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Fruit flies can learn non-elemental olfactory discriminations

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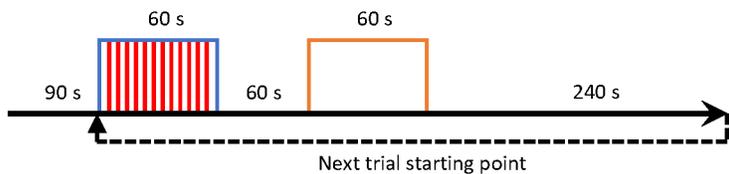
MD carried out all the experiments, analysed data, participated in the design of the study and drafted the manuscript; AW participated in the design of the study and critically revised the manuscript; PA performed the semi-automated device; MG participated in the design and conceptualization of the study and critically revised the manuscript; GI conceived, designed and coordinated the study, and helped draft the manuscript.

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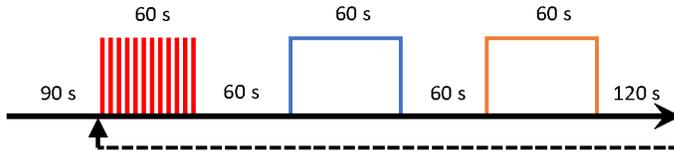
a)

DC & NF protocols

Paired training procedure

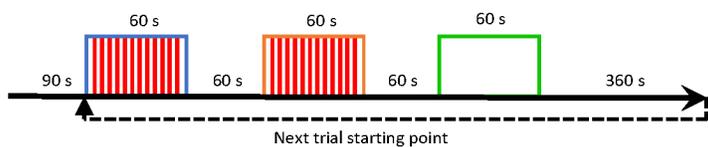


Unpaired training procedure

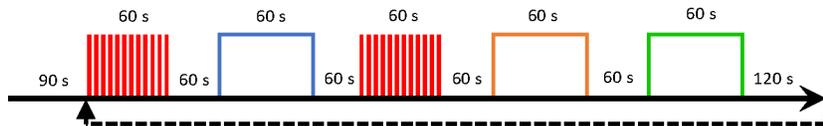


NP protocol

Paired training procedure



Unpaired training procedure



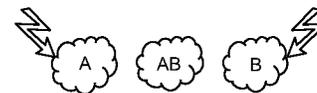
b)

Differential Conditioning

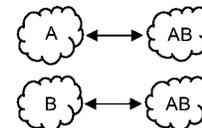
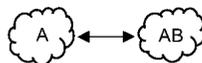
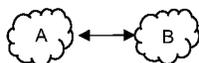
Feature Negative

Negative Patterning

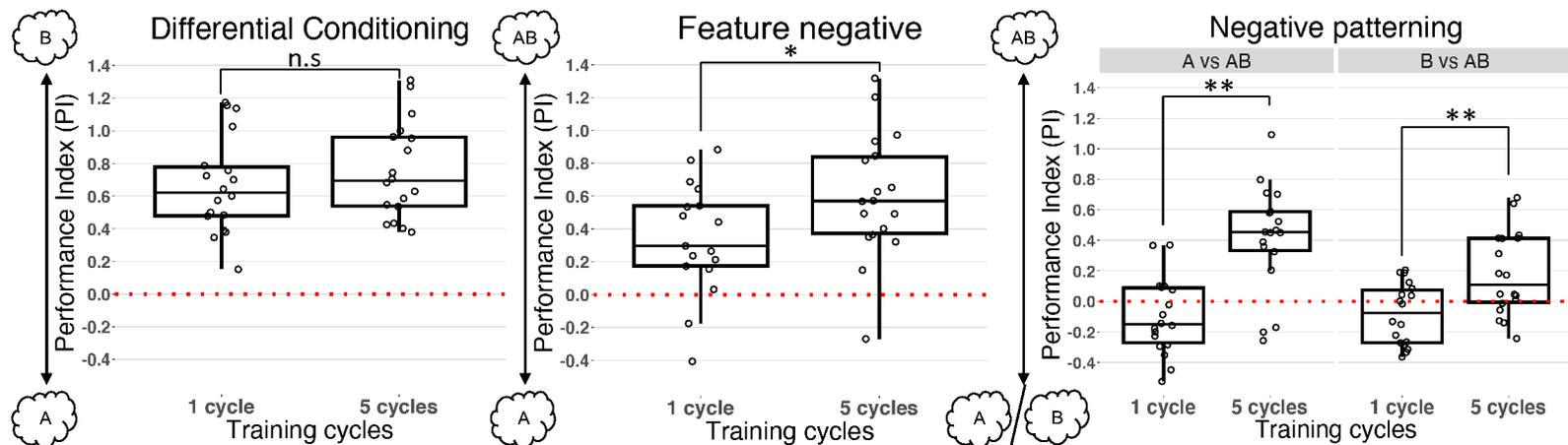
Training



Test



c)



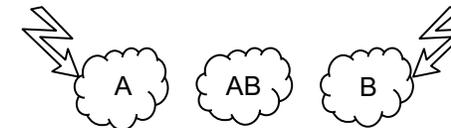
a)

Differential conditioning

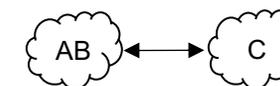
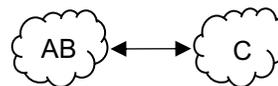
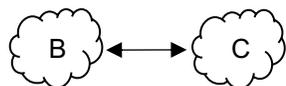
Feature Negative

Negative Patterning

Training



Test

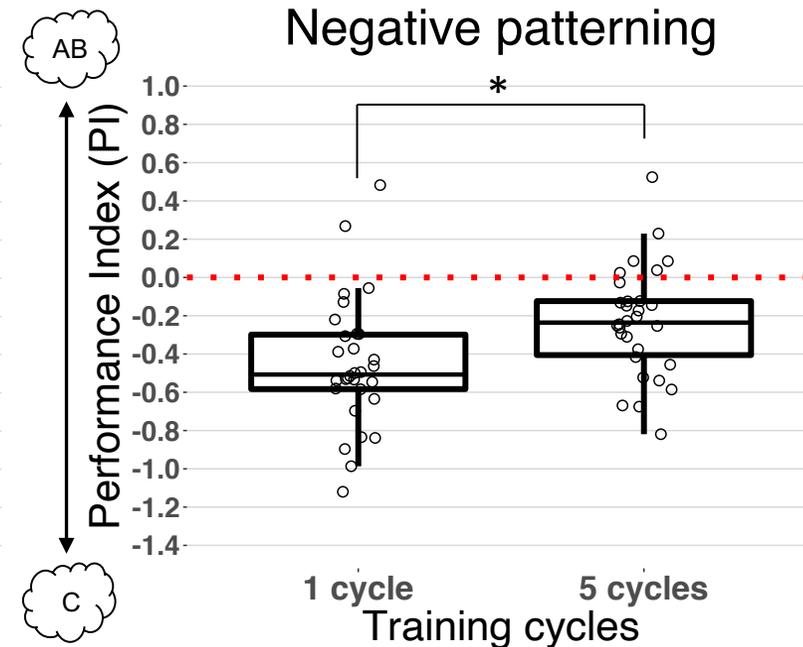
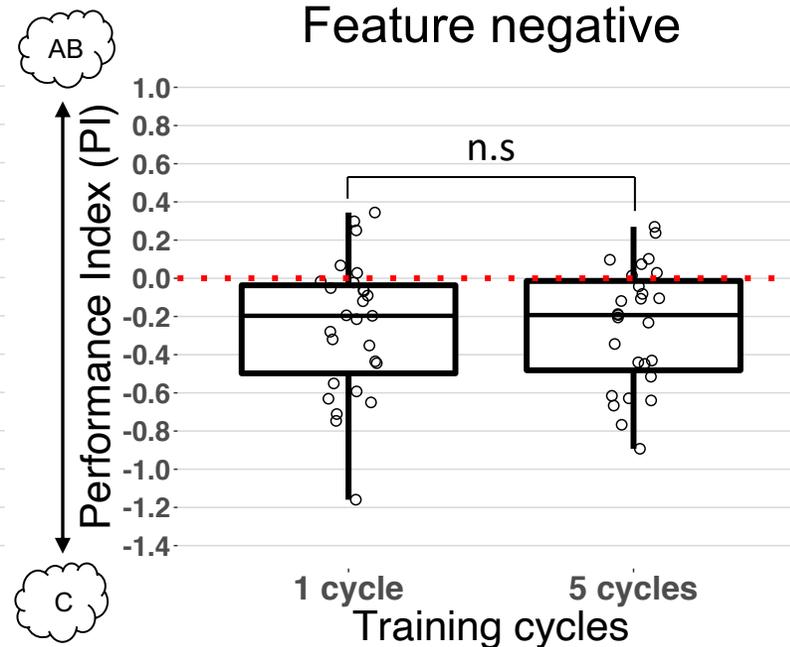
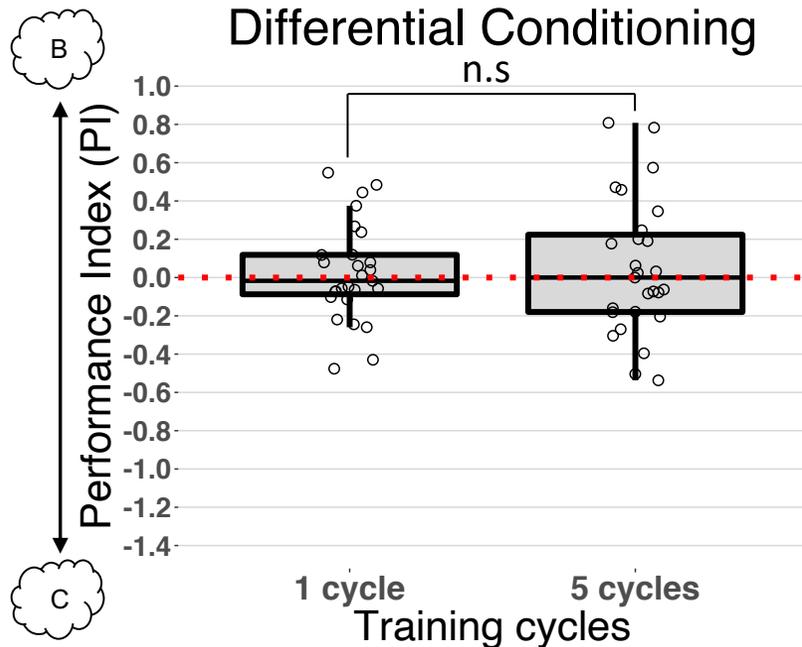


b)

Differential Conditioning

Feature negative

Negative patterning



Fruit flies can learn non-elemental olfactory discriminations

Matthias Durrieu, Antoine Wystrach, Patrick Arrufat, Martin Giurfa, Guillaume Isabel

Key words: Negative Patterning, Feature Negative Discrimination, Associative Learning, Pavlovian Conditioning, Insect, *Drosophila melanogaster*

Abstract

Associative learning allows animals to establish links between stimuli based on their concomitance. In the case of Pavlovian conditioning, a single stimulus A (the conditional stimulus, CS) is reinforced unambiguously with an unconditional stimulus (US) eliciting an innate response. This conditioning constitutes an 'elemental' association enabling to elicit a learnt response from A⁺ without US presentation after learning. However, associative learning may involve a 'complex' CS composed of several components. In that case, the compound may predict a different outcome than the components taken separately, leading to an ambiguity and requiring the animal to perform a so-called 'non-elemental' discrimination. Here we focus on such a non-elemental task, the negative patterning (NP) problem, and provide the first evidence of NP solving in *Drosophila*. We show that *Drosophila* learn to discriminate a simple component (A or B) associated to electric shocks (+) from an odour mixture composed either partly (called 'feature-negative discrimination' A⁺ vs. AB⁻) or entirely (called 'NP' A⁺B⁺ vs. AB⁻) of the shock associated components. Furthermore, we show that conditioning repetition results in a transition from an elemental to a configural representation of the mixture required to solve the NP task, highlighting the cognitive flexibility of *Drosophila*.

Introduction

The ability to form a link between meaningful events is the cornerstone of associative learning. One of the most studied forms of associative learning is Pavlovian conditioning, where animals learn a conditional relation between a conditioned stimulus (CS) and an unconditioned stimulus (US) (1). Several protocols can be used to study Pavlovian conditioning. One of them is differential conditioning, which allows studying how animals learn to associate different CSs with different outcomes (e.g. a reinforced stimulus A⁺ from a non-reinforced stimulus B⁻) (2). In this case, each CS is associated with a distinct US through a simple, non-ambiguous link so that they are learnt independently of each other. In consequence, this type of learning is also referred to as 'elemental learning'(3,4).

40 However, stimuli in nature may not appear as isolated, distinct elements. Usually, they are
41 compounds constituted of multiple elements. For the elemental-learning theory, a
42 compound AB is the linear sum of its elements. In other words, an animal presented with AB
43 would learn the independent relation of A and of B with reinforcement. Yet, several
44 compounds with shared elements and different outcomes could co-exist, creating thereby
45 ambiguity at the level of the single elements. This possibility led to the proposal of the
46 configural (non-elemental) theory, which states that a compound is not learned as the linear
47 sum of its components but as a distinct configuration in which elements would not be fully
48 recognized (5–7). The validity of this hypothesis has been studied using a discrimination task
49 termed ‘Negative Patterning’ (NP) in which a subject has to learn to respond to two single
50 elements reinforced (A^+ , B^+) and to inhibit its response to their non-reinforced compound
51 (AB^-). For the elemental-learning account, solving this task is impossible as animals
52 reinforced on A and B should respond twice as more to AB. In contrast, for the configural
53 account, NP learning is possible as AB would be perceived as a configuration different from A
54 and B, thus facilitating differentiation (5). Other studies explored alternative explanations for
55 NP solving and argued that the compound AB would be perceived as the sum of A and B plus
56 a unique cue specific to the AB compound (therefore termed ‘unique cue theory’) (8). Within
57 this framework, joint presentation to the animal may result in perceptual interferences such
58 as overshadowing, which suggests that in that case, only part of A and B are perceived within
59 the compound (9). In either Configural and Unique cue theory, animals would learn the NP
60 task by focusing on an unambiguous compound-specific cue.

61
62 Another task that has received wide attention among learning scholars is the ‘Feature
63 Negative discrimination’ (NF) in which subjects learn to respond to a single element
64 reinforced (A^+) but not to a non-reinforced compound AB^- (10). NF is interesting as it admits
65 both elemental and configural explanations: for the elemental theory, focusing on B (the
66 negative feature) brings an elemental solution to the problem. As stimulus A has an
67 ambiguous valence, being as often reinforced and non-reinforced, B alone suffices as a
68 conditioned inhibitor to respond appropriately. For the non-elemental theory, discrimination
69 is straightforward as the compound AB is a configuration that is unrelated to its elements
70 (10,11).

71
72 Studying if and how animals solve the NP and NF discriminations is important to access the
73 mechanisms of associative learning in the animal brain. Given the importance of
74 invertebrates for studies on learning and memory (12,13), research on lobsters (14) and
75 honeybees (15–17) has focused on the capacity of these animals to learn these
76 discrimination problems. In other insect species, other forms of non-elemental learning such
77 as multimodal NF (18), biconditional discrimination (19,20) or contextual learning (21) have
78 been described, yet the solving of NP has remained circumscribed to the honeybee.
79 Attempts to study these phenomena in the fruit fly *Drosophila melanogaster*, an insect
80 model that is used recurrently for studying elemental learning forms (22,23) have failed (24).
81 This lack of success is regrettable as the fruit fly offers a vast spectrum of neurogenetic tools,
82 which would allow comparing circuits and mechanisms underlying elemental and non-
83 elemental forms of learning. This approach has allowed identifying the olfactory pathways
84 and learning circuitry of the fly, both from an anatomical (a full connectome of these circuits
85 has been published recently (25)) and functional perspective (23). Robust computational
86 models based on said circuits have been developed and predict that non-elemental learning

87 should be achievable by fruit flies, as normalization events at the perceptual level support
88 the emergence of configural representation of compound stimuli (26). Thus, studying non-
89 elemental learning from a mechanistic perspective in the fly represents an attractive and
90 accessible goal.

91 Here we achieved the first demonstration of the fruit fly's capacity to solve NP and NF in the
92 olfactory domain, thus showing that beyond simple discrimination learning, flies can also
93 solve non-elemental discriminations. In showing this capacity, we determined the
94 associative strategies used by the insects and demonstrated that the processing of olfactory
95 compounds moves along a continuum between elemental and configural processing.
96 Increasing the number of conditioning trials promoted configural processing, enabling flies
97 to solve the NP task.

98 Material and Methods

99 Fly rearing: The wild-type line used in this study was a *Canton-Special* (*Canton-S*) strain. Flies
100 were raised on standard medium at 25°C, ~60% humidity and a 12h/12h light/dark cycle. The
101 flies were kept in 36x82mm plastic tubes containing ~20mL of medium.

102
103 Olfactory conditioning: Odours were diluted in a bottle of mineral oil. Odours used were 3-
104 Octanol (termed 'A' for the sake of simplicity, 2.27mM) and 4-Methylcyclohexanol ('B',
105 2.62mM) (figure 1 and figure 2). Benzaldehyde ('C', 1.89 mM) was used as a novel odour in
106 some conditions (figure 2). IsoamylAcetate ('D') and EthylButyrate ('E') were used as
107 alternative odours (electronic supplementary material, figure S2) to determine if
108 discrimination solving was independent of the pair of odours used. Odours were delivered at
109 the same concentration whether as components (A, B, D or E) or as mixtures (AB or DE). In
110 the case of mixtures, odours were diluted together in the same bottle of mineral oil. All
111 odours and the solvent were from Sigma Aldrich (France). The US consisted of twelve 1.5s
112 pulses of 60V electric shock every 5s delivered through a metallic grid. Each experiment
113 included two groups of ~30 flies (2 – 4 days old) and was performed using a semi-automated
114 device based on a previous work (27). In a T-Maze, two main phenomena drive the
115 preference of flies toward a compartment or another: on the one hand, the learnt
116 information about the stimuli acquired during conditioning and on the other hand, the fact
117 that odours are rarely completely neutral; at the concentrations used in our work, they are
118 in fact repulsive to naïve flies. When two odorants are opposed in the absence of
119 punishment in the T-maze, repulsion balances; yet, if one odorant is opposed to a
120 compound, which is more repulsive, a bias towards the less repulsive stimulus is visible. To
121 disentangle learning from non-learning behavioural components, one of the groups
122 experienced an explicit pairing of CS and US ('paired group') while the other group
123 experienced both stimuli unpaired to prevent their association ('unpaired group').
124

125
126 Training: Each training trial consisted of 90 s of acclimatisation, after which flies were
127 subjected to their respective conditioning protocol. Each of the two odours (CS) was
128 presented once for 1min with an intertrial interval of 1min. For the paired group (figure 1a),
129 one of the olfactory stimulus (CS⁺) was paired with the US, while the other stimulus (CS⁻)

130 remained unpunished. In the unpaired group, flies were exposed to 1min of either shocks or
131 odours, separated by an interval of 1min. This sequence formed one conditioning cycle.

132

133 Flies were subjected to one of three training protocols (figure 1b): a differential conditioning
134 (DC) in which they had to learn to discriminate a punished from a non-punished odour (A⁺
135 vs. B⁻), a NF discrimination in which they had to learn to discriminate a punished odour from
136 a non-punished odour compound (A⁺ vs. AB⁻) and a NP discrimination in which they had to
137 learn to discriminate two punished odours from a non-punished odour compound (A⁺, B⁺ vs.
138 AB⁻). 3-Octanol was always used as the CS⁺ for the DC and NF protocols (with 4-
139 Methylcyclohexanol (DC) and 3-Octanol+4-Methylcyclohexanol (NF) as CS⁻). For the NP
140 protocol, both 3-Octanol and 4-Methylcyclohexanol were used as CS⁺ when presented alone
141 and as CS⁻ when presented as a compound. Thus, flies subjected to DC training faced a pure
142 elemental discrimination. On the contrary, flies trained in the NP protocol could only solve
143 the problem if they adopted a non-elemental strategy. Finally, flies subjected to NF training
144 could solve the problem using either an elemental or a non-elemental strategy. For each
145 protocol, training consisted of either one or five cycles, which allowed studying if the
146 amount of experience gathered by flies promoted a particular discriminations strategy.

147

148 Test: After training, flies were transferred to a T-maze (28) where they could choose
149 between the CS⁺ and the CS⁻ in the absence of shock during 1min. Flies from paired and
150 unpaired protocols were sequentially tested. At the end of the test, flies in each arm of the
151 T-maze were counted. If paired flies learned the discrimination, they should be mostly
152 located in the CS⁻ arm, the arm presenting the odour stimulus that was not associated to the
153 shocks during the training. A Performance Index (PI) was calculated as: (Number of flies in
154 the CS⁻ arm – Number of flies in the CS⁺ arm) / total Number of flies. To control for any
155 experimental bias, each replicate consisted of a 'paired-group' PI (reflecting associative
156 learning + bias) from which an 'unpaired-group' PI (reflecting bias only) was subtracted
157 (electronic supplementary material, figure S1).

158

159 Statistical analysis: PI data were plotted and analysed using R software (3.5.0 version). Group
160 distributions were tested for normality using the Shapiro-Wilkinson test; homoscedasticity
161 was tested using Bartlett's test. All our experiments met the requirements for parametric
162 statistics. For one or two-sample analyses, we applied one sample or two samples two-tailed
163 Student's tests, respectively. For comparisons involving more than two samples, we used
164 analysis of variance (ANOVA) followed by Tukey's (HSD) post hoc tests. A significance level of
165 $p < 0.05$ was set for every experiment.

166

167 Results

168

169 Figure 1c (left panel) shows that flies trained in the DC protocol learned the discrimination
170 and preferred the non-punished odour B⁻ to the punished odour A⁺. Comparing their PIs
171 against 0 by means of a one-sample t test yielded significant differences after both one
172 training cycle ($t = 9.49$, $df: 17$, $p = 3.33e-8$) and five training cycles ($t = 10.83$, $df = 17$, $p =$
173 $4.76e-9$). Both PIs did not differ from each other (two-sample t-test : $N = 18$ and 18 , $t = -$
174 0.87 , $df = 34$, $p = 0.39$), thus ~~providingshowing that the amount of experience of flies did not~~

175 ~~affect the learning of this elemental discrimination~~ no evidence for an effect of amount of
 176 experience.

177
 178 Flies trained in the NF protocol (figure 1c, middle panel) also learned the discrimination
 179 between the single odour punished A+ and the non-punished odour-compound AB-. Note
 180 that AB is innately more repulsive than A but the relative PI shows the expected effect of
 181 learning (figure 2b, and electronic supplementary material, figure S1). Performance was
 182 significantly different from zero after both one cycle of training ($t = 4.13$, $df = 16$, $p = 7.78e-4$)
 183 or after five cycles ($t = 6.69$, $df = 17$, $p = 3.77e-6$). In contrast with DC training, performance
 184 improved significantly with the amount of training ($N = 17$ and 18 , $t = -2.11$, $df = 33$, $p =$
 185 0.042). We also tested the flies' performance after the DC or NF protocols balancing odor
 186 contingencies, i.e. using B as CS⁺ and A as CS⁻ (electronic supplementary material, figure S3).

187
 188 In the case of flies trained in the NP protocol (figure 1c, right panel), the type of odour used
 189 during tests had no significant effect on performance (i.e. 'A vs. AB' or 'B vs. AB'; 2-way
 190 ANOVA: $F(1,69) = 2.72$, $p = 0.10$). Yet, the PI varied significantly depending on the number of
 191 training cycles ($F(1,69) = 34.92$, $p = 1.18e-7$). After 1 training cycle, the PI was negative, i.e.
 192 biased towards the single odours associated with the shocks during training (A⁺ or B⁺) ($t = -$
 193 2.47 , $df = 35$, $p = 0.018$). However, after 5 training cycles, preference was reversed and flies
 194 preferred the non-punished compound AB- over the single punished odorants A⁺, B⁺ ($t =$
 195 5.37 , $df = 35$, $p = 5.16e-6$; figure 1c, right panel). These findings show that training repetition
 196 is crucial for NP solving as it improved the ability of flies to discriminate the odours with
 197 different outcome. To determine if NP solving is independent of the pairs of odours used, we
 198 repeated the experiment using different odours (D⁺, E⁺ vs. DE⁻; electronic supplementary
 199 material, figure S2). The flies learned the NP discrimination also in this case, showing that
 200 the type of odour used during the test phase had no significant effect on performance (i.e.
 201 'D vs. DE' or 'E vs. DE'; 2-way ANOVA: $F(1, 69) = 3.005$, $p = 0.088$). After 1 trial, flies were
 202 not attracted by the single odours ($t = -0.73$, $df = 35$, $p = 0.47$). After five trials, they were
 203 able to solve the task and showed a significant preference for the compound ($t = 5.21$, $df =$
 204 35 , $p = 8.59e-06$). This result demonstrates that the ability to solve NP is not specific to the
 205 type of odour used in the protocol.

206
 207 *Figure 1 (a) schematic representation of a typical training cycle. Blue and orange boxes show CS presentation while red bars*
 208 *show US delivery. (b) schematic representation of the conditioning protocols. Clouds represent the CS odorants while light*
 209 *bolts indicate the delivery of electric shock during training. A = 3-Octanol, B = 4-Methylcyclohexanol (c) ~~Corrected~~Relative*
 210 *performance indices (PI) computed as the difference between paired and unpaired scores. Performances were compared*
 211 *within the same protocol (i.e. 1 cycle vs. 5 cycles) but not between protocols. Data are plotted as boxplots. The middle line*
 212 *represents the median while the upper and lower limits of the box are the 25 and 75 percent quantiles. The whiskers are the*
 213 *maximum and minimum values of the data that are respectively within 1.5 times the interquartile range over the 75th*
 214 *percentile and under the 25th percentile. Raw data are superimposed as jittered dots.*

215 *"n.s." stands for "non-significant", * indicates $p < 0.05$, ** indicates $p < 0.01$ after a t test (DC and NF) or after a 2-way anova*
 216 *(NP)*

217
 218 Despite their different complexity, the three protocols assayed opposed one or two CS⁺ to a
 219 CS⁻. Under these conditions, an animal may learn to avoid the CS⁺, to be attracted to the CS⁻,
 220 or both. To determine the nature of the associations inculcated by DC, NF and NP, we
 221 studied if the CS⁻ (not reinforced) was learnt as a safe stimulus (inducing attraction),
 222 remained neutral (being indifferent to trained animals) or in the case of compounds sharing
 223 elements with the reinforced CS⁺, gained also inhibitory strength, thus eliciting avoidance. To

224 test these options, flies were first trained under the DC, NF or NP protocols (Figure 2a) and
 225 then given a choice between the CS⁻ and a novel, neutral odour C. Here again, the
 226 preference of trained flies was compared to preference of flies after an unpaired protocol.
 227 After being trained in the DC protocol (figure 2b, left panel), flies tested with the non-
 228 punished odour B vs. the neutral odour C did not show any preference either after one
 229 training cycle ($t = 0.48$, $df = 26$, $p = 0.64$) or after five training cycles ($t = 0.73$, $df = 26$, $p =$
 230 0.47). There was no significant difference between PIs corresponding to these different
 231 training amounts ($N = 27$ and 27 , $t = -0.31$, $df = 52$, $p = 0.75$). This result thus ~~shows-suggests~~
 232 that the non-punished odour was not perceived as attractive after DC training ~~and that it~~
 233 ~~conserved this status irrespective of the amount of training, and that the number of training~~
 234 ~~cycles has little impact if at all-~~.

235
 236 After NF conditioning (figure 2b, middle panel), flies preferred significantly the novel odour C
 237 to the non-punished compound AB both after one ($t = -3.78$, $df = 26$, $p = 8.41e-4$) and five
 238 training cycles ($t = -4.15$, $df = 26$, $p = 3.16e-4$). This preference was maintained despite the
 239 increase in the amount of training ($N = 27$ and 27 , $t = -0.03$, $df = 52$, $p = 0.97$), thus showing
 240 that in learning this discrimination, flies assigned an aversive valence to the CS⁻ despite the
 241 fact that it was not paired with shocks. This inhibitory strength must have been acquired *via*
 242 the presence of the punished element A in the compound AB.

243
 244 Finally, after NP training (figure 2b, left panel), flies also ~~preferred~~ significantly preferred the
 245 new odour C to the non-punished compound AB both after one ($t = -7.36$, $df = 29$, $p = 4.12e-$
 246 8) and five training cycles ($t = -4.46$, $df = 29$, $p = 1.12e-4$). Yet, differently from NF training, the
 247 flies' aversion for the CS⁻ was reduced after 5 training cycles ($N = 30$ and 30 , $t = -2.74$, $df = 58$,
 248 $p = 8.10e-3$), thus showing that increasing the amount of experience diminished the
 249 inhibitory strength of the compound. This variation is consistent with a non-linear processing
 250 of the compound, which became less similar to the two single odour components, both
 251 being inhibitory after having been paired with shocks.

252
 253
 254 *Figure 2 (a) Schematic representation of the tests performed after the three conditioning protocols to determine the nature*
 255 *of the CS⁻ representation. A = 3-Octanol, B = 4-Methylcyclohexanol, C = Benzaldehyde (b) ~~Corrected~~Relative performance*
 256 *indices (PI) computed as the difference between paired and unpaired scores. Performances were compared within a given*
 257 *protocol (i.e. 1 cycle vs. 5 cycles) but not between protocols. Data are plotted as boxplots. the middle line represents the*
 258 *median. The upper and lower limits of the box are the 25 and 75 percent quantiles. The whiskers are the maximum and*
 259 *minimum values of the data that are respectively within 1.5 times the interquartile range over the 75th percentile and under*
 260 *the 25th percentile. Raw data are superimposed as jittered dots. "n.s" stands for "non-significant", * indicates $p < 0.05$.*
 261 *after a t test. Gray shading indicates performances that were not significantly different from chance level while white filling*
 262 *indicates a significant difference from chance level (t test against zero).*

263

264 Discussion

265
 266 Our results provide the first evidence of Negative-Patterning solving in an insect other than
 267 the honey bee (15,24). Our results show that flies solved both a NF and a NP discrimination
 268 and preferred, in both cases, the odour compound AB that was unpunished during training.
 269 In both cases, the compound acquired some inhibitory strength as shown by its avoidance
 270 when confronted with a novel stimulus C, a fact that could reflect an influence of the

271 punished element (A in NF, A and B in NP) in it. Such an influence would be consistent with
272 an elemental processing of the compound ($AB = A+B$). Yet, in NP, increasing the amount of
273 training diminished this influence, thus promoting a configural processing ($AB = \text{new odour}$).
274

275 When interpreted in the light of these theories, our results cannot accommodate a strictly
276 elemental or configural account. Even after five training cycles in NP, the flies still exhibited
277 some avoidance of the CS⁻ mixture AB, implying that AB⁻ still carried some of the inhibitory
278 strength associated with its constituents, a result that goes against a purely *configural*
279 compound representation (figure 2b, right panel). In other words, the compound remained
280 repulsive, but was preferred to the “more repulsive” elements taken alone. This result
281 differs from the response of naïve flies, for which a compound is more repulsive than the
282 single odorants, at the concentrations used ([electronic supplementary material, figure S1](#)).
283 Our result could thus be interpreted as supporting the ‘unique cue theory’, which is
284 consistent with observations both in honeybee and *Drosophila*. In vivo imaging of the
285 Antennal Lobes, the primary olfactory center in the insect brain, suggests that binary
286 mixtures are not coded as the linear sum of their constituents but rather show both
287 similarities and features that are unique to the compound considered (29–32). However, the
288 fact that the flies’ initial choice, which was closer to an elemental representation, was
289 reversed (although not entirely) after NP training shows that increasing training gradually
290 changed the odour representation.

291
292 Our results show that the experience of an individual modulates the kind of processing
293 adopted. This modulation is consistent with previous work on honeybees showing, in both
294 the visual and the olfactory modality, that increasing the number of training trials promotes
295 a configural stimulus representation (33,34). Such a plastic stimulus representation has also
296 been described in other learning paradigms (in crustaceans (14); larvae (35) and adults
297 *Drosophila* (21,36); rabbits(37); humans (38)).

298
299 How such a change of odour representation could be implemented in the fly’s brain?
300 Firstly, associative learning has been shown to modulate neural representation of odours in
301 the honeybee Antennal Lobes, a structure involved in olfactory processing, also present in
302 *Drosophila*. Over the course of CS/US associations, odours representation was modified,
303 amplifying the CS⁺ representation and reducing CS⁺/CS⁻ correlation. This could be a first
304 explanatory mechanism for enhanced discrimination abilities necessary for NP resolution
305 (39). Moreover, pioneer work in the field identified the mushroom bodies as critical
306 structures for the encoding of learnt behaviour in insect brain (40–42). Interestingly, bees
307 without functional mushroom bodies learn simple olfactory discriminations but are unable
308 to solve NP tasks (43). Such a failure in NP tasks (as well as in other tasks such as reversal
309 learning) was related to the pharmacological blockade of a specific subset of feedback
310 neurons providing GABAergic signaling to the mushroom bodies (43,44). The functional
311 equivalent of these neurons in *Drosophila*, the Anterior Paired Lateral neurons (APL), are
312 equally necessary for reversal learning (45). Together, this suggests that APL neurons play a
313 crucial role for the modulation of odour representation, and thus for NP solving in flies.
314 The inhibitory feedback mediated by APL neurons enables a sparse encoding of sensory
315 information by maintaining a low level of activity in the MB, which is needed to discriminate
316 between similar olfactory stimuli (46). Previous modeling work suggested that normalization
317 at the Antennal Lobes level is crucial for non-elemental forms of learning as it leads to

318 elements-specific features inhibition, thus enhancing compound-specific (unique cue)
319 salience (26). APL neurons activity also acts as a gain control mechanism and thus further
320 enhances discrimination at the MB level, which would confirm a hypothesis already proposed
321 in a previous work on NP in honeybee (43). Moreover, by modulating its inhibitory activity
322 depending on the task at hand, the APL neurons could in theory mediate a shift along the
323 generalization / discrimination balance as observed here. Indeed, after 1 cycle of NP
324 conditioning, flies respond to AB as if it were A or B, consistent with odour generalization. Yet,
325 after 5 cycles, flies change their behaviour and choose AB even though the second experiment
326 clearly shows that they still perceive the A and B constituents as aversive. This result, in
327 contrast can be attributed to better discrimination abilities. Interestingly, dopamine signals
328 originating from PPL1 neurons (responsible for aversive US transmission) inhibit APL activity
329 through D2-like receptors, thus modulating olfactory learning (47). Moreover, APL neurons
330 are activated differently by the CS+ and CS- (48). More specifically, the association between
331 the CS and the US results in a diminished APL activity (leading to a reduction of inhibition
332 towards the CS+, facilitating -in principle- its reinforcement). Thus, it is possible that during
333 trials, a differential activation of APL in response to the CS+ vs CS- produces a form of pruning,
334 leading to the unequivocal reinforcement of the KC associated only with A or B while inhibiting
335 the KC associated both to A/B (the CS+) and AB (the CS-). If this process is slow compared to
336 the acquisition of the original CS-US association, APL activity would gradually modulate
337 stimulus representation to facilitate the task resolution. In the case of NP, it would help
338 segregating the representation of AB from those of its elements. APL neurons could thus
339 continually adjust and update representation along a generalization/discrimination
340 continuum based on learning events, besides their role as regular gain control system.
341 Understanding how APL activity might modulate non elemental olfactory learning constitutes
342 an interesting future research agenda.

343 So far, olfactory learning in fruit flies was limited to DC protocols, thus reducing the study of
344 associative learning and its neural and molecular mechanisms to its most simple form. Our
345 results thus open new perspectives to explore these mechanisms for higher-order forms of
346 learning and determine if the same or different circuits intervene when different learning
347 strategies are employed.

348

349 *Data accessibility*

350 Data available from the Dryad Digital Repository (temporary link,
351 doi:10.5061/dryad.v9s4mw6t1):

352 https://datadryad.org/stash/share/gXEA6n_fjLubzjdmB3VvKakm1WBAqJohvqvjTOeTTg5U.

353

354 *Authors' contributions*

355 [MD carried out all the experiments, analysed data, participated in the design of the study](#)
356 [and drafted the manuscript; AW participated in the design of the study and critically revised](#)
357 [the manuscript; PA performed the semi-automated device; MG participated in the design](#)
358 [and conceptualization of the study and critically revised the manuscript; GI conceived,](#)
359 [designed and coordinated the study, and helped draft the manuscript.](#)

360 [All authors gave final approval for publication and agree to be held accountable for the work](#)
361 [performed therein.](#)

362 ~~M.D. carried out all experimental work and analysed the data. P.A. devised an electric~~
363 ~~generator and its programming. M.D., A.W., M.G. and G.I. wrote the manuscript. G.I.~~
364 ~~supervised the project and designed all experiments with M.D., A.W. and M.G.~~

365

366 *Competing interests*

367 The authors declare no competing interests.

368

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379

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382

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