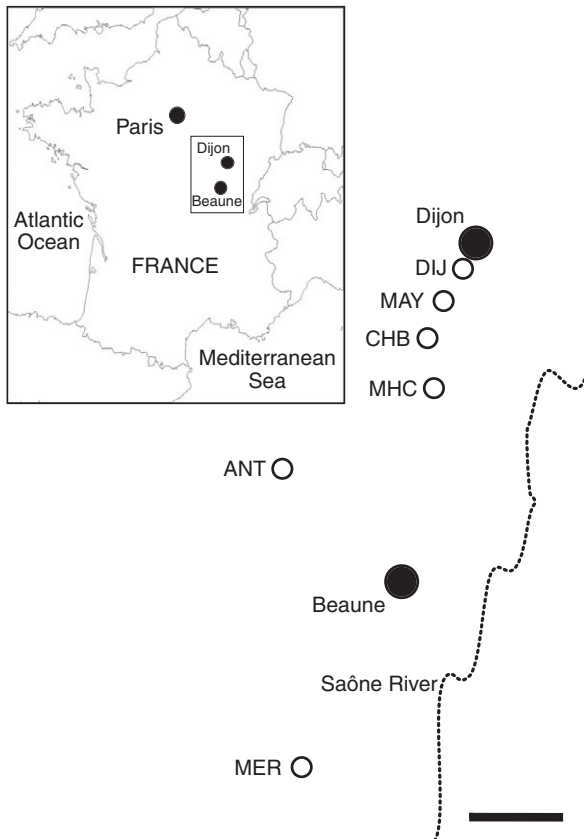


Fig. 1. Simplified map of the *Drosophila* collection area. Flies were picked up in France, in north-east Burgundy around the cities of Dijon and Beaune. Abbreviations represent the following strains: DIJ, Dijon; MAY, Marsannay; CHB, Chambertin; MHC, Morey-Hautes-Côtes; ANT, Antigny-la-Ville; MER, Mercurey. The scale bar represents 10 km.

anesthesia) with a mouth aspirator and individually stored in a vial containing fresh food. To verify that mated females brought back to the laboratory belonged to the *D. melanogaster* species, a sample of their progeny was mated with a *D. melanogaster* laboratory strain (Canton-S). Depending upon this verification, F<sub>2</sub> progenies of 10–15 verified *D. melanogaster* females were pooled and kept during one extra generation to allow populations to breed and to slightly expand before the first series of tests took place. All strains were raised in 150 ml glass vials containing 50 ml of yeast/cornmeal/agar medium and kept in a breeding room at 24±0.5°C with 65±5% humidity on a 12h:12h light:dark cycle. Each strain consisted of three or four independent replicates (vials) with crowded population conditions. To avoid overlap between generations, flies were transferred every 12–13 days to fresh food vials. Laboratory conditions and food composition were kept constant between 2002 and 2007.

### Behavioral assays

#### Courtship and mating tests

All flies were screened and isolated under light CO<sub>2</sub> anesthesia, 0–4 h after eclosion. To avoid sexual experience with other males (Svetec and Ferveur, 2005), tester male flies were held individually whereas females were kept in groups of five flies, in fresh glass food vials for five days, before testing.

All behavioral experiments, performed with heterosexual pairs of 5 day old flies, were carried out in the breeding room with conditions described above. For each strain, and to mix experimental

variability, tests were performed over several days, while the six strains were simultaneously tested. Tester males were individually aspirated (without anesthesia) under a watch glass used as a courtship observation chamber (1.6 cm<sup>3</sup>). After 10 min, necessary for the male habituation to the chamber, a virgin intact female was introduced and the observation period started (=t<sub>0</sub>). During the first 10 min of each behavioral test, we precisely measured the male latency to court (lapse of time between t<sub>0</sub> and the onset of courtship) and the total duration of male courtship expressed as the courtship index (CI). CI is the proportion of time that the male spends in active courtship (tapping, wing vibration, licking and attempting copulation). The CI was measured during a 10 min period unless copulation occurred before. Only males showing a minimum activity (CI>5) were taken into account to calculate the active courtship index (Act-CI). Moreover, to estimate the intrinsic sexual ardor of courting males during their period of active behavior (after courtship latency), we also weighted the Act-CI. The weighted Act-CI or W-Act-CI=[Act-CI/(courtship latency–courtship end)×total observation period]. CI was always measured in males paired with intact females. N≥30.

#### Copulation

We used the same pairs of courting flies to determine their copulatory behavior, during a one-hour observation period. Basically, we measured the male latency to copulate (the lapse of time between t<sub>0</sub> and the copulation onset), the duration of copulation (time in min from the copulation onset until disengagement) and the mating frequency for each strain. N≥25.

#### Locomotor activity

To evaluate male and female general activity, we measured the locomotor activity of single 5 day old flies. Each fly was introduced in a courtship chamber (1.6 cm<sup>3</sup>) placed over a pattern delimited in 12 equal areas. After a habituation period of 5 min, we counted the number of lines crossed (or of areas visited) during five periods of 10 s, and the total count number (during 50 s) was used as the individual locomotor activity index. This measurement was simultaneously and sequentially performed on four flies of different genotypes during a total period of 5 min. We only measured the locomotion of DIJ, MAY, CHB and MHC flies because the two former strains, respectively, showed the highest and lowest courtship intensity whereas the two latter strains showed intermediate values. Locomotion and mating tests always took place 1–4 h after lights on. N=16–39.

#### Hydrocarbon extraction and analysis

Cuticular hydrocarbons (CHs) were extracted from 5 day old intact individual flies by gas chromatography following a brief wash in hexane according to standard procedures (Ferveur, 1991). Analyses were performed with a Varian CP3380 chromatograph (Walnut Creek, CA, USA), equipped with a Cp-sil 25 m capillary column with hydrogen as the carrier gas. All the *D. melanogaster* predominant CHs have already been identified and characterized (Antony and Jallon, 1982; Pechiné et al., 1985). Twenty-four CHs were systematically detected in female flies, and 14 in male flies, both with a chain length ranging from 23 to 29 carbons (Marcillac et al., 2005). Each CH was characterized both by its percentage relative to the sum of all CHs (ΣCH) and to the area of an internal standard (hexacosane) used to calculate its absolute amount (in nanograms). For the sake of clarity, we only show the amounts of the predominant compounds: 7-tricosene (7-T, 23C), 7-pentacosene (7-P, 25C), 7,11-heptacosadiene (7,11-HD, 27C), 7,11-nonacosadiene (7,11-ND, 29C), n-tricosane

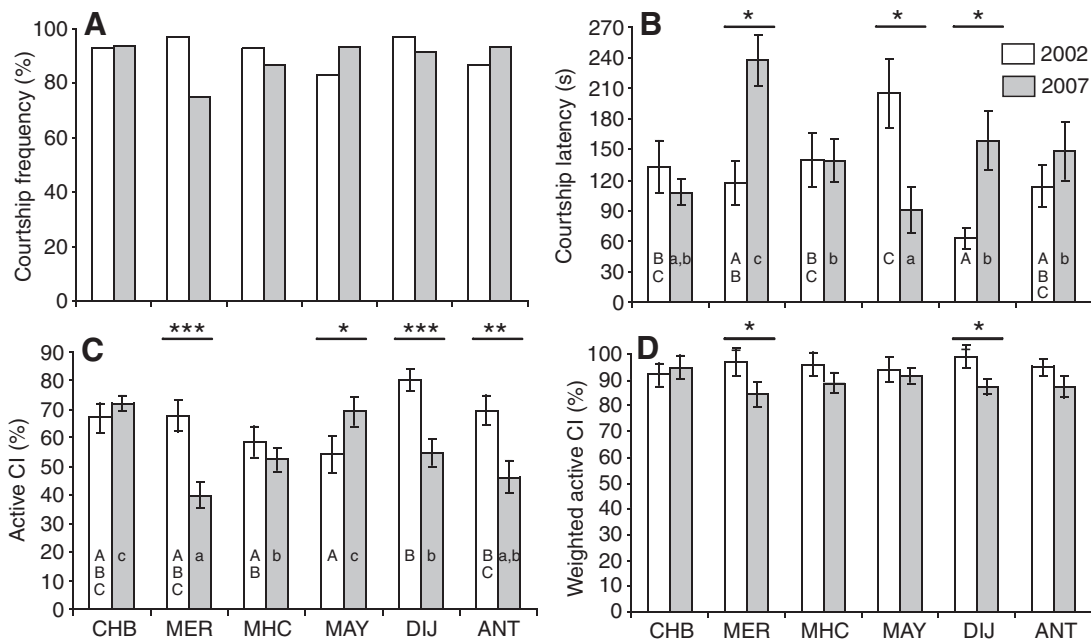


Fig. 2. Heterosexual courtship in six wild-type strains before and after long-lasting laboratory acclimation. Courtship tests always involved heterosexual pairs of intact 5 day-old flies observed during 10 min. We measured the frequency of courting pairs (%; A), their latency to initiate courtship (in s; B), the male courtship intensity (or courtship index=CI), which was calculated only from actively courting males (active CI; C) and was 'weighted' during the period of behavioral activity (weighted active CI; D). The histograms shown on items B, C and D represent the means ( $\pm$ s.e.m.) in the various strains. The data were obtained in 2002 (empty bars) and in 2007 (shaded bars). For each item, ANOVA (with Bonferroni *post-hoc* tests) was carried out to estimate the difference between strains compared for each date (differences are indicated by different – uppercase for 2002, lowercase for 2007 – letters inside the bars) and within each strain between years (shown over the relevant histograms bars with significance at  $P < 0.001$ \*\*\*;  $P < 0.01$ \*\*;  $P < 0.05$ \*). For strain abbreviations, see Fig. 1.  $N = 31$ –43.

(23Lin), n-pentacosane (25Lin). We also show the sums of all CHs ( $\Sigma$ CH), of all unsaturated CHs ( $\Sigma$ Desat) and of the three principal saturated CHs ( $\Sigma$ Lin=n-tricosane[23Lin]+n-pentacosane[25Lin]+n-heptacosane[27Lin]). A more complete analysis of male and female CHs is shown, respectively, in Tables S1 and S2 in the supplementary material.

#### Statistical analysis

To compare CIs (Act-CI, W-Act-CI) and copulation duration, we used two-way analyses of variance (ANOVAs) completed by a multiple pair-wise comparisons using Bonferroni *post-hoc* tests. The data obtained for latencies (courtship and copulation), for locomotor activity and for CHs levels were compared with Kruskal–Wallis tests. Each data point obtained for copulation kinetics was compared with an homogeneity Chi-square test. The variance and coefficient of variation (c.v.=standard deviation/mean) were compared between 2002 and 2007 flies with a Mann–Whitney test (Excel Stats, Microsoft Corporation, Redmond, WA, USA). Only  $P$  values  $< 0.05$  were considered to be statistically significant.

#### RESULTS

The two principal objectives of our study were (1) to measure and compare several reproduction-related characters (male courtship, mating ability, sex-pheromone production) in six *D. melanogaster* populations freshly collected in nature in October 2002 ('2002 strains'), and (2) to assess whether these characters (measured in November 2007; '2007 strains') could change without experimental selection after about 100 generations spent in the laboratory.

#### Male courtship behavior

In 2002 strains (empty bars; Fig. 2), between 83% and 97% males courted homotypic target females. The latency to initiate courtship was three times faster for DIJ males (63 s) than for MAY males (205 s) whereas the four other strains showed median values (114–140 s; Fig. 2B). The Act-CI (see Materials and methods) variation was reciprocal to that noted for courtship latency: MAY males showed a much lower intensity (54%) than DIJ and ANT males (respectively, 80% and 69%) whereas the three other strains showed median values (59–68%). This 'Act-CI' difference may largely result from the variation of courtship latency between strains because the W-Act-CI revealed no strain difference (range=92–99%).

2007 strains (shaded bars; Fig. 2) significantly varied for specific courtship aspects both (1) between strains, and (2) with the respective 2002 strains. Moreover, the differences initially noted between 2002 strains were not constant in 2007. Globally, 75–94% 2007 males courted intact homotypic target females; These frequencies were similar to those observed in 2002 strains ( $\chi^2 = 15.84$ , d.f.=11,  $P = 0.1689$ ). If no significant difference was detected for courtship frequency either between 2007 strains or within strains (between 2002 and 2007), 2007 MER flies showed a marked tendency to decrease their courtship frequency (75%) in comparison (1) with the five other 2007 strains (range: 86–94%), and (2) with the 2002 MER strains (97%). Moreover, 2007 MER flies drastically increased their courtship latency (237 s) – whereas 2007 MAY flies showed a much shorter latency (91 s) – than the four other strains ( $K_{11,d.f.} = 41.910$ ;  $P < 0.0001$ ). 2007 MER, DIJ and ANT males showed a decreased 'Act-CI' compared with respective 2002 males ( $F_{5,339} = 6.25$ ;  $P < 0.0001$ ) whereas this slightly increased in 2007

MAY males. The W-Act-CI of 2007 MER and DIJ males also decreased relatively to 2002 males ( $F_{5,339}=9.951$ ;  $P=0.0018$ ). A comparison of the variance between 2002 and 2007 (carried out with two statistical tests; see Materials and methods) revealed that only the W-Act-CI slightly increased in 2007 ( $P=0.041$ ; Table 1).

### Copulatory behavior

In 2002 strains, the mean copulation latency ranged between 9.5 min and 14.6 min ( $K_{11d.f}=43.693$ ;  $P<0.0001$ ). The only significant difference was noted between MHC flies, which mated much faster (9.5 min) than ANT flies (14.2 min; Fig. 3A). This difference is reflected by the different mating frequency between MHC and ANT

flies (respectively, 70% and 40%) during the first 10 minutes of mating (Fig. 3C). However, no difference was observed after one hour. Among 2002 strains, CHB flies showed a longer copulation duration (19.6 min; Fig. 3B) than MAY and ANT flies (respectively, 17.6 min and 17.7 min).

All copulation parameters showed enhanced variations in 2007 strains. Copulation latency was significantly longer in CHB and MER flies (respectively, 22.3 min and 16.3 min) than in MAY flies, which mated faster (8.9 min; Fig. 3A). These variations are consistent with the marked difference of copulation frequency noted between MAY and CHB flies during the first 10 min (respectively, 63% and 12%; Fig. 3D). If some intra-strain frequency differences were also

Table 1. Comparison of the variance between 2002 and 2007 for several sex-related characters

	Coefficient of variance			Variance		
	2002	2007	<i>P</i>	2002	2007	<i>P</i>
<b>Courtship behavior</b>						
Courtship latency	0.95 (0.04)	0.91 (0.05)	NS	4.26 (0.54)	3.69 (0.94)	NS
Act-CI	0.42 (0.05)	0.48 (0.07)	NS	734.94 (84.15)	645.95 (99.55)	NS
W-Act-CI	0.15 (0.03)	0.24 (0.02)	0.041	236.73 (70.64)	459.51 (66.83)	0.041
<b>Copulatory behavior</b>						
Copulation latency	0.83 (0.06)	0.79 (0.01)	NS	103.07 (24.99)	110.20 (27.56)	NS
Copulation duration	0.21 (0.10)	0.15 (0.01)	0.004	14.28 (1.98)	6.64 (0.90)	0.002
<b>Locomotor activity</b>						
Male	0.27 (0.05)	0.29 (0.03)	NS	216.84 (37.40)	223.06 (37.68)	NS
Female	0.35 (0.03)	0.29 (0.02)	NS	243.09 (49.21)	163.20 (27.05)	NS
<b>Male cuticular hydrocarbons</b>						
7-T	0.19 (0.08)	0.09 (0.01)	NS	63.62 (38.71)	16.33 (3.26)	NS
23Lin	0.17 (0.03)	0.11 (0.01)	NS	5.15 (2.16)	3.51 (1.28)	NS
7-P	0.33 (0.03)	0.24 (0.03)	NS	4.97 (1.91)	5.35 (1.46)	NS
ΣDesat	0.14 (0.07)	0.08 (0.01)	NS	58.51 (42.34)	24.21 (3.17)	NS
ΣLin	0.19 (0.05)	0.13 (0.01)	NS	12.28 (5.71)	6.68 (1.95)	NS
ΣCH	0.22 (0.03)	0.21 (0.02)	NS	100525.42 (46367.53)	78919.65 (17567.15)	NS
<b>Female cuticular hydrocarbons</b>						
7.11-HD	0.19 (0.09)	0.26 (0.03)	NS	18.48 (6.91)	12.71 (6.40)	NS
23Lin	0.17 (0.04)	0.16 (0.05)	NS	6.77 (3.58)	6.07 (3.98)	NS
7.11-ND	0.26 (0.07)	0.36 (0.06)	NS	7.77 (3.29)	5.51 (0.96)	NS
ΣDesat	0.10 (0.01)	0.06 (0.03)	NS	7.77 (2.63)	23.89 (8.01)	NS
ΣLin	0.16 (0.02)	0.16 (0.02)	NS	9.21 (1.72)	16.03 (7.36)	NS
ΣCH	0.63 (0.03)	0.30 (0.08)	NS	83674.51 (29267.45)	279925.65 (142901.30)	NS

For each parameter, we measured the variance coefficient (c.v.; left columns) and the variance (right columns). The parameters are either related to courtship behavior (courtship latency; active courtship index=Act-CI; weighted active courtship index=W-Act-CI), to copulation (copulation latency; copulation duration), to locomotor activity (in male and female flies), to male and female cuticular hydrocarbons (7-tricosene=7-T; n-tricosane=23Lin; 7-pentacosene=7-P; 7, 11-heptacosadiene=7,11-HD; 7, 11-nonacosadiene=7,11-ND; sum of unsaturated hydrocarbons=ΣDesat; sum of saturated linear hydrocarbons=ΣLin; sum of detected hydrocarbons=ΣCH). Each value represents the mean (± s.e.m.). We show the probability value to estimate acclimation effect in the laboratory between 2002 and in 2007: it was only significant for W-Act-CI and copulation duration. For all other parameters, it was not significant (NS).

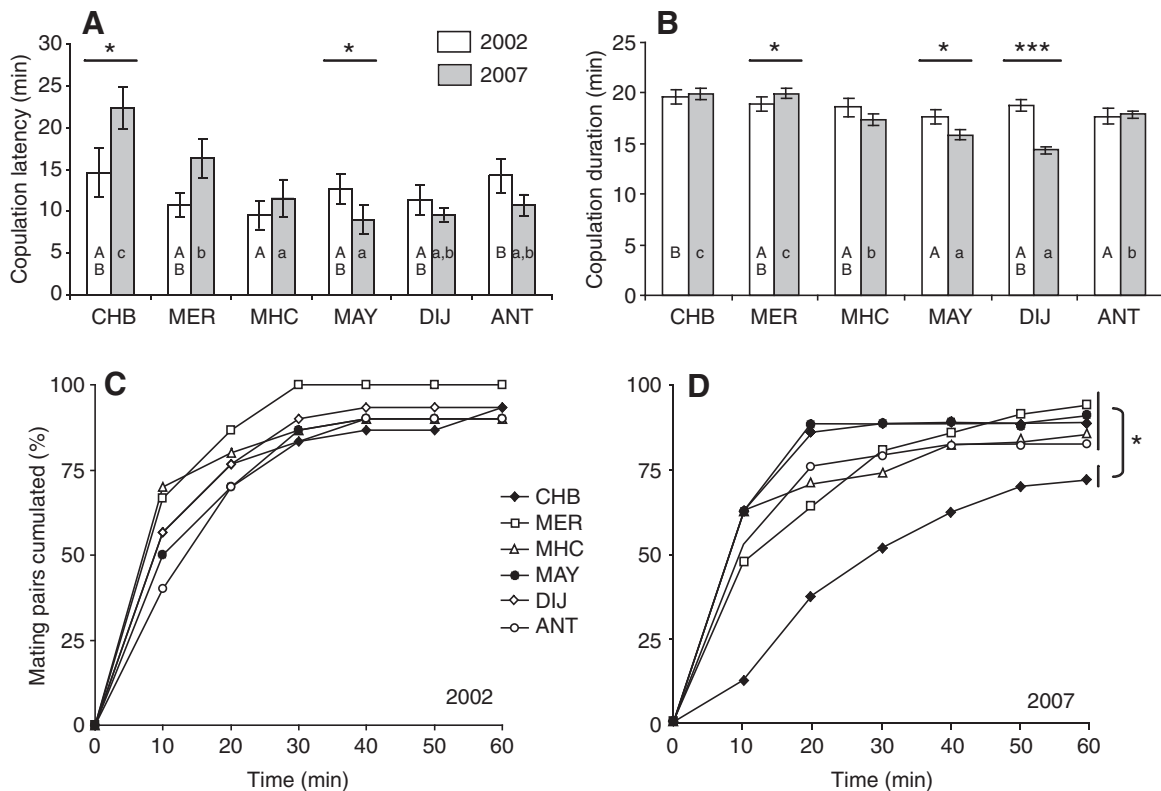


Fig. 3. Copulatory behavior in six wild-type strains before and after long-lasting laboratory acclimation. Mating tests always involved the heterosexual pairs of intact 5 day-old flies previously used for courtship tests (see Fig. 2), and lasted one hour. For each strain, we measured the latency to initiate copulation (in min; A), their duration of copulation (in min; B), and the cumulated frequency of mating pairs during one hour (shown every 10 min) in 2002 (C) and in 2007 (D). The histograms shown on items A and B represent the means ( $\pm$ s.e.m.) copulation latency and duration obtained in the various strains in 2002 (empty bars) and in 2007 (shaded bars). For each item, we tested the difference between strains for a given year (indicated by uppercase or lowercase letters inside the bars) and the difference between years within each strain (shown over the relevant histogram bars). In 2007, a difference (D;  $P < 0.05^*$ ) of mating frequency was only found after one hour between the Chambertin (CHB) strain and the five other strains. For abbreviations of strains, see Fig. 1; for statistical tests, see Fig. 2.  $N = 31-39$ .

sporadically detected between 2002 and 2007 (at 20 min for MAY; at 30 min and 40 min for MER), they tended to vanish before the end of the one-hour observation period. Conversely, 2007 CHB flies showed a strongly and durably decreased copulation frequency compared with both 2002 CHB and other 2007 strains: in 2002, 57% CHB flies mated within 10 min while in 2007 they needed 30 min to reach a similar frequency.

2007 flies also showed enhanced differences for their copulation duration (Fig. 3B). CHB and MER showed a longer duration (19.9 min) – and MAY and DIJ flies showed a shorter copulation duration (15.9–14.4 min) – than ANT and MHC flies (17.9–17.3 min). Moreover, the copulation duration strongly decreased between 2002 and 2007 ( $F_{5,341} = 6.939$ ;  $P < 0.0001$  – from 18.8 min to 14.4 min) in DIJ flies. A strong reduction of the variance was observed for the duration of copulation in the 2007 strains ( $P = 0.002-0.004$ ; Table 1).

#### Locomotor activity

In 2002, MHC males and females showed a lower locomotor activity than same-sex flies of the three other strains. Note that MHC male locomotor activity was only significantly different with CHB males (Fig. 4A). In 2007, only CHB males and females showed a high locomotor activity, which was similar to that of 2002 CHB flies, while the locomotion of most other flies tended to decrease. The decrease noted between 2002 and 2007 was only significant for MAY male and female flies (respectively,  $K_{7,d.f.} = 48.482$ ;  $P < 0.0001$

and  $K_{7,d.f.} = 21.386$ ;  $P = 0.003$ ). No difference of variance was detected with time (Table 1).

#### Cuticular hydrocarbons in male flies

2002 males showed a substantial variation for their  $\Sigma$ CH (empty bars; Fig. 5). These data are based on the statistical comparison of only four strains because no, or not enough, data were available, respectively, for 2002 ANT and DIJ males. The comparison of  $\Sigma$ CH between the four other 2002 males shows that CHB males produced about 2.5 more CHs ( $>1950$  ng) than MAY males ( $<800$  ng). In particular, CHB and MER males roughly produced three times more 7-T (1056–949 ng) than MAY males (356 ng). The former males also produced more 23Lin (233–270 ng) and 7-P (138–196 ng) than MAY males (respectively, 119 ng and 33 ng). However, the percentage of predominant CHs showed no, or much less, difference between strains. CHB showed a lower %23Lin – and MER males a higher %7-P – than other males. MAY males showed a (non-significant) tendency to decrease their overall % of desaturated CHs ( $\Sigma$ Desat) and to increase their overall % of saturated linear CHs ( $\Sigma$ Lin) compared with other males. Overall, these data reveal a great homogeneity of the male CH profiles despite a substantial variation of absolute amounts between strains.

In 2007, males showed significant CH differences that were not parallel to those found in 2002 (filled bars; Fig. 5). The  $\Sigma$ CH found in DIJ males was almost double (1941 ng) that of MER and MAY males (respectively, 1069 ng and 1247 ng). The  $\Sigma$ CH shown by 2007

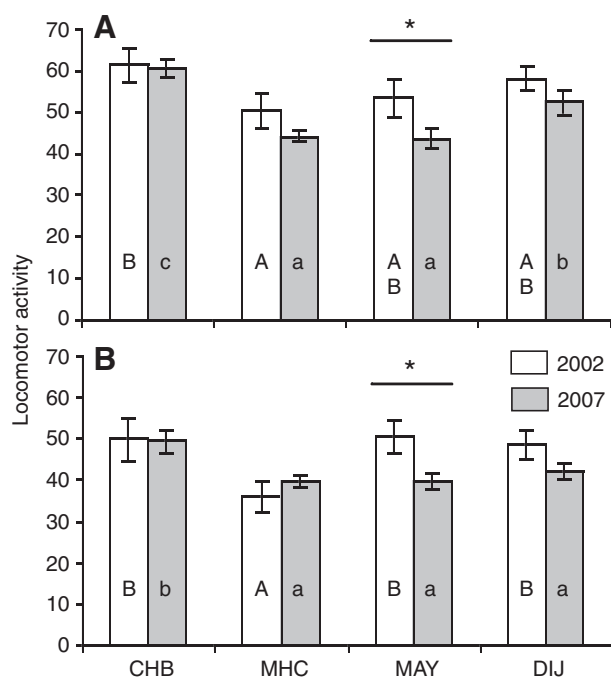


Fig. 4. Locomotor behavior in four wild-type strains before and after long-lasting laboratory acclimation. Locomotor activity tests always involved single intact 5 day-old flies observed during 5 min. We measured the number of lines crossed during a total period of 50 s (see Materials and methods) by male (A) and female flies (B) in 2002 (empty bars) and in 2007 (shaded bars). Data represent the means ( $\pm$ s.e.m.) in the various strains. For each item, we tested the difference between strains for a given year (indicated by uppercase or lowercase letters inside the bars) and the difference between years for each strain (shown over the relevant histogram bars with significance at  $P < 0.05^*$ ). For abbreviations of strains, see Fig. 1; for statistical tests, see Fig. 2.  $N = 16$ –39.

DIJ males was close to that shown by 2002 CHB and MER males. Between 2002 and 2007, the  $\Sigma$ CH of MER males decreased by almost 50% ( $K_{10d.f.} = 57619$ ;  $P < 0.0001$ ). This effect may be the result of the strongly decreased absolute amount of 7-T which dropped (from 949 ng to 434 ng) between 2002 and 2007 in MER males. Moreover, the amount of 23Lin doubled in MAY males (from 119 ng to 277 ng) during this period of time. These absolute quantity variations could explain the weak difference of  $\Sigma$ Desat and  $\Sigma$ Lin between 2007 strains, at the notable exception of 2007 MAY males, which significantly decreased their  $\Sigma$ Desat and increased their  $\Sigma$ Lin compared with both (i) other 2007 males and (ii) 2002 MAY males.

#### Cuticular hydrocarbons in female flies

Several CH parameters diverged between 2002 MAY and other 2002 females (empty bars; Fig. 6). First, 2002 MAY showed a high  $\Sigma$ CH (+350–750 ng), which was probably caused by the increased amount of CHs with shorter chains (23 carbons: 7-T, 23Lin; 25 C: 9-P, 7-P and 25Lin). Conversely, MAY females produced similar amounts or less CHs with longer chains (27C: 7,11-HD; 29C: 7,11-ND) than other 2002 females. MAY females produced 1004 ng of unsaturated CH representing a slightly lower  $\Sigma$ Desat (46%) than other females (50–52%). The amount of saturated linear CHs showed a reciprocal pattern in MAY (610 ng;  $\Sigma$ Lin=27.7%) and in other females (265–408 ng; 16.4–24.7%).

In 2007, females showed no difference for their  $\Sigma$ CH (filled bars; Fig. 6), indicating that the variation of absolute amounts noted in

2002 vanished in the laboratory. However, MAY females showed a CH profile that was highly different to that shown by other 2007 females ( $K_{10d.f.} = 25.266$ ;  $P = 0.0083$ ). This difference seems to be somewhat similar – but enhanced – to that detected between 2002 females. In particular, 2007 MAY females produced about half 7,11-HD and 7,11-ND (representing a total of 330 ng) if compared with other 2007 females (538–723 ng). Reciprocally, 2007 MAY females roughly produced much more saturated CHs (23Lin + 25 Lin + 27 Lin=642 ng) than other 2007 females (206–360 ng). This may explain why  $\Sigma$ Desat decreased (35.5%) and  $\Sigma$ Lin increased (38.8%) in 2007 MAY females compared with other 2007 females (respectively, 46.6–56.8% and 14.1–23.6%). No variance difference was detected between both dates, for any male or female CH parameter (Table 1).

#### DISCUSSION

Several studies have compared sex-specific characters between *D. melanogaster* populations, either in nature, or in the laboratory. The present study combines the two aspects. We found that traits involved in complex reproduction-related characters can (1) diverge between natural populations collected in neighbouring geographic locations, and (2) change in a population-specific manner after many generations in the laboratory. Differently to other studies (Harshman and Hoffmann, 2000; Sgro and Partridge, 2000), these changes were induced without any experimental selection – our principal goal consisted to determine the general effects induced by acclimation in the constant conditions of our laboratory.

#### Natural variability in distant and local environments

On a large geographic scale (>100 km), the existence of allelic variability – or of a natural polymorphism – for behavioral genes allows populations to react to environmental change within a few generations. This is particularly true for species (such as *D. melanogaster*) with a short generation cycle and living in a geographic area with marked climatic amplitude between seasons. The relationship between behavior and genetic variability was studied in genes such as *period* (*per*), *foraging* (*for*) and *desaturase* (*desat*). The clinal distribution of *per* alleles noted between populations collected in temperate areas is an adaptation to the climatic variation in these regions (Sawyer et al., 1997). The two natural *for* alleles coexist both in natural and laboratory populations and show a frequency-dependent selection (Fitzpatrick et al., 2007). The *desat1/desat2* tandem genes are involved in intraspecific variation of sex-specific CHs (Jallon and Pechiné, 1989; Ferueur et al., 1996; Coyne et al., 1999; Takahashi et al., 2001), and this variation may reflect the adaptation to climatic conditions (Gibbs; 2002; Savarit and Ferueur, 2002; Rouault et al., 2004).

A study revealed that *D. melanogaster* strains sampled from neighboring vineyards (in Germany) varied for their ability to learn associated sexual cues after 21 generations in the lab but the natural variation between these strains was not shown (Reif et al., 2002). The six populations studied here were collected in neighboring sites (<20 km), and their phenotypic variations may either reflect local adaptation to micro-ecological conditions or result from a random sampling within larger populations maintaining a substantial genetic variation. Future sampling of these natural populations in the exact same sites should indicate to which extend these characters remain stable over time (Sgro and Partridge, 2000). If the inter-population variation remains stable, this will reflect a constant environmental effect and be used as an ecological marker. The ecology and natural history of most *Drosophila* species seem to be yet insufficiently characterized to permit predictions with respect to population

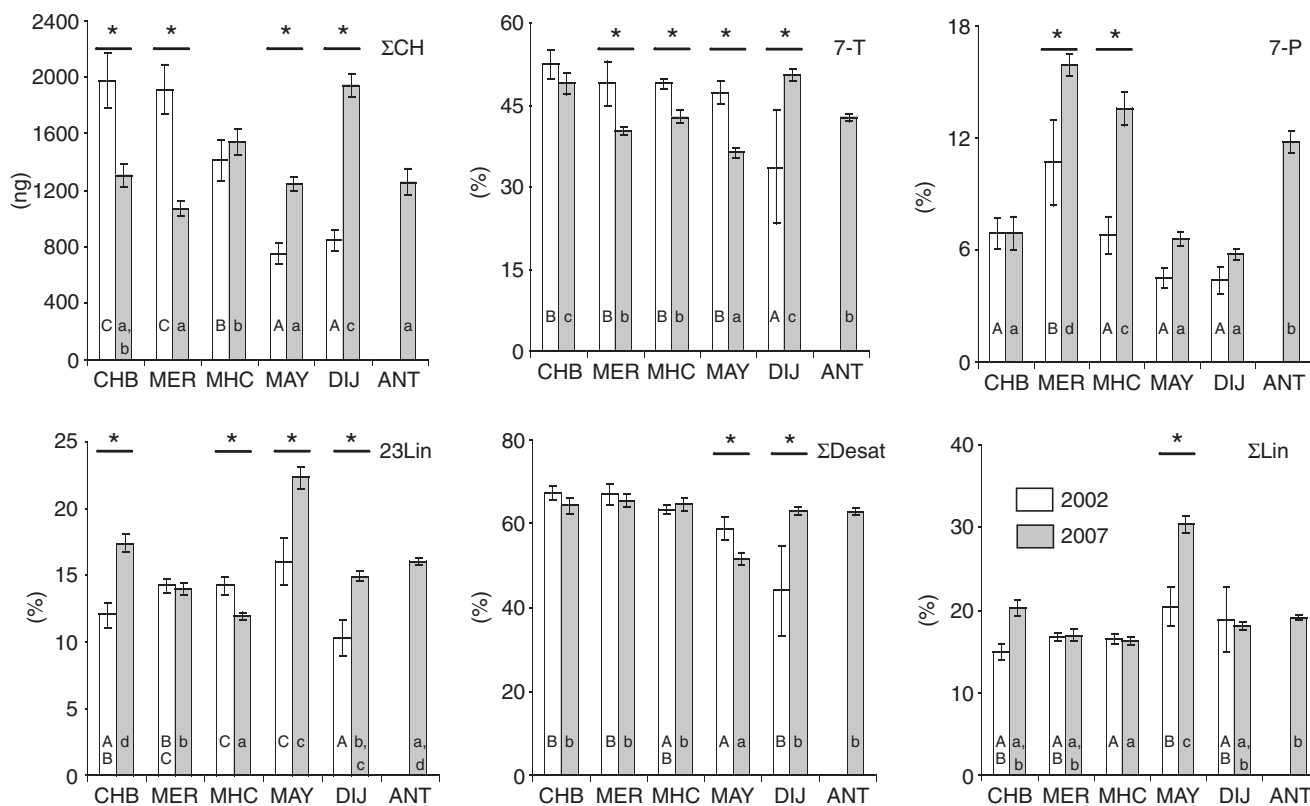


Fig. 5. Production of the principal cuticular hydrocarbons (CH) in males of six strains before and after long-lasting laboratory acclimation. We calculated the proportion (%) of the principal (group of) CH from the total amount of CH ( $\Sigma$ CH, in ng) detected in each individual fly, in the six strains in 2002 (empty bars) and in 2007 (shaded bars). CHs are 7-tricosene (7-T), 7-pentacosene (7-P), n-tricosane (23Lin), the sum of unsaturated CHs ( $\Sigma$ Desat) and the sum of saturated linear CHs ( $\Sigma$ Lin). Data represent the means ( $\pm$ s.e.m.) in the various strains. For each item, Kruskal–Wallis tests were carried out to compare the difference between strains for each date (differences are indicated by different – uppercase for 2002, lowercase for 2007 – letters inside the bars), and for each strain between years (shown over the relevant histogram bars with significance at  $P < 0.05$ ).  $N = 11–17$  (except for 2002 DIJ=3 and 2002 ANT=0). For strain abbreviations, see Fig. 1. A detailed analysis of male CHs is provided in Table S1 in the supplementary material.

genetic structure. An exception is *D. nigrospiracula* that has not population subdivision (Pfeiler et al., 2005). However, it is not surprising that the behavior of *D. melanogaster* adults diverge between natural populations because larval behavior can also vary between Chilean natural *D. simulans* populations only separated by a few meters (Godoy-Herrera et al., 1997). However, although the geographic distance between our strains ranged between 5 km and 40 km, we are not completely sure that these populations were indeed separated without gene flow between them. In this case, their apparent difference could only result of a sampling bias.

The variation of CH profiles noted either (i) between closely related species of flies of three *D. mojavensis* species living in the Mojave Desert, on varied cactus plants, or (ii) between populations of these species reflects the adaptation to different host plants (Etges and Jackson, 2001). In these flies, the ratio of the principal CHs rapidly changed with laboratory acclimation, and influenced courtship and mating (Stennett and Etges, 1997). These CH changes depend on enzymes whose level could represent a metabolic adaptation to host-plant chemicals (Higa and Fuyama, 1993; Jones, 2001).

#### Does laboratory acclimation induce specific or general effects?

A natural variability for the production of CHs was found either between larvae or adults, in several mosquito species. Some of these variations resulted from an exposure to insecticide or to a short-

term acclimation in the laboratory (Kamhawi et al., 1992; Anyanwu et al., 1997; Anyanwu et al., 2000). Here, the flies of six wild-derived populations differentially varied for their sexual behavior and CH production after many generations spent in the laboratory. If long-term acclimation to laboratory conditions affected, in a strain-specific manner, one or few trait(s) of these two complex phenotypes, no general change was observed. For example, both 2007 MER and DIJ flies showed a lower general ability to court than their 2002 ancestors but this had no detrimental consequence on copulatory ability. Moreover, MER and DIJ strains showed a reciprocal variation of copulation duration (respectively, increased and decreased between 2002 and 2007) but DIJ flies did not change their locomotor activity. Conversely, 2007 CHB flies strongly decreased their mating ability but this effect was apparently not related to any courtship defect. Taken together these results indicate that laboratory acclimation (i) affected, in a strain-dependent manner, some genetic factors underlying the realization of these behaviors, and (ii) these genetic factors are involved in specific – rather than in general – aspects of sexual behavior. As these complex phenotypes are polygenetically controlled, this suggests that the natural variation of a single gene is partly compensated by the action of several other genes acting in a common network (van Swinderen and Greenspan, 2005).

Moreover, the general stability observed for the variance of most characters (except copulation duration) indicates that the genetic variability of these strains was not drastically reduced with laboratory

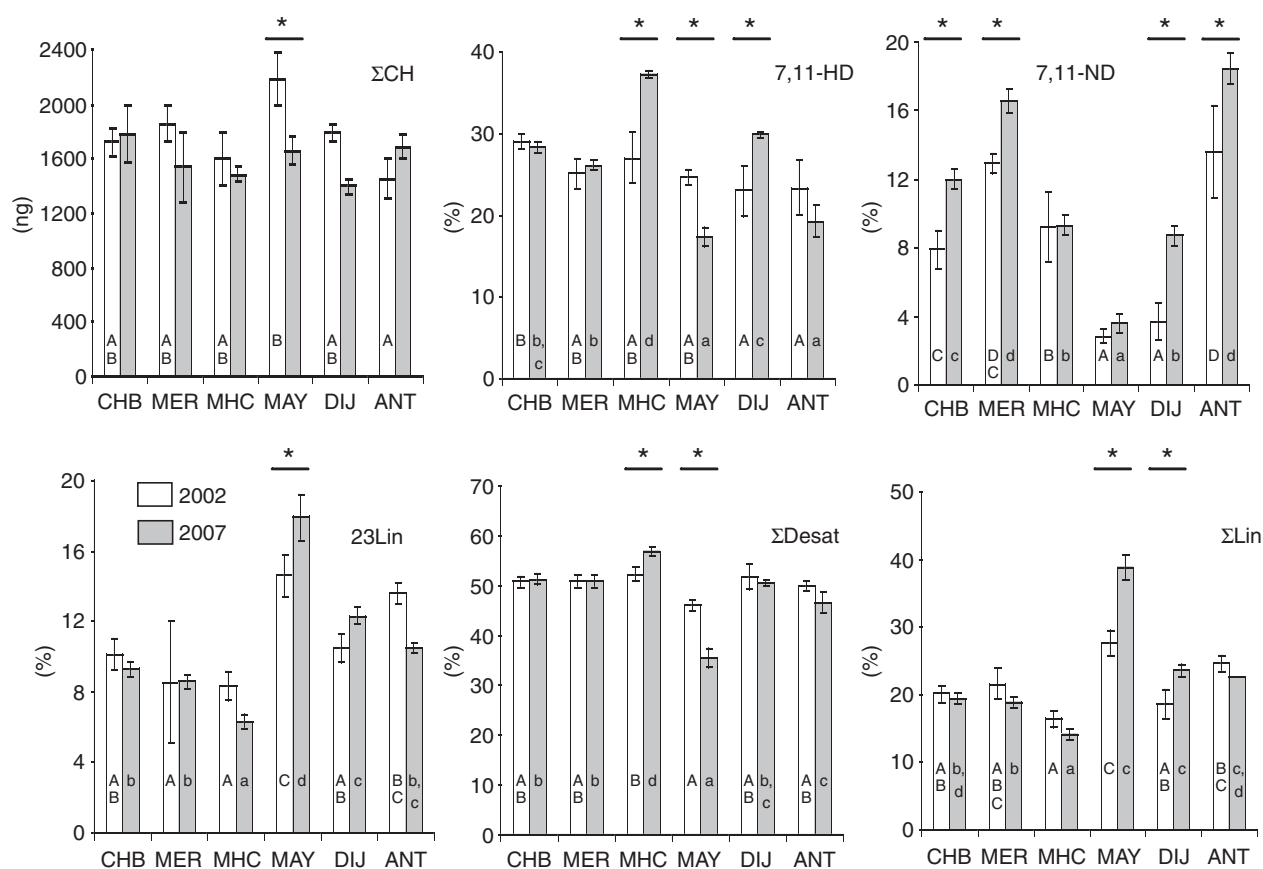


Fig. 6. Production of the principal cuticular hydrocarbons (CH) in females of six strains before and after long-lasting laboratory acclimation. We calculated the proportion (%) of the principal (group of) CH from the total amount of CH ( $\Sigma$ CH, in ng) detected in each individual fly, in the six strains in 2002 (empty bars) and in 2007 (shaded bars). These CHs are 7,11-heptacosadiene (7,11-HD), 7,11-nonacosadiene (7,11-ND), n-tricosane (23Lin), the sum of unsaturated CHs ( $\Sigma$ Desat) and the sum of saturated linear CHs ( $\Sigma$ Lin). Data represent the means ( $\pm$ s.e.m.) in the various strains. For strain abbreviations and statistical tests, see Figs 1 and 2.  $N=11-16$ . A detailed analysis of female CHs is provided in Table S2 in the supplementary material.

acclimation. Given that similar experimental conditions were kept between 2002 and 2007, the general phenotypic stability of these populations reflects a great stability of their genetic variability. This can be verified, for example, by comparing the variance of courtship frequency that varied between 0.79 (in ANT flies, Fig. 2A) and 0.91 (DIJ). This indicates that our method took into account at least 79% of the behavior (with less 21% errors or measurement), which is a satisfactory figure with this kind of experiment (Broadhurst, 1960).

However, laboratory acclimation induced a strongly decreased copulation duration in DIJ and MAY flies. The variability of the genes involved in this phenotype (MacBean and Parsons, 1967; Acebes et al., 2004) was possibly reduced with laboratory acclimation. Alternatively, the 'shorter copulation effect' may be linked to the increased overall percentage of linear CHs ( $\Sigma$ Lin) found in both DIJ and MAY females. This result, supported by a previous study (Marcillac and Ferveur, 2004), suggests that desaturated and linear female CHs induced different effects on the copulating male.

Among other changes occurring with laboratory acclimation, 2007 MAY male and female flies showed a decreased locomotor activity and this may explain their increased latency to court and mate. However, this variation had no detrimental consequence on both their courtship intensity and mating kinetics. Two major CH changes also occurred after laboratory acclimation. First, the global absolute amount of CH ( $\Sigma$ CH) changed with time but this may be related to parameters, such as larval density, that were not strictly

controlled [see the discussion in Ferveur (Ferveur, 1991)]. For this reason, we used CH relative amounts (%), which are more reliable than CH absolute quantities to compare strains raised in asynchrony (between 2002 and 2007). Second, MAY females and males showed a specific variation; while 2002 MAY flies showed a (non-significant) tendency to skew their ' $\Sigma$ Desat: $\Sigma$ Lin' ratio to a lower value, this variation was significantly enhanced in 2007 MAY flies. This suggests that the MAY strain originally contained genetic factors affecting the degree of CH desaturation (such as the *desat1* gene) (Marcillac et al., 2005), and their expression was increased in 2007 MAY flies.

This CH variation – possibly linked to the decreased copulation duration noted in MAY flies (see above) – represents the unique putative 'behavioral CH' link found either between populations compared at the same date or within populations between 2002 and 2007. This suggests that long-lasting laboratory acclimation separately affected the factors underlying the realization of specific traits involved in reproductive behavior from those governing the production of cuticular pheromones. The general behavioral and hydrocarbon phenotypic values obtained after lab acclimation remained stable, at least in the DIJ strain, which was used in several other studies (Sveteć et al., 2005; Grosjean et al., 2008; Houot, 2009).

Other authors showed that strains selected for a specific character can also vary for other unrelated characters, depending upon the experimental design (Harshman and Hoffmann, 2000; Sgro and



Partridge, 2000). Thus, the changes observed in our study may be at least partly contingent on the experimental conditions used in our lab. Moreover, as our mating experiment involved one pair of flies, we hypothesize that the use of two (or more) males with a single female – a situation that may often happen in nature – would have probably changed our behavioral data, as a result of a competition between males with different experience (Svetec and Ferveur, 2005). A strong effect of competition between *Drosophila* flies of different strains was indeed shown to change the relative isolation between strains (Coyne et al., 2005).

#### Selection or genetic drift?

After 23 generations of laboratory acclimation, *D. melanogaster* isofemale lines also showed a constant range of natural genetic variability, while the variability rate varied independently between genetic loci (Delpuech et al., 1993). *Drosophila subobscura* strains sampled from a small geographic area revealed that the laboratory evolution of traits related to fitness is less contingent on the foundation circumstances than traits reflecting an adaptation to the environment (Simoes et al., 2008). Here, many generations spent in the laboratory have apparently not reduced the potential range of variability of the genes involved in sex-specific characters. We do not know whether the ‘acclimation effect’ especially noted for the duration of copulation resulted from a selection process and/or from a genetic drift, which allowed some alleles to fix in 2007 populations.

We chose to initiate our populations with 10–15 founder females. With such a relatively small size (compared with the size of most meta-populations in the wild), the effect of the genetic drift should be more important than selection. However, when combining the theoretical data (Boulétrau, 1978) with the number of founder females and replicates (3–4) per strain, we assume that each strain was originated by about 150–200 F<sub>2</sub> individuals. Under the assumption that this number was kept more or less constant during the next 100 generations, with a sex ratio close to 1, this relatively higher number of founders (compared with single females initiating isofemale lines) should have minimized the effect of the genetic drift (Wright, 1969) and limited the impact of the bottleneck effect occurring immediately after laboratory acclimation (Swindell and Bouzat, 2005). In this case, the selection process should tend to decrease inter-population variability to allow phenotype convergence to an ‘optimal’ point. Such a selection hypothesis seems to be true only for the ‘female ΣCH’ trait which showed a general convergence between 2007 strains. All other traits, showing a strain-specific variation unrelated to a decrease of variance, could have been influenced by a genetic drift of a limited number of genes with reduced effects. This suggests that some alleles were randomly eliminated whereas others were fixed. Finally, we can almost formally rule out the effect of spontaneous mutation whose rate in *D. melanogaster* is infinitesimally low (between  $1.28 \times 10^{-6}$  and  $3.86 \times 10^{-6}$  per gene and per generation) (Halliburton, 2004).

In conclusion, we found that neighboring *D. melanogaster* populations can significantly diverge for complex reproduction-related characters. If long-lasting laboratory acclimation strain-dependently affected some sex-specific traits, the relatively moderate effect induced on CH variation contrasted with the dramatic changes reported in other *Drosophila* species. Therefore, and given the rapidly increasing number of studies dealing with *D. melanogaster* courtship, mating and sex pheromones in long-laboratory established wild type strains, it seems timely to assess the variability of these phenotypes in nature and to follow their evolution in the laboratory.

#### LIST OF ABBREVIATIONS

7,11-HD	7,11-heptacosadiene
7,11-ND	7,11-nonacosadiene
7-P	7-pentacosene
7-T	7-tricosene
23Lin	n-tricosane
25Lin	n-pentacosane
Act-CI	active courtship index
ANOVA	analysis of variance
ANT	Antigny-la-Ville
c.v.	coefficient of variation
CH	cuticular hydrocarbons
CHB	Chambertin
CI	courtship index
DIJ	Dijon
MAY	Marsannay
MER	Mercurey
MHC	Morey-Hautes-Côtes
W-Act-CI	weighted active courtship index
ΣCH	sum of cuticular hydrocarbons
ΣDesat	sum of unsaturated hydrocarbons

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