Title

Large-scale assessment of olfactory preferences and learning in *Drosophila melanogaster*: behavioral and genetic measures

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Abstract

In the Evolve and Resequence method (E&R), experimental evolution and genomics are combined to investigate evolutionary dynamics and the genotype-phenotype link. This approach requires many replicates with large population sizes, which imposes severe restrictions on the analysis of behavioral phenotypes. Aiming to use E&R for investigating the evolution of behavior in *Drosophila*, we have developed a simple and effective method to assess spontaneous olfactory preferences and learning in large samples of fruit flies using a T-maze. We tested this procedure on (a) a large wild-caught population and (b) 11 isofemale lines of *Drosophila melanogaster*. Compared to previous methods, this procedure reduces the environmental noise and allows for the analysis of large population samples. Consistent with previous results, we show that flies have a spontaneous preference for orange *vs*. apple odor. With our procedure wild-derived flies exhibit olfactory learning in the absence of previous laboratory selection. Furthermore, we find genetic differences in the olfactory learning with relatively high heritability. We propose this large-scale method as an effective tool for E&R and genome-wide association studies on olfactory preferences and learning.

Key words

Olfactory learning, olfactory preferences, *Drosophila melanogaster*, T-maze, experimental evolution, Evolve and Resequence, heritability

Introduction

Ongoing evolutionary dynamics and genotype-phenotype mapping can be studied during experimental evolution through subsequent phenotyping and genomic sampling [1,2]. This method is known as Evolve and Resequence (E&R) [3] and can be applied to entire populations by sequencing at the same times hundreds of individuals (Pool-seq, see Futschik and Schlötterer 2010) of the same population. Thanks to the advancements of high-throughput sequencing techniques, the E&R method has been used to track the changes in genomic composition not only across thousand of generations in bacteria [2] but also in eukaryotes with a fast life cycle, such as yeast [5] and fruit flies [6]. This approach provides a very promising opportunity to investigate the evolution of complex traits and their genetic architecture with a limited budget [7], thus paving the way to the analysis of the evolutionary dynamics of traits that cannot be inferred through fossil records, including complex behavioral phenotypes [8].

The first implementation of E&R on a complex behavior focused on phenotypic and genomic change in response to artificial selection for shorter/longer inter-pulse interval in male courtship song in *Drosophila melanogaster* [9]. In this study thousand of loci have been identified that responded to artificial selection and differed between populations selected for different behaviors. Similar outcomes, with thousand of alleles that significantly change in frequency between generations and treatments, have been found also for morphological or physiological traits [10,11], showing that the same methodological issues apply to behavioral and other traits. Despite the success in identifying some causative genes [e.g. 12,13], theoretical [6,14] and empirical evidence [11,15] has clarified that many of the significantly changed variants are in fact false positives derived by short or long-distance linkage disequilibrium. Another limit that E&R shares with other genome-wide approaches, is low

52 statistical power in identifying unknown causative variants [e.g. 14,16].

53 Although haplotype-blocks can be used to study the dynamics of selected genomic regions during
54 experimental evolution [15], an effective E&R study should primarily minimize the false positives
55 rate and maximize statistical power. As shown in recent theoretical and simulation work
56 [6,14,17,18], to reach this aim several issues have to be taken into account in the design of the
57 experiment: (a) use a large starting population (possibly hundreds or thousands of individuals); (b)
58 use a large population size; (c) use at least 5-10 replicate populations; (d) run the experiment for
59 dozens of generations; (e) reduce linkage disequilibrium. In the light of this, during E&R
60 researchers should phenotype and propagate thousands of individuals in multiple replicate
61 populations for many generations [8]. In E&R studies a practical limitation is imposed by the
62 stages of propagation and phenotyping: when flies have to be individually phenotyped and
63 manipulated, the time and working load required can force a reduction of the census size. For this
64 reason investigating behavioral traits, that often require a large effort in phenotyping, poses a
65 methodological challenge.

66 In this work we focus on the development of a fast and reliable method for phenotyping and
67 propagating fruit flies in E&R of olfactory behavior, in particular spontaneous olfactory
68 preferences and olfactory learning. Fruit flies show complex behaviors, can be easily maintained
69 at a large census size, have a fast generation cycle and low linkage disequilibrium [19], thus are a
70 convenient model to investigate the evolutionary dynamics and genotype-phenotype map of
71 behavioral traits.

72 Olfactory behavior (olfactory preferences and olfactory learning) is a good candidate for E&R
73 investigation of both spontaneous and learned responses, because of its remarkable conservation
74 between *Drosophila* and vertebrates [20,21] and the presence of standing variation for both
75 olfactory preferences and olfactory learning [22–24]. Genetic variability in the experimental
76 population is crucial to apply E&R to fruit flies, because within the time scale of feasible
77 experiments (50 generations of selection take about two years to be completed) new mutations

78 have little impact on evolutionary change.

79 Different preferences for specific odors and odor concentrations have been documented in *D.*
80 *melanogaster* using T-mazes [e.g. 25,26,27]. Phenotypic variability in olfactory behavior is
81 associated with polymorphisms that influence reactions to different compounds [28,29] but to date
82 E&R has not been applied to odor preferences [see 30 for a similar idea]. Olfactory learning has
83 been extensively studied at the behavioral, genetic and neurobiological level [20,31–33]. Wild and
84 mutant flies have been tested in associative conditioning tasks, typically the association between an
85 olfactory conditioned stimulus and an electric or mechanical aversive stimulus. In a first paradigm
86 developed to measure olfactory learning, Quinn and colleagues [34] tested groups of about 40 flies.
87 From a starting tube flies could approach a light source at the end of a second tube painted with odor
88 A (or B). When flies entered the second tube an electric shock was delivered and could be associated
89 with odor A (or B). After being returned to the starting tube, flies could enter a third tube containing
90 odor B (or A), that was not associated with any electric shock. At test flies could choose to enter
91 either a tube with odor A or one with odor B. A performance index compared the fraction of flies
92 that avoided the unpaired odor and those which avoided the shock-paired odor. Not all flies explored
93 the tubes, and their performance was affected also by phototaxis, thus this small-scale assay
94 produced low learning scores. Tully and Quinn [35] modified the paradigm to test groups of about
95 100 flies in an apparatus in which odor A matched with pulses of electric shock was followed by
96 odor B in the absence of electric shock. The odors were delivered by vacuum so that all flies were
97 exposed to the odor-shock contingencies. After training, the flies were tested in a T-maze where
98 could choose to approach either odor A or B. The learning scores for this procedure ranged between
99 0.7 and 0.9 [31] but the need of dedicated machines and hands-on operations on the flies limit the
100 application of this method to large-scale long-term experiments.

101 To date the main method used in experimental evolution for enhanced learning [36,37] is the
102 oviposition paradigm. This method is a medium/large-scale procedure based on the habit of flies to use
103 the same medium for foraging and egg laying. The procedure starts exposing hundreds of free ranging

104 flies to olfactory (or visual) stimuli associated to palatable or aversive media displaced in petri dishes
105 located in a box (e.g. orange juice smell associated with palatable food, apple juice smell associated
106 with aversive food). After exposure to olfactory (or visual) and associated gustatory stimuli flies are
107 tested for their olfactory (or visual) preferences for olfactory stimuli previously associated or not
108 associated with the aversive food. Compared to flies that do not remember the association, flies that
109 remember the contingencies presented during the exposure phase are expected to lay more eggs in the
110 substrate whose smell (or color) was never associated with aversive food. The proportion of eggs laid
111 in the substrate associated with the palatable flavor is used as a proxy for learning.
112 To select for enhanced learning across generations, Mery & Kawecki [36] rinsed, moved to a neutral
113 medium and propagated only the eggs laid in the medium previously associated with palatable food
114 (alternatively Dunlap & Stephens [38] displaced eggs individually with a needle). As effect of this
115 regime, in about 15 generations the proportion of flies which made the "correct" choice significantly
116 increased. In spite of this, several aspects make the oviposition procedure less than ideal for E&R
117 studies: (a) this method is prone to experimental noise, as shown by the lack of learning effects
118 before selection; (b) only females are exposed to selection, because learning is measured using laid
119 eggs, thus reducing the selective pressure to half of the propagated individuals and preventing to
120 investigate behavioral domains different from oviposition and sex effects; (c) the oviposition
121 paradigm imposes selection for fertility, egg laying during the few hours of the test and resistance to
122 egg washing; (d) this paradigm does not control for the experience provided during the conditioning
123 phases (many flies might not experience all stimuli before making a choice in the test phase); (e)
124 extensive work to rinse/displace the eggs and propagate the flies is required, in turn reducing the
125 number of experimental replicates that can be propagated.
126 Aiming to use E&R for investigating the evolution of behavior in fruit flies, we have developed a
127 simple and effective method based on a T-maze to assess spontaneous olfactory preferences and
128 learning in large samples (hundreds) of fruit flies of both sexes as well as in smaller samples (dozens
129 of individuals). We find evidence for spontaneous and conditioned preferences for olfactory stimuli

130 in a large population of *D. melanogaster* originally caught in South Africa and in a population of
131 inbred lines originally caught in Portugal. Our procedure reduces the impact of undesired selective
132 pressures and the effort in propagation and phenotyping. Furthermore this method is sensitive
133 enough to detect learning, spontaneous preferences and heritability of these traits. We discuss the
134 relevance of this T-maze based procedure for E&R and genome-wide association studies.
135
136 **Materials and methods**
137 ***Subjects***
138 All experiments were run on isofemale lines of the same species, *Drosophila melanogaster*. Flies
139 were maintained on standard cornmeal-soy flour-syrup-yeast medium, except during the
140 experimental assays. Before the beginning of the experiments we kept all lines for at least two
141 generations at 22°C in a constant 14:10 h light:dark cycle.
142 The population-wide experiments were ran on 670 lines derived from a natural population of *D.*
143 *melanogaster* collected in Paarl (South Africa) in March 2012. In each trial we used a group of
144 250 adult flies (males and females 2 days old or older), randomly picked from the 670 isofemale
145 lines of the South African population.
146 The individual-line experiments were ran on 11 inbred lines derived from a *D. melanogaster*
147 population originally collected in Povoa de Varzim (Portugal) in July 2008. Before inbreeding,
148 B101, B192 and B211 were maintained in the laboratory as isofemale lines. R1-R10 were derived
149 from the same original population after being exposed to an experimental evolution procedure at
150 different temperature regimes. The hot and cold temperature regimes are described in Orozco-
151 terWengel et al. [10] and Tobler et al. [11]. Before inbreeding, R1-R5 entered the hot temperature
152 treatment, whereas R6-R10 entered the cold temperature treatment. Inbred lines were generated
153 through full-sib mating for 17 or more generations (B101: 17 generations; B192: 18 generations;
154 B211: 19 generations; R1 and R3: 27 generations; R2: 29 generations; R5: 21 generations, R6 and
155 R9: 20 generations; R7 and R10: 22 generations). For each line a virgin female and a randomly

156 collected male were allowed to mate and from their offspring another virgin female and a random
157 male were used to create the next generation. After inbreeding, these lines were kept as isofemale
158 lines until the experimental assays. In each trial we used a group of 40 flies (males and females 2
159 days old or older) of the same line.
160
161 ***Apparatus and stimuli***
162 The T-maze (31 x 17.5 cm) used for the experimental assays (Fig. 1A) consisted of a starting
163 chamber and a central chamber (12 x 8 x 1.5 cm) connected on each side to a food chamber. The
164 starting chamber (9.5 x 2.5 cm) contained the flies at the beginning of each experimental phase.
165 Food chambers (9.5 x 2.5 cm) were filled with experimental food. In each experimental phase flies
166 begun the exploration of the apparatus from the starting chamber. The central chamber was
167 connected to the food chambers with a funnel that prevents flies to re-enter the central chamber
168 once they have approached the food. A similar trapping technique has been previously used for
169 fruit flies.
170 Experimental media prepared with juice fruit (either orange or apple juice from 100% concentrate)
171 and agar (14 g/l). Aversive food was obtained adding 8 g/l of quinine to the experimental medium.
172
173 **Procedure**
174 ***Unconditioned olfactory preferences***
175 We assessed unconditioned preferences for apple and orange odor by using the same procedure
176 described for the learning assays (see below), with the only difference that no food supplemented
177 with quinine was provided during the exposure phases. This similarity enables us to investigate
178 the role of the conditioning procedure on spontaneous preferences.
179
180

Olfactory learning assays

We used CO_2 anesthesia to collect flies and starve them 15-16 hours before the beginning of the conditioning procedure. After starvation flies were moved to the starting chamber for Exposure 1. During Exposure 1, for two hours flies were exposed to the odor associated with the aversive flavor and the aversive flavor (e.g. orange odor and orange juice supplemented with quinine). Flies who entered the food chambers during Exposure 1 were moved to the starting chamber for Exposure 2. During Exposure 2, for two hours flies were exposed to the odor associated with the palatable flavor and the palatable flavor (e.g. apple odor and apple juice). Flies who entered the food chambers during Exposure 2 were starved for four hours prior to the Test.

In half trials we conditioned flies on apple odor associated with aversive food and orange odor associated with palatable food (A-/O), in half trials we conditioned flies on orange odor associated with aversive food and apple odor associated with palatable food (O-/A). It has been shown that in *D. melanogaster* appetitive long-term memory occurs after single-cycle training also in the absence of fasting [39], while aversive long-term memory by single-cycle training requires previous fasting [40]. For this reason the exposure to the aversive stimulus was always conducted immediately after fasting (Exposure 1).

Flies began the Test from the starting chamber. Differently from the exposure phases, during the Test the odor associated with the aversive flavor and the odor associated with the palatable flavor were presented simultaneously, each on a different food chamber (Fig. 1C). We alternated the right/left side in which the two odors were presented. No food was supplemented with quinine during this phase. We counted flies that chose to enter either the orange odor side or the apple odor side.

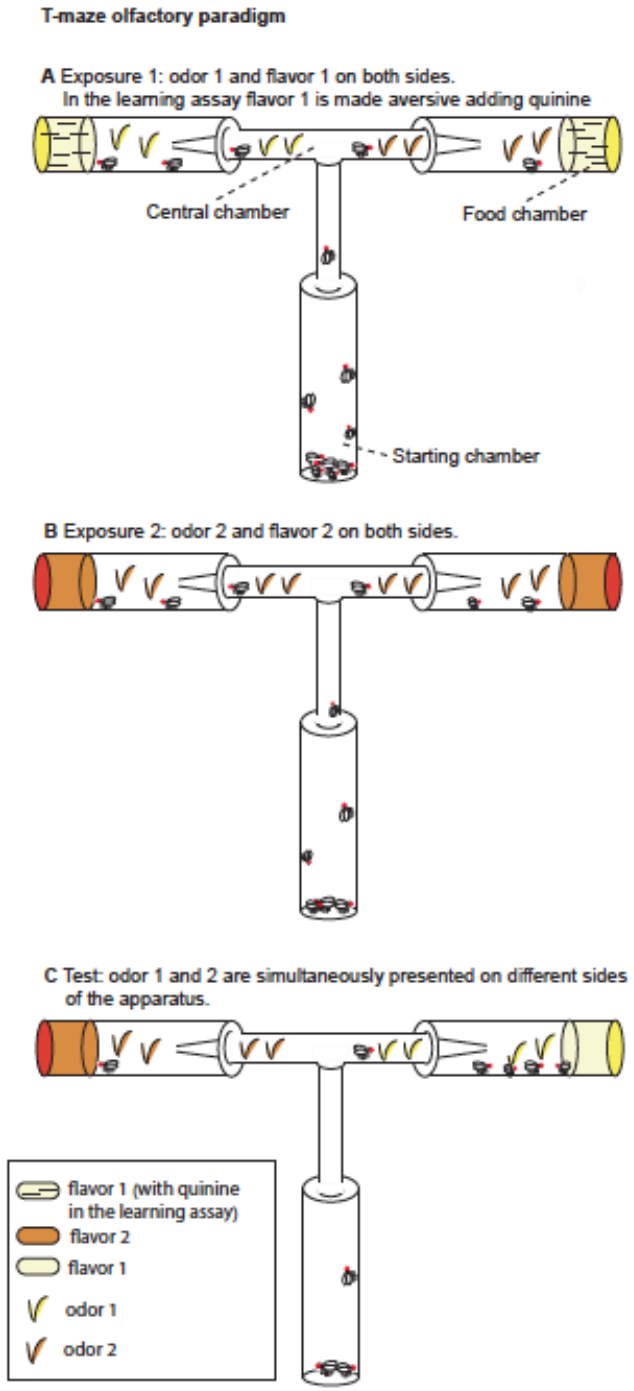


Fig. 1 T-maze apparatus and experimental paradigm. A During Exposure 1 flies are presented with one odor (apple or orange) and the aversive food (apple or orange juice supplemented with quinine in the learning assay, without quinine in the spontaneous preference assay). **B** In Exposure 2 flies are presented the second odor (orange or apple) and the second flavor (orange or apple juice without quinine). **C** At Test both stimuli (orange and apple odor) are presented (without quinine) on different sides of the apparatus.

Data analysis

In the test for spontaneous preferences between the orange and apple odor we compared the proportion of flies that across 28 trials chose the orange odor *vs*. the random choices level using a t-test single sample against the random choices proportion of 0.5. Beforehand we controlled for deviations from the normal distribution of the data using the Shapiro-Wilk normality test. To check for any effect of the order of presentation (O/A: Orange followed by Apple odor, A/O Apple followed by Orange odor) we calculated the order score *o* as the difference in the proportion of flies that in each trial chose orange odor after being exposed to O/A *vs*. A/O:

o = (proportion orange choices O/A flies) – (proportion orange choices A/O flies).

An order score significantly different from zero was expected if the order of presentation of the two odors/flavors had an effect.

Similarly, in the test for conditioned preferences between the orange and apple odor we compared the proportion of flies that across 28 trials chose the orange odor *vs*. the chance level using a t-test single sample against the random choices proportion of 0.5. Beforehand we controlled for deviations from the normal distribution of the data using the Shapiro-Wilk normality test.

To obtain a measure of learning (learning score, *l*) we calculated the difference in the proportion of flies that in each trial chose orange odor after being conditioned on apple flavor *vs*. orange flavor:

l = (proportion orange choices O-/A flies) – (proportion orange choices A-/O flies).

A learning score significantly different from zero was expected if the conditioning had an effect.

To investigate the heritable component of learning we repeatedly tested the same inbred lines (n=10) and derived the intraclass correlation t, which is an estimate of the genetic heritability (h^2) of learning for the tested population, using the variance between (Vb) and within (Vw) inbred lines [41]:

235 $$t = \frac{Vb - Vw/n}{Vb + (n-1)(Vw/n)} = \frac{nVb - Vw}{nVb + (n-1)Vw}$$

236

237 As discussed by David et al. [42] the intraclass correlation can be used as a proxy for heritability

238 [see also 41,43].

239

240 **Results**

241 ***Population experiments: spontaneous preferences and learning assays***

242 To evaluate the sensitivity of our method in detecting learning and spontaneous preferences in

243 large groups of naturally derived fruit flies, we investigated the differences in olfactory learning

244 and spontaneous preferences in a large South African *D. melanogaster* population. In each trial we

245 tested 250 flies of both sexes.

246 In the spontaneous preference experiment, across 28 test trials the overall population showed a

247 marginally significant preference for the orange odor (t_{27}=1.953, p=0.06). This preference is

248 consistent with the preference for citrus previously documented in *D. melanogaster* [36 but see

249 Betti et al. 2014,e.g. 44], that is likely a behavioral strategy against the infection of parasitic

250 wasps [44].

251 Before testing flies, we exposed them to both odors/flavors: in half trials flies were exposed first

252 to orange then to apple (O/A), in half trials first to apple then to orange (A/O). We have derived

253 the order effect score *o* to investigate the effect of the order in which the orange/apple stimuli had

254 been presented. We observed a significant order effect score (t_{13}=3.09, p=0.009; Fig. 2B),

255 indicating that A/O flies (flies first exposed to Apple, then to Orange) had a significantly higher

256 preference for orange odor than O/A flies (flies first exposed to Orange, then to Apple). A post-

257 hoc t-test on A/O and O/A flies *vs*. the chance level (0.5) revealed that only A/O flies had a

258 significant preference for the orange odor: for A/O flies t_{13}= 3.18, p= 0.007; for O/A flies t_{13}= -

259 0.242, p=0.81.

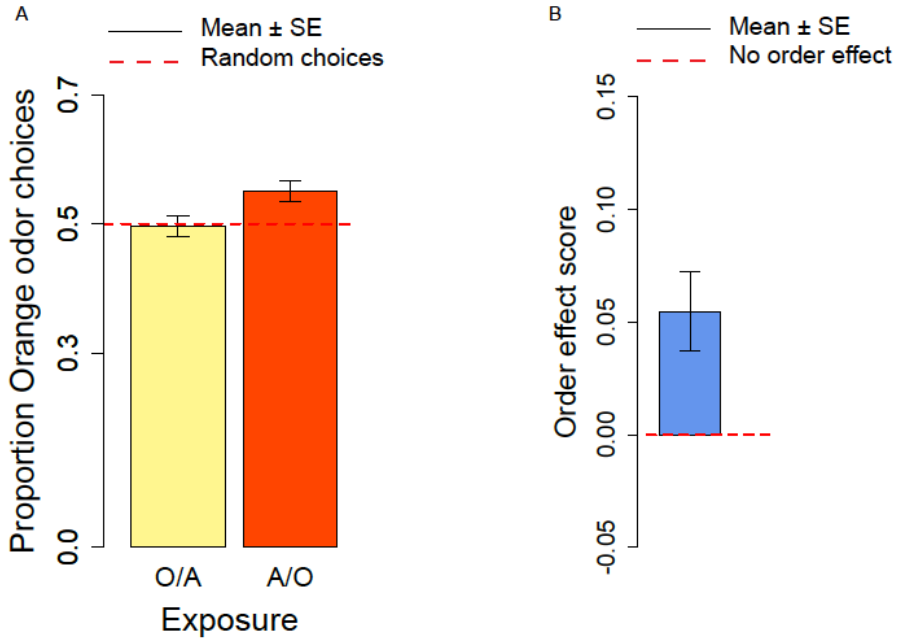


Figure 2. Spontaneous orange choices and order effect score. A Proportion of orange choices of flies exposed to Orange as first, Apple as second stimulus (O/A), and to Apple as first, Orange as second stimulus (A/O). **B** Order effect score (difference in orange odor choices between flies exposed to A/O and O/A).

In the conditioning experiment the overall population showed a preference for the orange odor (mean=0.59, t_{57}=3.95, p<0.001). Flies that previously experienced Apple as aversive/Orange as palatable (A-/O) were more likely to choose orange than flies exposed to the opposite contingency (O-/A) (t_{56}=2.24, p=0.029; Fig. 3A). The population showed a significant learning score (t_{28}=2.88, p=0.007; Fig. 3B).

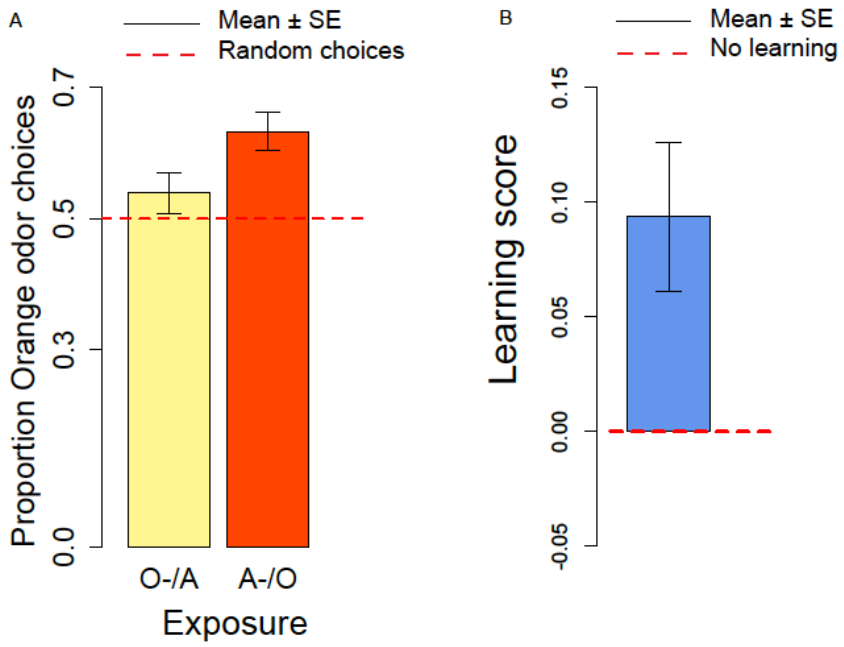


Figure 3. Orange choices after conditioning and learning score. A Proportion of orange choices of flies conditioned with Orange aversive/Apple palatable (O-/A, yellow) and Apple aversive/Orange palatable (A-/O, orange). **B** Learning score: difference in the proportion of orange odor choices between flies conditioned A-/O (Apple aversive, Orange palatable) and O-/A (Orange aversive, Apple palatable).

We also checked for differences in the proportion of orange choices after the conditioning procedure and after the spontaneous preference exposures. Overall, after conditioning flies had a stronger preference for orange odor than in the absence of conditioning (t_{81}=2.50, p=0.014). These results suggest that exposure to aversive stimuli can influence the preferences of flies towards specific odors/flavors.

When comparing samples that had the same order of presentation of apple and orange odor in the conditioning and spontaneous preferences procedure, we observed a significant difference for the A/O but not for the O/A presentation (A-/O: t_{40}=2.49, p=0.017; O-/A: t_{39}=1.24, p=0.22). These results indicate that the conditioning procedure is more effective for the A-/O exposure than for the O-/A exposure.

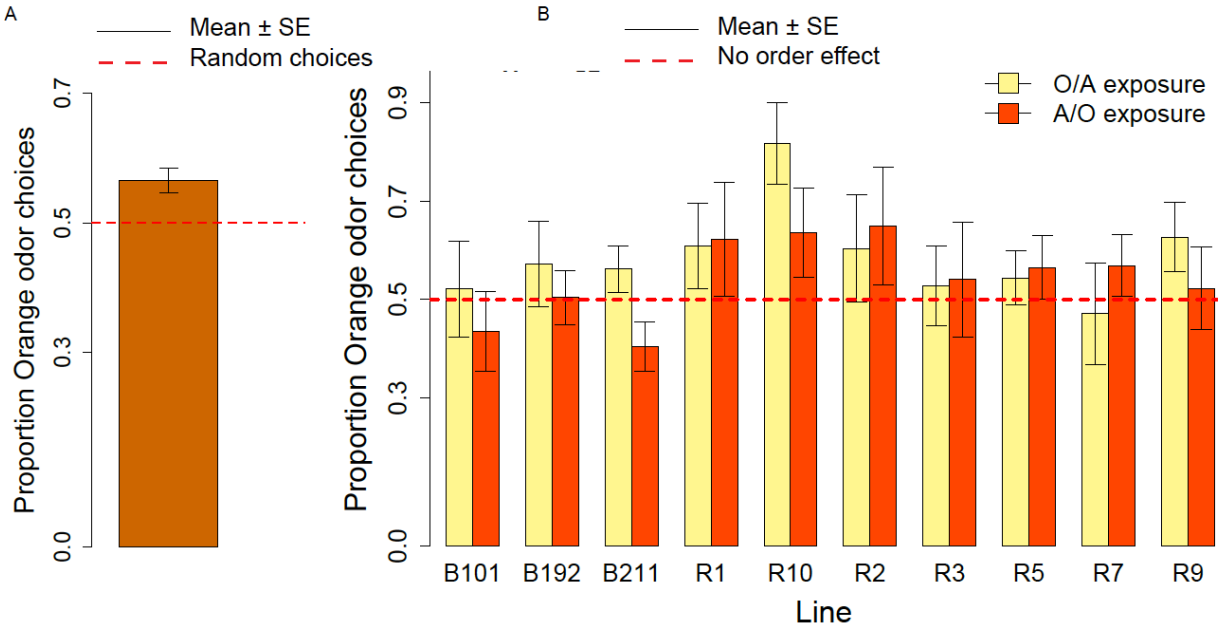
289 ***Inbred lines: spontaneous preferences and learning assays***

290 To study olfactory preferences and learning in 11 inbred lines of *D. melanogaster* derived from a
291 population collected in Portugal we used the same procedure adopted for the large population
292 using 40 flies from the same isofemale line in each trial. Line R6 consistently did not enter the
293 food chambers in both experiments, so we excluded this line and run the analyses in the ten
294 remaining lines.

295 In the spontaneous preference assay the overall distribution of the orange odor choices was
296 significantly different from the normal distribution (Shapiro-Wilk normality test: W=0.98, p=0.03)
297 and we analyzed the data using non-parametric tests (Wilcoxon signed-rank test and Kruskal-
298 Wallis test). Overall, the group of ten responsive lines showed a spontaneous preference for the
299 orange odor (mean=0.56; V=7862, p<0.001; Fig. 4A). We did not observe significant differences
300 across lines (Kruskal-Wallis Chi squared$_9$=14.14, p=0.12; see Fig. 4B) and in the effect of the
301 order of presentation (A/O *vs*. O/A) (Kruskal-Wallis Chi squared$_9$= 1.48, p=0.22).

302 We calculated the order effect score – (proportion of orange odor choices after A/O exposure) -
303 proportion of orange odor choices after O/A exposure – for the overall sample of ten lines tested
304 and found no significant effect (V=1300.5, p=0.23).

305



306

307 **Figure 4. Spontaneous orange odor choices for a population of ten inbred lines and individual lines. A** Overall
308 proportion of spontaneous orange odor choices for a population of ten inbred lines. **B** Orange odor choices after
309 exposure to Orange first Apple second (O/A) and to Apple first, Orange second (A/O) in each of ten inbred lines.

310

311 In the learning assay, the overall distribution of the orange odor choices was significantly different
312 from the normal distribution (Shapiro-Wilk normality test: W=0.98, p=0.005) and we analyzed the
313 data using non parametric tests (Wilcoxon signed-rank test and Kruskal-Wallis test). Overall, after
314 the conditioning procedure the ten responsive lines showed a preference for orange odor
315 (mean=0.59, Wilcoxon signed-rank test, V=8630, p=9.159 x^{10-6}), Fig. 5A. No significant
316 difference in the overall choices was observed between the spontaneous preference and the
317 learning assay (W=13655.5, p=0.30). Differently from the spontaneous preference assay though.
318 significant differences in the proportion of orange choices were apparent between the two
319 conditioning treatments (A-/O *vs*. O-/A: Kruskal-Wallis Chi square: 34.93, p=3.424 x^{10-9}) (Fig.
320 %A), and we documented a significant learning effect (Fig. 5B). We also detected significant
321 differences in the proportion of orange choices between lines (Kruskal-Wallis Chi square: 23.45,
322 p=0.005).

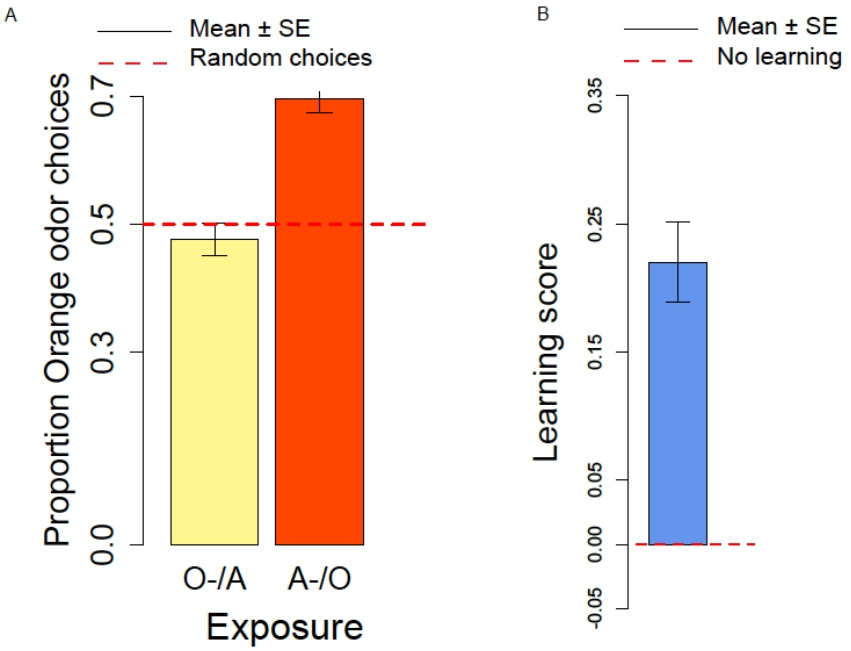
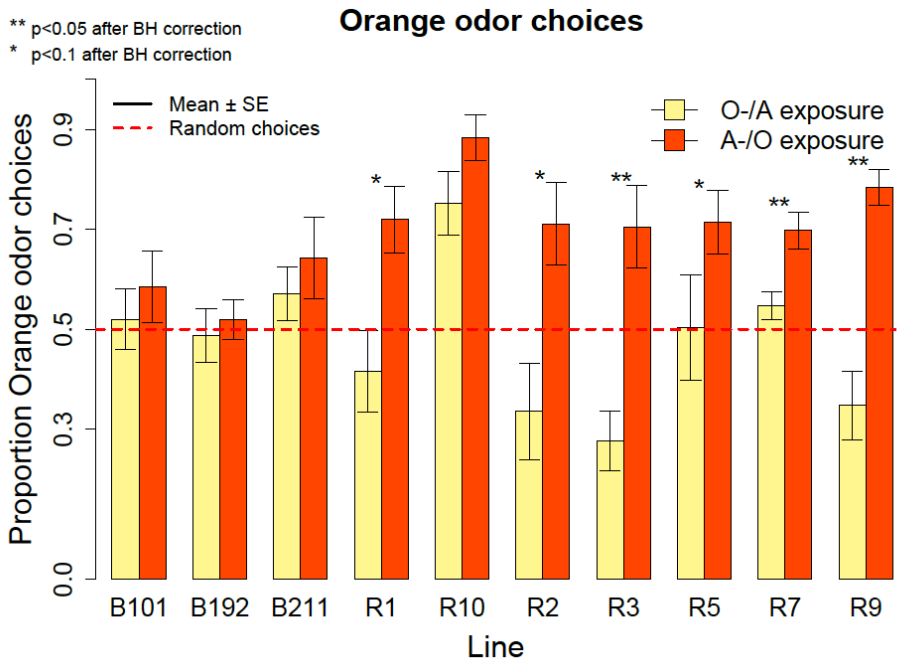


Figure 5. Conditioned orange odor choices for a population of ten inbred lines and learning score. A Proportion of orange odor choices for flies conditioned with Orange aversive/Apple palatable (O-/A) and Apple aversive/Orange palatable (A-/O) in the overall sample of ten inbred lines. **B** Learning score: difference in the proportion of orange odor choices between flies conditioned on A-/O (Apple aversive, Orange palatable) and O-/A (Orange aversive, Apple palatable).

We ran post-hoc tests to evaluate the performances of each line in the A-/O and O-/A conditioning procedure. For each line we measured the proportion of orange choices after conditioning with A-/O and O-/A (fig. 6A and B). After using the Bonferroni-Holmes correction for multiple comparisons, we found that in the A-/O procedure three lines (R10, R7, R9) had a preference significant at 5% level for orange and four lines (R1, R2, R3, R5) had a preference significant at 10% level, whereas in the O-/A procedure only R5 had a preference significant at 10% level. These results indicate that most of the tested inbred lines are able to discriminate between apple and orange odor.

We calculated the learning score – (proportion of orange odor choices after A-/O exposure - proportion of orange odor choices after O-/A exposure) – for the overall population and found a significant effect of learning (mean=0.22, V=2752.5, $p<0.001$). Since there was a significant effect of Line in the learning score (Kruskal-Wallis Chi square=22.15, p=0.008) we ran also post-hoc

341 tests on each line (fig. 6). All lines had an higher proportion of orange choices after being
342 conditioned with Orange as palatable stimulus (and all lines increased the proportion of orange
343 odor choice after conditioning compared to the spontaneous choice assay, see Fig. 4B). Using the
344 Bonferroni-Holmes correction for multiple comparisons we found that three lines (R3, R7, R9)
345 showed a learning score significant at 5% level and three lines (R1, R2 and R5) that were
346 significant at 10% level. These results suggest that most of the tested lines are able to learn
347 through our conditioning procedure.
348



349

350 **Figure 6** Proportion of orange odor choices for each tested line conditioned with Orange aversive/Apple palatable (O-
351 /A exposure) and Apple aversive/Orange palatable (A-/O exposure).
352

353 ***Inbred lines: heritability of olfactory behavior***

354 We derived an estimate of the genetic heritability of olfactory preferences and olfactory learning
355 using the variance between (Vb) and the variance within (Vw) lines and calculating the intraclass
356 correlation *t* as a proxy for heritability in inbred lines [41,42].

357 In the olfactory preferences, the variability between lines (Vb=0.09) was larger than the variability
358 within lines (Vw=0.06) and the intraclass correlation is t=0.6. The same pattern holds true for
359 olfactory learning: the variability between lines (Vb=0.017) is much higher than the variability
360 within lines (Vw=0.004), thus leading to t=0.80. The high intraclass correlations show a moderate
361 to high heritability of olfactory preferences and learning and suggest that our method is suitable to
362 investigate these traits.
363
364 **Discussion**
365 Historically the evolutionary dynamics of behavioral traits have been particularly hard to catch.
366 This is not only due to lack of fossil record as a tool to help reconstructing evolutionary change
367 but also to limits in investigating organisms with a complex behavior for hundreds or thousands of
368 generations, as can be done with yeast [e.g. 45] and bacteria [2]. *Drosophila* is a model system
369 which shows either complex behavior and a life cycle fast enough for being studied in
370 experimental evolution. For instance, in few generations of targeted selection it has been possible
371 to obtain a significant increase in learning in a wild-derived population of *Drosophila*
372 *melanogaster* [36], or to change its responsiveness to specific (odor-flavor or color-flavor)
373 associations [37]. These behavioral findings have not been accompanied by a correspondent
374 genomic investigation, partly due to the costs and difficulties associated until recently to this
375 enterprise. The recent development of high-throughput sequencing technologies, together with
376 advancements in statistical and bioinformatics tools, has changed this scenario. In particular, using
377 the Evolve and Resequence method [3] entire populations can be propagated and investigated for
378 genomic changes at subsequent time points by sequencing collectively hundreds of individuals (a
379 method known as Pool-seq, see Futschik and Schlötterer [4]). This approach paves the way to the
380 analysis of complex behavioral phenotypes such as olfactory preferences and learning in
381 *Drosophila* [8].

382 Empirical [9–11,15] and theoretical studies [6,14] have shown the current limits of E&R in terms
383 of false positive and false negative errors. Different strategies have been suggested to reduce the
384 error rate and increase the efficiency of this method in investigating evolutionary dynamics and
385 the genotype-phenotype link [6,14,17], including propagate and phenotype large samples of
386 several replicate populations for multiple generations. The oviposition method [36] is the
387 experimental paradigm currently used for experimental evolution of learning in fruit flies [36–38].
388 This procedure though is not optimal for E&R due to several drawbacks: the effort required for
389 propagation (e.g. eggs have to be rinsed and/or individually displaced on culture media), the fact
390 that selection is imposed only on half of the propagated subjects (females) and males or
391 male/female interactions cannot be investigated, the presence of selection for fast egg laying and
392 survival to egg washing. With the aim of overcoming these limitations we have established a
393 method based on subsequent exposure and test in a T-maze used to assess olfactory preferences
394 and learning in large (hundreds or even thousands of individuals) and small (dozens) samples of
395 fruit flies. This simple procedure can impose selection on both sexes and does not entail selection
396 for fast egg laying and egg washing to propagate flies.
397 We have used the T-maze procedure to investigate spontaneous and learned olfactory choices in a
398 large population of *D. melanogaster* originally caught in South Africa and in 11 inbred lines of
399 another population of *D. melanogaster* originally caught in Portugal. Overall both populations
400 show a spontaneous preference for orange *vs*. apple odor. The preference for citrus media had
401 been previously documented in *D. melanogaster* [36, but see 43,44]. We find a significant effect
402 of learning in both populations.The presence of olfactory learning in the absence of selection for
403 this trait shows the sensitivity of our method.
404 Small population size and inbreeding negatively affect the resolution of genomic scans [6,14,15],
405 thus limiting the power of E&R and genome-wide association studies. This limitation could be
406 overcome using the T-maze procedure on large samples, since no individual handling of eggs is
407 necessary in this method.

Moreover, repeatedly testing inbred lines we have detected genetic differences in olfactory behavior between lines. Differences between wild-derived inbred lines have been previously documented [46,47]. We have also calculated the intraclass correlation *t* [41,42] as an estimate of heritability, showing a medium/high heritability for the investigated traits.

We have showed that our method is suitable to be used with large samples to investigate the evolution of spontaneous preferences and learning performances in large groups of fruit flies with limited effort. On this basis we suggest to use of T-mazes in large-scale experiments as a tool for E&R and genome-wide association studies on olfactory preferences and learning and for other traits. Our experimental paradigm can be easily adapted to assess visual behaviors of fruit flies by changing the color/texture of the central and food chambers, and navigation performance by increasing the number of food chambers, as well the role of social information transmitted [see for instance 48,49] by one or both sexes. Varying the delay between conditioning and test it is possible to investigate the duration of memory. We suggest the use of large-scale T-mazes to widen the investigation of behavioral traits and cognitive abilities in invertebrates at the behavioral and genomic level.

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