

1 **Learning of pathogenic bacteria in adult *C. elegans* bidirectionally regulates pathogen**
2 **response in the progeny**

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11 **Parental experience can generate adaptive changes in the behavioral and physiological**
12 **traits of the offspring¹⁻³. However, the biological properties of this intergenerational**
13 **regulation and the underlying molecular and cellular mechanism are not well understood.**
14 **Here, we show that the experience of learning to avoid pathogenic bacteria in *C. elegans***
15 **alters the behavioral response to the pathogen in the progeny through the endogenous**
16 **RNA interference (RNAi) pathway. We previously show that the adult *C. elegans* learns to**
17 **avoid the smell of pathogenic bacteria, such as the *Pseudomonas aeruginosa* strain**
18 **PA14, after feeding on the pathogen for a few hours^{4,5}. Here, we report that this learning**
19 **experience can bidirectionally regulate the olfactory response to PA14 in the progeny**
20 **that are never directly exposed to the pathogen. The olfactory preference for PA14 in**
21 **these progeny is linearly correlated with the learned avoidance of PA14 in their mothers.**
22 **If the mothers show strong learning of PA14, their progeny avoid PA14; intriguingly, if**
23 **the mothers show weak learning of PA14, the progeny prefer PA14, suggesting that the**
24 **PA14-trained mothers transmit both the negative and positive information of PA14 to**
25 **their progeny. The intergenerational behavioral effect results from an altered behavioral**
26 **decision regulated by an olfactory sensorimotor neural circuit. Learning to avoid the**
27 **pathogen also influences the development of the progeny, which is regulated**
28 **independently from the behavioral change. Animals mutated for the RRF-3/RNA-directed**
29 **RNA polymerase, a master regulator for the synthesis of the small interfering RNAs that**
30 **are maternally inherited or in the soma^{6,7}, display the normal naive and learned response**
31 **to PA14 but are defective in regulating the olfactory response to PA14 in their progeny.**
32 **Our results characterize an intergenerational effect that allows the progeny to rapidly**
33 **adapt to an environmental condition that is critical for survival.**

34
35 In many organisms, the parental experience generates adaptive changes in the behavior and
36 the physiology of the progeny¹⁻³. However, whether the parental interaction with the
37 environment regulates the same behavioral modalities in the parents and the progeny,
38 indicating the transmission of specific sensory information, is not clear. In addition, the signaling
39 pathways underlying the transmission of the parental information to the progeny are poorly
40 understood. Olfaction plays critical roles in animal behaviors that are essential for survival, such
41 as searching for food and avoiding dangers. While genetically encoded^{8,9}, the olfactory system
42 is highly adaptable. Experience can profoundly shape the meaning of an odorant to an
43 animal^{10,11}. *Caenorhabditis elegans* feeds on bacteria and is often attracted by the smell of the
44 bacteria in its habitat^{4,12,13}. Meanwhile, some bacteria are pathogenic. Ingesting the pathogenic

45 bacteria makes the worm ill through intestinal infection¹⁴. Previous studies show that after briefly
46 feeding on certain pathogenic bacteria, such as the *Pseudomonas aeruginosa* strain PA14,
47 adult *C. elegans* reduces the preference for the smell of the pathogen, even if it is initially
48 attractive^{4,5}. This form of learning is contingent on the pathogenesis of the training bacteria^{4,5}
49 and resembles the Garcia effect, a robust form of conditioned aversion that allows animals to
50 learn to avoid the smell or taste of a food that makes them ill¹⁵.

51
52 Although the intestinal infection caused by ingesting PA14 results in a slow death of the worm
53 over a course of few days¹⁴, the bacteria in the *Pseudomonas* genus are abundant in the natural
54 habit of *C. elegans* and serve as common food sources for the worm¹³. Therefore, the
55 pathogenic *Pseudomonas* PA14 contains the information of food and danger, both of which are
56 critical for the survival of the worm. We asked whether learning to avoid PA14 regulated the
57 olfactory response to the smell of PA14 in the offspring. We cultivated the worms under the
58 standard conditions on the benign bacteria strain *Escherichia Coli* OP50 until the adult stage¹⁶.
59 We then trained the adult hermaphrodite worms (F0s) by feeding them on PA14 for 8 hours
60 (Figure 1A). We quantified the preference between the smell of OP50 and the smell of PA14 in
61 the trained and the naive F0 worms using an automated assay⁵. In this assay, a positive choice
62 index indicates a preference for PA14 and a negative choice index indicates a preference for
63 OP50 (Methods)⁵. After randomly sampling the preference in F0s, we harvested the embryos
64 (F1s) from the trained and the naive F0s with a bleach solution that dissolved the adult bodies
65 and the associated bacteria. We cultivated the F1 worms on the benign bacteria *E. Coli* OP50
66 under the standard conditions. Thus, the F1 progeny of the PA14-trained F0s and the F1
67 progeny of the naive F0s were cultivated under the same condition and neither was directly
68 exposed to PA14. We quantified the preference of F1s between the smell of OP50 and the
69 smell of PA14 after they reached the adult stage. We measured the olfactory preference of F1
70 worms using a two-choice assay, in which a worm chooses between a drop of supernatant of an
71 OP50 bacteria culture and a drop of supernatant of an PA14 bacteria culture by navigating
72 towards the drop (Figure 1A). We recorded the behavior of the F1 worms during the two-choice
73 assay and analyzed their chemotactic movement and their choices. A positive choice index in
74 the two-choice assay indicates a preference for PA14 and a negative choice index indicates a
75 preference for OP50 (Figure 1A and Methods). While the automated assay allows us to rapidly
76 measure the olfactory preference in F0s before handling the F1 embryos, the two-choice assay
77 allows us to examine the olfactory behavior in F1 worms in detail (Methods).

78

79 We found that consistent with our previous findings^{4,5}, training the F0 adult worms with PA14 for
80 8 hours strongly reduced the preference for PA14 (Extended Figure 1). This experience-
81 dependent change is indicated by a learning index (LI), which is the difference between the
82 choice index (CI) of the naive F0s and the choice index of the trained F0s in the automated
83 assay (Figure 1B and Methods). A positive LI indicates learned avoidance of PA14 (Methods).
84 Next, we examined whether the training experience of the F0s generated any difference in the
85 olfactory preference of their F1 progenies. Thus, we measured the difference between the
86 choice index of the F1 progeny of the naive F0 worms and the choice index of the F1 progeny of
87 the trained F0 worms, and defined this difference as the parental-experience dependent
88 learning index (P_LI) (Figure 1B and Methods). A positive P_LI indicates increased avoidance
89 of PA14 in F1 induced by the parental experience with PA14 (Methods). We found that P_LI in
90 F1s were significantly correlated with the LI in their F0 mothers (Figure 1B). On one end of this
91 spectrum, the more the trained F0s learned to reduce their preference for the PA14 smell, the
92 more their F1 progeny avoid the PA14 smell in comparison with the F1s of the naive F0s that
93 were tested in parallel (Figure 1B), indicating that a strong aversive learning of PA14 in the F0s
94 generates the avoidance of the PA14 smell in F1. Surprisingly, on the other end of the
95 spectrum, we found that for F0s that only weakly learned to reduce their preference for the
96 PA14 smell, the less the trained F0s learned to suppress their preference for PA14, the more
97 their F1s preferred the PA14 smell in comparison with the F1s of the naive F0s tested in parallel
98 (Figure 1B), suggesting that the F0s that weakly learned transmit the beneficial information of
99 PA14. These results show that the maternal learning experience with PA14 bidirectionally
100 regulates the olfactory response to PA14 in the progeny.

101

102 Next, we further examined the bidirectional effect of the F0's learning experience on the
103 olfactory choice of F1s. We separated all the experiments into two groups, the experiments in
104 which F0s displayed a high level of learning of PA14 and the experiments in which F0s
105 displayed a low level of learning of PA14 (Methods). We found that the F1 progeny of the F0s
106 with strong learning avoided PA14 in the two-choice assay, indicated by their negative choice
107 indexes (P_CI, Figure 1C). In contrast, the F1 progeny of the F0s with weak learning preferred
108 PA14 in the two-choice assay, indicated by their positive choice indexes (P_CI, Figure 1C). The
109 extent of the F0 mothers' learning significantly regulated the preference in their F1 progeny.
110 This regulatory effect is also evident in a significant correlation between the learning indexes of
111 F0s (LIs) and the choice indexes of the F1s (P_CIs) of the trained F0s (Figure 1E). In
112 comparison, the choice indexes of the F1s (P_CIs) of the naive F0 mothers in the experiments

113 where training generated strong aversive learning were similar to the P_CIs of the F1s of the
114 naive F0 mothers in the experiments where training generated weak aversive learning (Figure
115 1D). Consistently, there is no correlation between the choice indexes of the F1s (P_CI) of the
116 naive F0s with the learning indexes of the F0 generation (LIs) (Figure 1F). Thus, it is
117 conceivable that the parental experience with PA14 signals two types of information, aversive
118 information such as pathogenesis and appetitive information such as food, and the weight of
119 these information transmitted to the progeny depends on the degree of the parental learning.
120

121 To examine whether the difference in the olfactory response to PA14 in the F1 generation
122 passed onto the F2s, we isolated the F2 worms from the F1 mothers and cultivated F2s under
123 the standard conditions on *E. coli* OP50. Similarly, we quantified the parental-experience
124 dependent learning index (P_LI) in F2s, which was defined as the difference between the choice
125 index of the F2s of the naive F0 worms and the choice index of the F2s of the trained F0 worms.
126 We found that the P_LIs of the F2s did not depend on the P_LIs of their F1 mothers (Figure
127 1G). These results together indicate that the parental experience with PA14 only regulates the
128 olfactory response of their first-generation progeny, leaving the flexibility for F2s to respond to
129 further changes in the environmental conditions.
130

131 To further characterize the parental-experience dependent olfactory learning in F1s, we asked
132 what behavioral changes gave rise to their altered olfactory choice. We analyzed the movement
133 of the F1s when they navigated toward OP50 or PA14 during the two-choice assay (Figure 2A).
134 We measured the navigation index, which is defined as the ratio of the radial speed towards the
135 target and the actual traveling speed to quantify the efficiency of the chemotactic movement
136 towards an odorant source¹⁷(Figure 2B). We divided the assay plate in sections along the axis
137 of the two drops of the bacteria cultures that were 2cm apart and quantified the navigation index
138 for each section in each worm. We analyzed the median value of the navigation indexes for all
139 the worms in each section as a function of the distance away from the target. We found that the
140 navigation index of the F1s of the trained F0s increased when the animals approached the
141 target, indicated by a significant correlation between the navigation index and the distance from
142 the target regardless of whether the worms chose OP50 or PA14 (Figure 2B); in contrast, the
143 navigation index of the F1 worms of the naive F0s did not correlate with the distance to the
144 target (Figure 2B). We also measured the locomotory speed, the distance that the worms
145 travelled and the time spent on travelling before reaching the target, the rate of reorienting
146 movements, the reversals and the large body bends, and did not find any difference between

147 the F1 worms of the trained F0s and the F1 worms of the naive ones (Figure 2C, 2D and
148 Extended Figure 2A-2C). These results identify different strategies for chemotactic movements
149 used by the F1 progeny of the trained mothers versus the F1 progeny of the naive mothers.
150 Previous studies show that the sensorimotor integration of a cholinergic neural circuit regulates
151 the efficiency of chemotactic movements towards odorants, indicated by the navigation index¹⁷.
152 These results together identify the neuronal substrates for the parental-experience dependent
153 olfactory plasticity in the F1s.

154
155 In addition, we asked whether the parental experience with PA14 regulated other physiological
156 traits of the F1s. We first examined the immune resistance to PA14 in F1s using a slow-killing
157 assay that measures the survival rate over time in worms cultivated on a lawn of PA14¹⁴. We
158 used the *nsy-1(k397)* animals as a control, because *nsy-1(k397)* is mutated for the worm
159 homolog of MAPKKK and displays significantly compromised resistance to the infection of
160 PA14¹⁸. We found that while the *nsy-1(k397)* animals showed a much reduced survival rate, the
161 F1s of the PA14-trained F0s and the F1s of the naive F0s showed similar survival rates (Figure
162 2E), indicating that training the adult worms with PA14 do not alter the resistance to PA14 in
163 their progeny. However, we noticed that the F1s of the trained mothers were larger in the body
164 size than the F1s of the naive F0 mothers (Figure 2F). To characterize this difference, we
165 examined the F1 embryos inside the F0s. While the trained F0 mothers and the naive F0
166 mothers contained the same amount of embryos in their bodies on average (trained F0s: $12 \pm$
167 0.8 eggs; naive F0s, 11 ± 0.7 eggs; $n = 7$ adults each, mean \pm sem, two-tailed Student's t-test, p
168 $= 0.3$), the F1 embryos inside the trained F0s were more advanced in the developmental stage
169 than the F1 embryos inside the naive F0s (Extended Figure 2D). In addition, the F1s of the
170 trained F0 mothers entered the L4-larval stage, a developmental stage with distinct anatomical
171 features¹⁹, sooner than the F1s of the naive F0 mothers (Figure 2G). Thus, the parental
172 experience with PA14 influences the development of the offspring. Notably, there is no
173 correlation between the body size of the F1 progeny of the trained mothers with either the
174 learning index of the mothers (LI) or the olfactory choice of the F1s (P_CI) (Figure 2H and 2I),
175 indicating that the parental-experience dependent changes in the olfactory choice of the F1s
176 and in the development of the F1s are independently regulated.

177
178 Next, we sought the signaling mechanisms whereby the information of the parental experience
179 is transmitted to the progeny. In *C. elegans*, the endogenous RNA interference pathway can
180 respond to environmental conditions to generate small interfering RNAs (endo-siRNAs) that

181 transmit the parental experience cross generations and to regulate the physiology of the
182 offspring²⁰. We tested the mutant animals that contained a deletion in *rrf-3*, which encodes a
183 RNA-directed RNA Polymerase(RdRP)²¹ that is needed for the biogenesis of endo-siRNAs that
184 are maternally inherited or found in the soma^{6,7}. We found that the *rrf-3(pk1426)* mutant animals
185 display normal naive and learned olfactory preference for PA14 in the F0 generation (Figure
186 3A), but are significantly defective in the parental-experience dependent learning in F1s (Figure
187 3B). While the parental-experience dependent learning index (P_LI) in F1s significantly
188 correlated with the learning index (LI) in F0s in wild-type animals, this correlation was
189 completely abolished in the *rrf-3(pk1426)* mutants (Figure 3B). Thus, the pathway of the endo-
190 siRNAs regulates the transmission of the parental experience to the progeny.

191
192 We were intrigued by the bidirectional effect of the parental experience with PA14 on the F1's
193 response to PA14. Previous studies show that although adult worms reduce their preference for
194 the smell of PA14 after training, they still prefer the PA14 smell more than non-food smells⁵,
195 indicating that PA14 represents as a less preferred food source to the trained F0s. Thus, we
196 tested the possibility that training F0s with a shorter duration, which reduces the infection of
197 PA14 to the F0s, induces a preference for PA14 in the F1s. Interestingly, we found that
198 shortening the training of adult F0s to 4 hours generated aversive learning of PA14 in F0s
199 (Figure 3C) and induced a significant increase in the preference for PA14 in the F1s of the
200 trained F0s in comparison with the F1s of the naive F0s (Figure 3D). Furthermore, we found that
201 although the *rrf-3(pk1426)* F0s were normal in learning of PA14 after 4-hour training (Figure
202 3C), their F1s were defective in the induced preference for PA14, indicated by the similar
203 preference displayed by the *rrf-3(pk1426)* F1s of the trained and the naive mothers (Figure 3D).
204 These results further demonstrate that the parental experience can generate the appetitive and
205 the aversive effects on the olfactory response of PA14 in the progeny and that both of the
206 intergenerational effects are regulated by the endogenous RNAi pathway.

207
208 Previously, we show that the adult *C. elegans* learns to avoid the smell of pathogenic bacteria,
209 such as the *Pseudomonas aeruginosa* strain PA14, after feeding on the pathogen for 4-8
210 hours^{4,5}. Meanwhile, it is shown that exposing the L1-stage larvae worms to PA14 generates the
211 avoidance of PA14 when the animals reach the adult stage and the imprinting of PA14 requires
212 12-hour training of the L1 larvae²². In this study we identify a new form of adaptive behavior
213 where briefly training the adult worms with PA14 alters the olfactory response to PA14 in their

214 progeny. Together, these results reveal versatile behavioral responses that *C. elegans* uses to
215 interact with pathogenic bacteria that the worm frequently encounters in its habitat.

216

217 Unexpectedly, the experience with PA14 in the adults can regulate the olfactory response of
218 their progeny bidirectionally. While the strong learning of the adult mothers leads to the
219 avoidance of PA14 in the progeny, weak learning of the mothers induces attractive response to
220 PA14 in their progeny. We found that training the mothers for 4 hours, the minimal amount of
221 training needed to induce significant aversive learning of PA14 in the adults⁵, significantly
222 increases the progeny's preference for the smell of PA14, suggesting the mother-to-offspring
223 transmission of beneficial information of PA14. The smell of PA14 is attractive to the worms that
224 never ingest PA14; even after training, the PA14-trained adult worms that display a reduced
225 preference for the PA14 smell still prefer the smell of PA14 more than the non-food odorants⁵.
226 The bacteria of the *Pseudomonas* genus are highly abundant in the habits of *C. elegans* with
227 many isolates being beneficial for the development and the reproduction of the worm¹³. Thus, it
228 is conceivable that the parental experience transmits information of food and pathogenesis of
229 PA14 to the progeny and the resulting behavioral effect is regulated by the extent of the
230 experience.

231

232 In many animals, the parental experience with the environmental conditions, especially those
233 associated with food and danger, regulates the development, physiology and behavior of the
234 offspring and the effect can last for various amounts of time^{1-3,23-29}. In *C. elegans*, the insulin-like
235 signaling acts in the soma or the germline of the mothers to regulate the development and the
236 stress responses in response to the parental experience with food deprivation or osmotic
237 stresses, respectively^{26,27}. However, in most cases, especially when the parental experience
238 generates adaptive changes in the same behavioral modality in both the mothers and the
239 progeny, the signaling mechanism underlying the transmission of the parental experience is
240 largely unknown. In *C. elegans*, it is shown that the pathways of the endogenous small
241 interfering RNAs (endo-siRNAs) can regulate the gene expression and the function of the
242 nervous system in response to the experience within the same generation^{30,31}. In addition, it is
243 shown that the production of endo-siRNAs is regulated by prolonged starvation and the altered
244 siRNA profile persists for several generations²⁰. In this study, we show that the RRF-3/RdRP²¹,
245 which is critically required for the biogenesis of endo-siRNAs that are produced in the soma or
246 maternally inherited^{6,7}, specifically regulates the parental-experience dependent changes in the

247 olfactory response to the pathogenic bacteria PA14, revealing a new function of the endo-
248 siRNAs in regulating intergenerational adaptive behavioral responses.

249

250 **Methods**

251 **Strains**

252 *C. elegans* hermaphrodites were used in this study and cultivated using the standard
253 conditions¹⁶. The strains used in this study include: N2 (Bristol), ZC2834 *rrf-3(pk1426)* II and
254 CX4998 klys 140 I; *nsy-1(k397)* II.

255

256 **The aversive olfactory training with PA14 in F0s and the cultivation of the F1s**

257 The aversive olfactory training was performed mainly as previously described⁴ with minor
258 modifications. To prepare the training plates, individual OP50 colonies or PA14 colonies were
259 used to inoculate 50 mL nematode growth medium (NGM, 2.5g/L Bacto Peptone, 3.0g/L NaCl,
260 1mM CaCl₂, 1mM MgSO₄, 25mM KPO₄ pH6.0), which were cultivated at 27°C for overnight.
261 800 mL (for 4h training protocol) or 400 mL (for 8h training protocol) OP50 or PA14 culture was
262 spread onto each NGM plate, which was incubated at 27°C for 2 days to prepare the naive
263 control and the training plates, respectively. To perform training, the first-day adult
264 hermaphrodites cultivated under the standard conditions with OP50 as food were transferred to
265 the control or the training plate, respectively, and kept at 20°C for 4 or 8 hours as specifically
266 described. By the end of the training, some of the naive control and the trained F0 worms were
267 randomly picked to measure the olfactory choice and learning in the automated assay and the
268 rest of the F0s worms were collected from the plates with S-basal medium and passed through
269 a cell strainer 40 micrometer nylon filter (Falcon) to remove laid eggs. Worms were then treated
270 with a bleach solution to isolate the F1 embryos, which were hatched and cultivated under the
271 standard conditions until the adult stage.

272

273 **Olfactory preference assay using the automated olfactory assay**

274 The automated assay that quantifies the olfactory preference in individual animals were
275 conducted as previously described⁵. Briefly, by the end of the training, the naive and trained F0s
276 were washed with buffer and individually placed in the droplets of 2 µL NMG buffer (1mM
277 CaCl₂, 1mM MgSO₄, 25mM KPO₄ pH6.0) in an enclosed chamber and subjected to alternating
278 airstreams that were saturated with the smell of OP50 or PA14 by blowing clean air through
279 freshly generated bacterial cultures. Each olfactory stimulation lasted for 30 second and each
280 assay contains 12 cycles of stimulation. The locomotion of the worms is video recorded and the

281 large body bends of the tested worms were identified with machine-vision softwares. Because
282 large body bends are followed by reorientation, a higher rate of the large body bends indicates a
283 lower preference to the tested airstream^{5,32}. The choice index (CI) is defined as the body bends
284 evoked by OP50 smell minus the body bends evoked by PA14 smell and normalized by the total
285 body bends [CI = (bends evoked by OP50 - bends evoked by PA14)/(bends evoked by OP50 +
286 bends evoked by PA14)] and the learning index (LI) is defined as the CI of the naive worms
287 minus CI of the trained worms (LI = CI of naive worms – CI of trained worms). A positive LI
288 indicates learned avoidance of PA14.

289

290 **Olfactory preference assay using the two-choice assay**

291 The two-choice assay is similar to the one described in Zhang et al., 2005, except for several
292 modifications. To measure the olfactory preference for bacteria, a drop of 5 μ L supernatant of
293 OP50 culture and a drop of 5 μ L PA14 culture were put 2cm apart on a 6 cm NMG plate. In
294 each assay, one worm was placed on a plate equidistant to the two drops of the bacteria culture
295 supernatant and allowed to crawl to the preferred stimulus. The movement and the choice of
296 each worm were recorded and later analyzed. The worms that did not make choice after 10
297 minutes are counted in the total number. The parental-experience dependent choice index
298 (P_CI) in the two-choice assay is defined as the number of worms that choose PA14 minus the
299 number of worms that choose OP50 normalized by the total number of worms tested [P_CI =
300 (number of worms that choose PA14 - number of worms that choose OP50)/total number of
301 worms] and the parental-experience dependent learning index (P_LI) for the two-choice assay is
302 defined as the P_CI of F1s of naive F0s minus P_CI of F1s of trained F0s (P_LI = P_CI of F1s
303 of naive F0s – P_CI of F1s of trained F0s). A positive P_LI indicates increased avoidance of
304 PA14 in F1 induced by the parental experience with PA14.

305

306 **Slow killing assay**

307 The slow killing assay was performed essentially as previously described¹⁴. 200 μ L freshly
308 prepared PA14 culture incubated at 27°C in the Luria-Bertani (LB) medium overnight were
309 spread into a 4cm diameter circle on a 6cm NMG plate and incubated at 37°C for 24 hours and
310 then left at room temperature for another 24 hours before the assay. 20 F1 young adult
311 hermaphrodites cultivated under the standard conditions (Figure 1A) were transferred onto each
312 slow killing plate and kept at 20°C. The dead and the alive worms were counted at the specific
313 time points as shown in Figure 2 over a course of 50 hours.

314

315 **The analysis of parental-experience dependent learning index**

316 To separate the experiments in which F0s exhibited high learning indexes from the experiments
317 in which F0s exhibited low learning indexes, we tested for a significant linear correlation
318 between the learning index (LI) of F0s and the parental-experience dependent learning index of
319 F1s (P_LI). We found that these 2 variables are linearly correlated as shown in Figure 1B. We
320 then used the point where the line of linear regression intersects with the x-axis (LI of F0) to
321 separate the experiments into two groups. The experiments in which the LI of F0 is larger than
322 the point of intersection are included in the group in which F0s exhibited a high level of PA14
323 learning; conversely, the experiments in which the LI of F0 is smaller than the point of
324 intersection are included in the group in which F0s exhibited a low level of PA14 learning.

325

326 **Quantification of the body size and the chemotactic movements**

327 The body size and the parameters of the chemotactic movements in the two-choice assays
328 were quantified by analyzing the recorded worms with the Wormlab tracker
329 (<https://www.mbfbioscience.com/wormlab>).

330

331 **Statistics**

332 The statistical methods, sample size and the number of the replicates are indicated in the
333 legend of each figure.

334

335 **Data Availability**

336 All the data are available upon request. Figure 1-3 and Extended Figure 1 and 2 are generated
337 based on raw data.

338

339 **Code availability**

340 The method used for the automated assay has been previously published⁵ and the code is
341 freely available upon request. The software used to analyze F1 chemotaxis is available at
342 <https://www.mbfbioscience.com/wormlab>.

343

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348

349 **Author contributions**

350 A.P. and Y.Z. designed the experiments, interpreted the results and wrote the manuscript. A.P.
351 performed the experiments and analyzed the data.

352

353 **Competing Interests**

354 The authors declare no competing interests.

355

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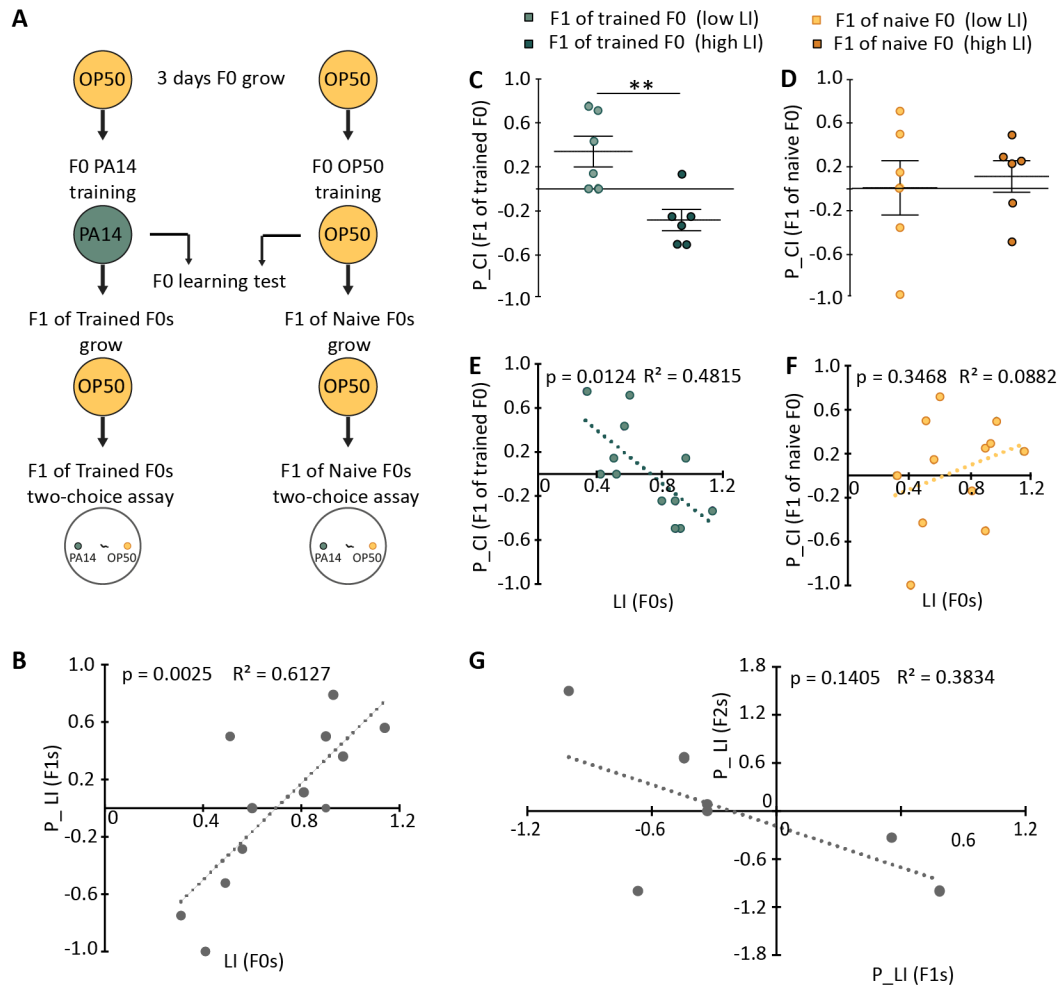
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434

Figure 1



435

436 **Figure 1. Training with PA14 bidirectionally regulates the olfactory response to PA14 in**
 437 **the progeny.**

438 (A) Schematics for the parental-experience dependent olfactory learning.

439 (B) Parental-experience dependent olfactory learning indexes (P_LI) in F1s are linearly
 440 correlated with the aversive olfactory learning indexes (LI) of the F0 mothers.

441 Choice index of F0 (CI) = (bends evoked by OP50 - bends evoked by PA14)/(bends evoked
 442 by OP50 + bends evoked by PA14);

443 LI = CI of naive F0s - CI of trained F0s;

444 Choice index of F1 (P_CI) = (number of worms that choose PA14 - number of worms that
 445 choose OP50)/total number of worms.

446 P_LI = P_CI of F1s of naive F0s - P_CI of F1s of trained F0s;

447 n = 12 independent experiments that contained 12 independent learning assays for F0s
 448 (274 animals) and 12 independent two-choice assays where 186 F1 worms were individually
 449 tested, p value denotes the significance of the linear correlation coefficient.

450 (C, D) The F1s of the trained F0 mothers in experiments in which F0s exhibited a high level of
 451 aversive learning of PA14 avoided PA14, indicated by a negative choice index (P_CI), and
 452 the F1s of the trained F0 mothers in experiments in which F0s exhibited a low level of
 453 aversive learning of PA14 preferred PA14, indicated by a positive choice index (P_CI) (C); in
 454 contrast, the F1s of the naive F0s tested in the same sets of experiments as shown in C

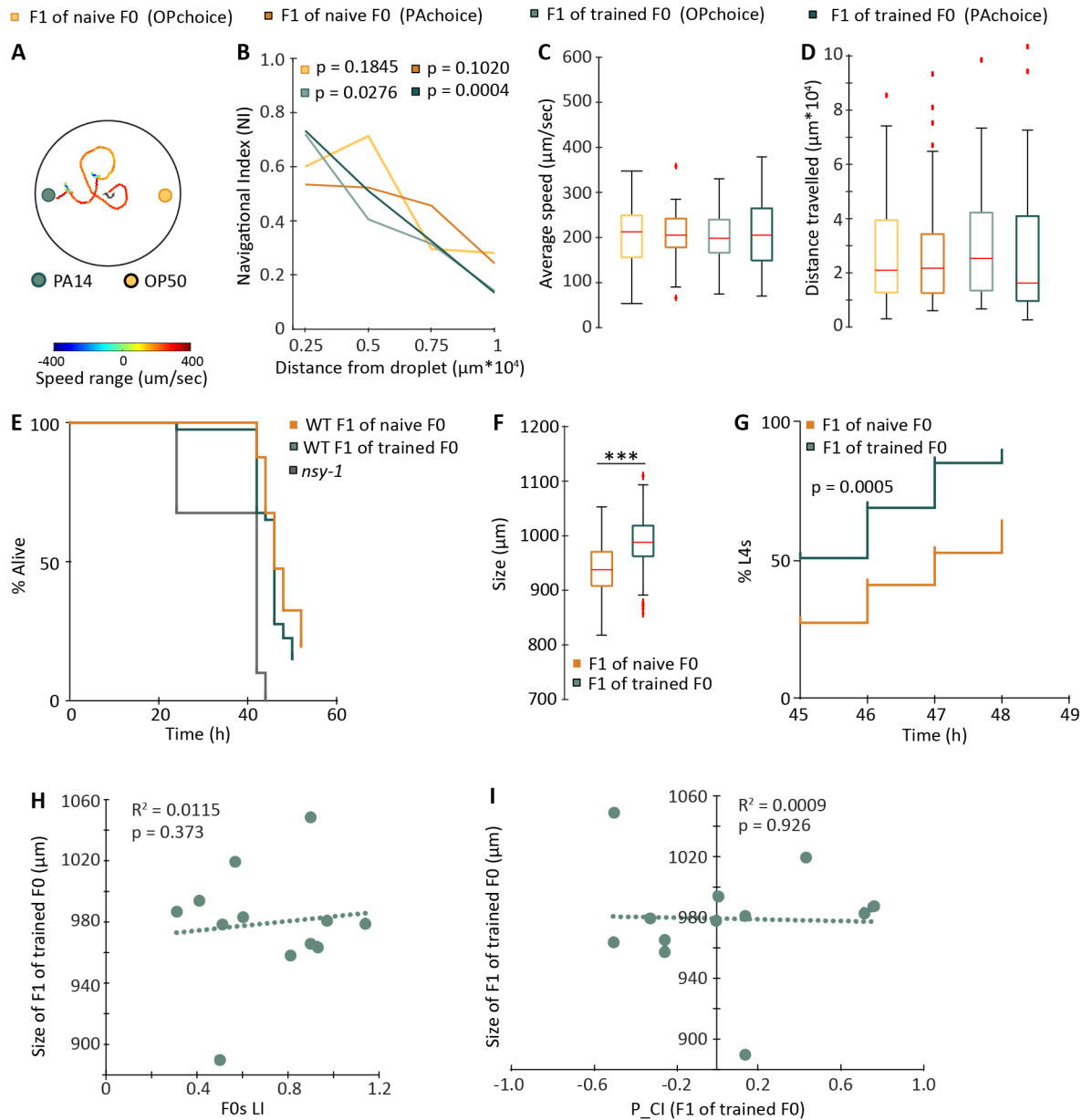
455 where F0s exhibited a high level or a low level of learning had similar choice indexes (P_CI)
456 **(D)**. n = 6 experiments for high F0 learning and 6 experiments for low F0 learning, where 95
457 (high F0 learning) and 91 (low F0 learning) F1 worms individually tested in the two-choice
458 assay for each group; Student's t- test (data are normally distributed), ** p = 0.004 for **C** and
459 p = 0.72 for **D**; Mean ± SEM.

460 **(E, F)** The choice indexes of the F1s (P_CI) of the trained F0s are inversely correlated with the
461 learning indexes (LI) of their trained F0 mothers **(E)**; in contrast, the choice indexes of the
462 F1s (P_CI) of the naive F0s have no correlation with the learning indexes (LI) of their naive
463 F0 mothers **(F)**. A positive or a negative P_CI respectively indicates a preference for PA14
464 or OP50, n = 12 learning assays for F0s (n = 274 animals) and n = 12 two-choice assays
465 where 186 F1 worms were individually tested, P values denote the significance of the linear
466 correlation coefficients.

467 **(G)** Parental-experience dependent olfactory learning indexes (P-LI) in F2s is not significantly
468 correlated with the P-LI of the F1s.
469 P-LI = choice index of F1s (or F2) of naive F0s – choice index of F1s (or F2) of trained F0s;
470 choice index of F1 (or F2) = (number of worms that choose PA14 - number of worms that
471 choose OP50)/total number of animals. n = 6 independent experiments that included 91 F1
472 worms and 88 F2 worms individually tested in the two-choice assays, P values denote the
473 significance of the linear correlation coefficient.
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Figure 2



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477 **Figure 2. The parental experience with PA14 regulates the olfactory behavior and the** 478 **development of the F1 progeny.**

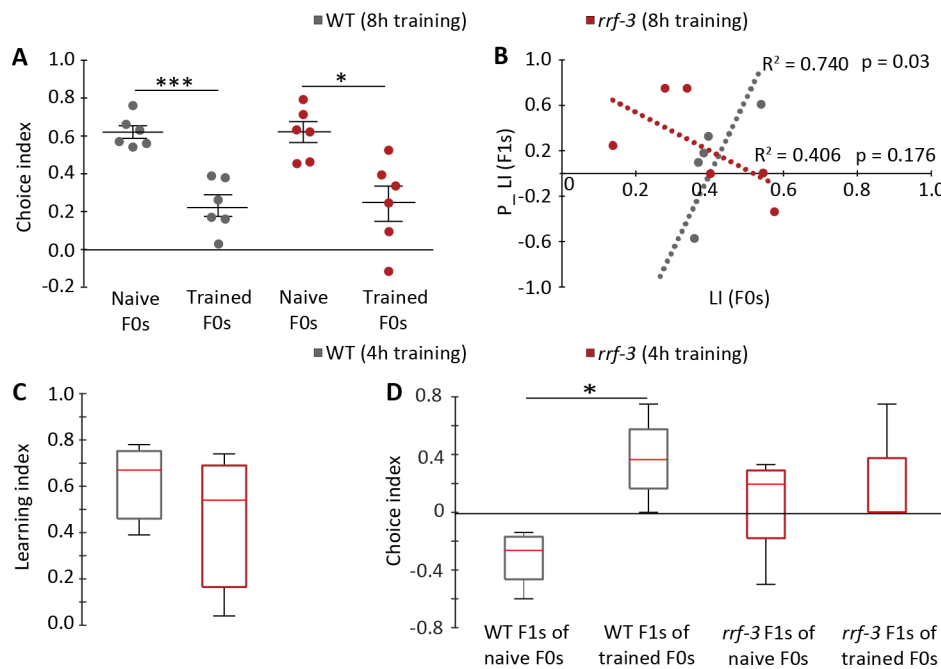
479 **(A)** Schematics showing the trajectory of a worm in the two-choice assay.

480 **(B)** The navigation indexes in the F1s of the trained F0s inversely and linearly correlate with the
 481 distance between the navigating F1s and the target drop of bacteria culture, regardless of
 482 the chosen bacteria; in contrast, the navigation indexes in the F1s of the naive F0 mothers
 483 do not have the similar correlations. The median value of the navigation indexes for each
 484 section was used to analyze linear regression; $n = 40$ F1s of naive F0 (OPchoice), $n = 47$
 485 F1s of naive F0 (PAchoice), $n = 42$ F1s of trained F0 (OPchoice), $n = 47$ F1s of trained F0
 486 (PAchoice). Data was not analyzed for worms that didn't made a choice at the end of the
 487 trial. Data is missing for 7 worms due to problems during recordings.

488 **(C, D)** The F1s of the trained F0s and the F1s of the naive F0s do not differ in the locomotory
489 speeds during the chemotactic movement towards bacteria cultures **(C)** and traveled similar
490 distances before reaching the target **(D)**. The box plots show the median, the first and the
491 third quantile, 1.5 IQR and the outliers; the number of worms for each group is the same as
492 in **B**, Wilcoxon rank-sum test with correction for multiple comparison (the data is not
493 normally distributed).
494 **(E)** In the slow killing assay, the wild-type (WT) F1s of the trained F0s and the wild-type F1s of
495 the naive F0s did not differ in their survival rates ($p = 0.104$), while the *nsy-1* mutant animals
496 were significantly weaker in resisting PA14 infection ($p < 0.0001$). Two independent
497 experiments generated the same conclusion and one experiment containing two replicates
498 ($n = 20$ animal each plate) was presented, Mantel-Cox log rank test.
499 **(F, G)** The F1s of the trained F0s are larger in the body size than the F1s of the naive F0s [**F**,
500 the box plots show the median, the first and the third quantile, 1.5 IQR and outliers; $n = 87$
501 F1s of naive F0 and $n = 89$ F1s of trained F0, Wilcoxon rank-sum test with correction for
502 multiple comparison (the data is not normally distributed)] and contain a higher percentage
503 of the L4-stage larvae after developing from the embryos extracted from F0s for the same
504 amount of time (**G**, $n = 5$ experiments with 10 animals for each group in each experiment,
505 Mantel-Cox log rank test).
506 **(H, I)** The body size of the F1s of the trained F0s do not correlate with the learning index (LI) of
507 their F0 mothers **(H)** or their own choice indexes (P_CI) **(I)**. $n = 12$ experiments in which the
508 learning index of F0s and the choice index of F1s are measured and $n = 89$ F1 worms were
509 measured for the body size.
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Figure 3



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Figure 3. The RNA dependent RNA polymerase RRF-3 regulates the parental experience-dependent olfactory learning of PA14.

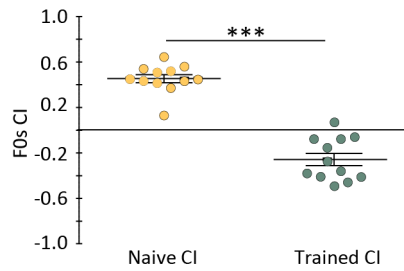
(A, B) Training with PA14 for 8 hours significantly decreased the preference for the smell of PA14, which is indicated by a positive choice index, in the F0 wild-type (WT) and the F0 *rrf-3(pk1426)* mutant animals (A, n = 6 learning assays for wild type (143 animals) and the *rrf-3* mutant (144 animals); two-tailed Student's t-test, *** p < 0.001 and * p < 0.05, Mean ± SEM); however, while wild-type animals displayed a strong correlation between the parental-experience dependent learning index (P_LI) of F1s with the learning index (LI) of F0s, the mutation in the *rrf-3(pk1426)* animals abolished the correlation (B, n = 6 learning assays for F0 (143 WT animals and the 144 *rrf-3* animals) and n = 6 two-choice assays where 88 WT F1s and 92 *rrf-3* F1s were individually tested; P values denote the significance of the linear correlation coefficients].

(C, D) Training with PA14 for 4 hours induced similar aversive learning of PA14 in the F0 wild-type (WT) and the F0 *rrf-3(pk1426)* (C, n = 3 learning assays for WT (36 animals) and *rrf-3* (36 animals), p = 0.71); however, while the wild-type F1 of the trained F0s displayed a significantly higher preference for PA14 than the wild-type F1s of the naive F0s in the two-choice assay, the *rrf-3(pk1426)* F1s of the trained F0s and the *rrf-3(pk1426)* F1s of the naive F0s displayed similar preference (D, n = 4 two-choice assays where 48 WT F1s and 51 *rrf-3* F1s were individually tested, * p = 0.0286 for WT and p = 0.91 for *rrf-3*). Wilcoxon rank-sum test because the data in C and D are not normally distributed, the box plots show the median, the first and the third quartile, 1.5 IQR.

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Extended Figure 1

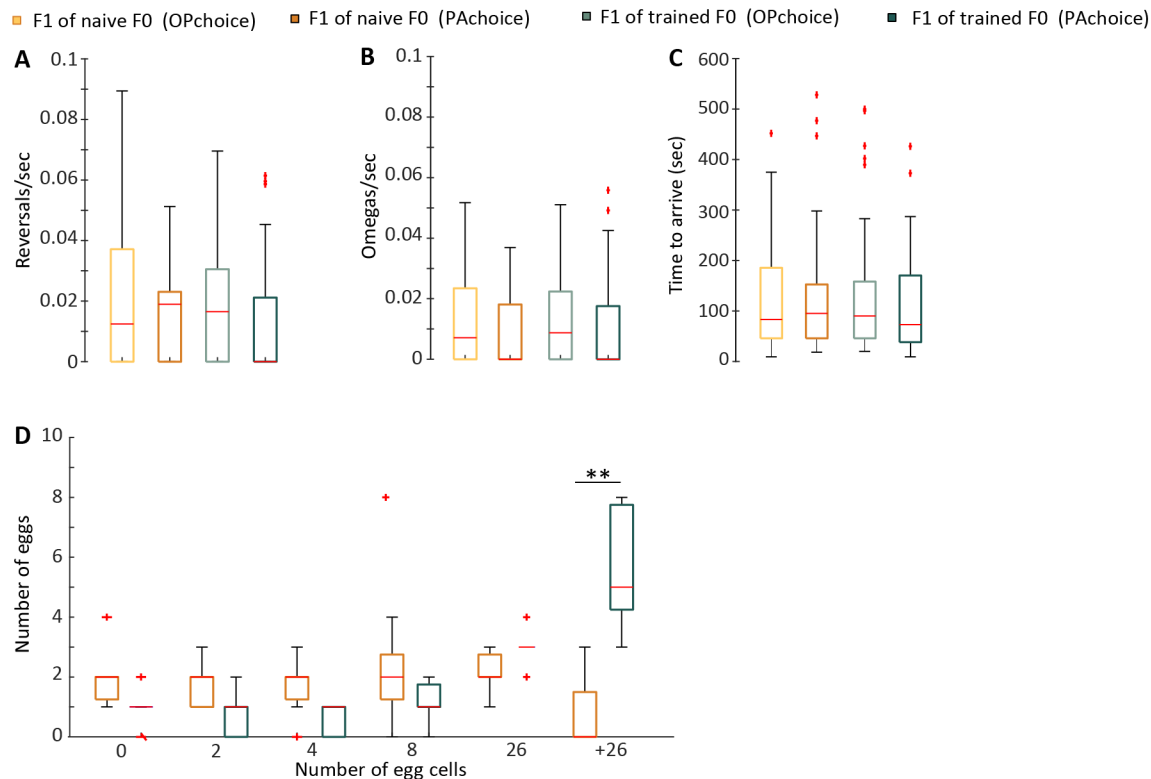


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540 **Extended Figure 1. Training with PA14 for 8 hours strongly reduced the preference for**
541 **the smell of PA14.** The preference of the naive and trained F0 worms were measured in the
542 automated assay. Choice index of F0 (CI) = (bends evoked by OP50 - bends evoked by
543 PA14)/(bends evoked by OP50 + bends evoked by PA14); therefore, a positive choice index
544 indicates a preference for the PA14 smell. n = 12 experiments (274 animals), two-tailed
545 Student's t-test, Mean \pm SEM, *** p < 0.001.
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Extended Figure 2



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Extended Figure 2. The parental experience with PA14 regulates the olfactory behavior and the development of the F1 progeny.

551 **(A, B, C)** The F1s of the trained F0s and the F1s of the naive F0s do not differ in the rate of
552 reversals **(A)** or omega bends **(B**, the large body bends resembling the Greek letter Ω that
553 lead to reorientation) during chemotactic movements towards a drop of bacteria culture in
554 the two-choice assay, or in the time needed to reach the target **(C)**. The box plots show the
555 median, the first and the third quartile, 1.5 IQR and outliers; the number of worms for each
556 group is the same as stated for Figure 2B, Wilcoxon rank-sum test with correction for
557 multiple comparison was used because the data is not normally distributed.

558 **(D)** The eggs inside the bodies of the trained F0s are older than the eggs inside the naive F0s.
559 The number of cells in each egg was counted. The box plots show the median, the first and
560 the third quartile, 1.5 IQR and outliers; all the eggs in $n = 7$ worms per treatment were
561 counted, Wilcoxon rank-sum test with correction for multiple comparison was used because
562 the data is not normally distributed, ** $p < 0.01$.

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