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1 # A memory of recent oxygen experience switches
2 # pheromone valence in *C. elegans*
3

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28 **ABSTRACT**

29 **Animals adjust their behavioral priorities according to momentary needs and**
30 **prior experience. We show that *C. elegans* changes how it processes sensory**
31 **information according to the oxygen environment it experienced recently. *C.***
32 ***elegans* acclimated to 7% O_2 are aroused by CO_2 and repelled by pheromones**
33 **that attract animals acclimated to 21% O_2. This behavioral plasticity arises from**
34 **prolonged activity differences in a circuit that continuously signals O_2 levels. A**
35 **sustained change in the activity of O_2 sensing neurons reprograms the**
36 **properties of their post-synaptic partners, the RMG hub interneurons. RMG is**
37 **gap-junctionally coupled to the ASK and ADL pheromone sensors that**
38 **respectively drive pheromone attraction and repulsion. Prior O_2 experience has**
39 **opposite effects on the pheromone responsiveness of these neurons. These**
40 **circuit changes provide a physiological correlate of altered pheromone valence.**
41 **Our results suggest *C. elegans* stores a memory of recent O_2 experience in the**
42 **RMG circuit and illustrate how a circuit is flexibly sculpted to guide behavioral**
43 **decisions in a context-dependent manner.**

44

45 **SIGNIFICANCE STATEMENT**

46 Animals use memories of their recent environment to regulate their behavioral
47 priorities. The basis for this cross-modal, experience-dependent plasticity is poorly
48 understood. *C. elegans* feeds on bacteria in rotting fruit. It monitors O_2 levels, and
49 switches behavioral state when O_2 approaches 21%. We show that *C. elegans*'
50 memory of its recent O_2 environment reconfigures how it processes sensory
51 information. Pheromones that attract animals acclimated to 21% O_2 repel animals
52 acclimated to 7% O_2. O_2 memory is encoded in the activity history of a circuit that
53 continuously signals O_2 levels. This circuit is connected to neurons driving pheromone
54 attraction and repulsion. O_2 experience changes the pheromone responsiveness of

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55 these sensors and their post-synaptic targets, correlating with the switch in pheromone
56 valence.
57

58 INTRODUCTION

59 The body comprises multiple highly integrated subsystems working together to sustain
60 life from moment-to-moment and over long time scales (1). Much of this coordination
61 involves dynamically interacting neural circuits that optimize responses to current
62 circumstances by taking into account sensory input, organismal state, and previous
63 experience (2–8). Circuit crosstalk enables animals to adjust their behavioral priorities
64 in response to a changing environment e.g. variation in temperature, humidity, day
65 length, or oxygen (O_2) levels (9–13). While some behavioral adjustments can be rapid
66 (14, 15), others develop over time, as animals adapt to changed conditions. How
67 animals store information about their recent environment, and use this information to
68 modify behavioral choices is poorly understood.
69 The compact nervous system of *Caenorhabditis elegans,* which comprises only
70 302 uniquely identifiable neurons (wormwiring.org) (16), provides an opportunity to
71 study the links between prior environmental experience, circuit plasticity, and
72 behavioral change. This nematode is adapted to a life feeding on bacteria in rotting fruit
73 (17, 18). It has sensory receptors for odors, tastants, pheromones, and respiratory
74 gases, as well as temperature, mechanical, and noxious cues (19–22). Despite this
75 simplicity, the mechanisms by which its nervous system marshals information about
76 past and present sensory experience to shape behavioral priorities have largely not
77 been dissected. While valuable (23), the anatomical connectome is insufficient to
78 explain or predict neuronal network function (24, 25), partly because neuromodulators
79 can dynamically reconfigure and specify functional circuits (26–28).

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80 When ambient O_2 approaches 21% *C. elegans* wild isolates become
81 persistently aroused and burrow to escape the surface (29–31). This state switch is
82 driven by tonically signalling O_2 receptors called URX, AQR and PQR (32, 33) whose
83 activity increases sharply when O_2 approaches 21% (29, 31, 34, 35). The URX neurons
84 are connected by gap junctions and reciprocal synapses to the RMG interneurons, and
85 tonically stimulate RMG to promote escape from 21% O_2 (wormwiring.org)(16, 36).
86 URX and RMG are both peptidergic, and at 21% O_2 tonically release neuropeptides
87 (29, 36). RMG is connected by gap junctions to several other sensory neurons besides
88 URX, including pheromone sensors (16, 37). Whether information communicated from
89 URX to RMG about the O_2 environment modulates other sensory responses is
90 unknown.
91 Here, we show that acclimating *C. elegans* to different O_2 environments
92 gradually reconfigures its response to sensory cues. Animals acclimated to 7% O_2 but
93 not 21% O_2 are aroused by CO_2. Pheromones that attract animals acclimated at 21%
94 O_2 repel animals acclimated to 7% O_2. These changes are driven by experience-
95 dependent remodelling of URX O_2 sensors, RMG interneurons, and the ASK and ADL
96 pheromone sensors.
97

98 **RESULTS**

99 **Acclimation to different O_2 environments reprograms CO_2 responses**

100 *C. elegans* escape 21% O_2, which signals that animals are at the surface, and
101 accumulate at 7% O_2, which indicates that animals are burrowed (29, 31, 32). We
102 speculated that *C. elegans* gradually change their sensory preferences when shifted
103 between these two environments.
104 To test our hypothesis, we first examined responses to CO_2. CO_2 is aversive to
105 *C. elegans*, and its concentration rises as O_2 levels fall, due to respiration. Animals

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106 escaping 21% O_2 will thus often encounter high CO_2, creating conflicting drives that we
107 thought could be ecologically significant. Previous work showed that *C. elegans*
108 immediately suppresses CO_2 avoidance when O_2 levels approach 21%, due to
109 increased tonic signalling from URX O_2 sensors (38–41). We speculated that not only
110 current but also prior O_2 experience remodels *C. elegans'* CO_2 responses. To test this,
111 we kept wild isolates from California, France, and Hawaii overnight at 21% or 7% O_2,
112 and compared their responses to 3% CO_2 on a thin lawn of bacteria kept at 7% O_2.
113 After halting briefly, animals acclimated to 7% O_2 became persistently aroused at 3%
114 CO_2 (**Fig. S1***A*–*C*), unlike animals acclimated to 21% O_2.

115 To probe this plasticity, we studied the N2 lab strain. Unlike natural isolates, N2
116 is aroused by 3% CO_2 regardless of prior O_2 experience (**Fig. 1***A*). In N2, output from
117 the RMG interneurons, a major relay of the circuit signalling 21% O_2, is blocked by a
118 hyperactive neuropeptide receptor, NPR-1 215V (36, 37). Natural *C. elegans* isolates
119 have a less active receptor, NPR-1 215F, which does not block RMG output (42). Does
120 this account for altered CO_2 responses? Disrupting *npr-1* caused N2 animals to behave
121 like natural isolates, and to inhibit CO_2-evoked arousal when acclimated to 21% O_2
122 (**Fig. 1***A*). The effects of acclimating *npr-1* animals to 7% O_2 developed over 16 hours,
123 and were reversed within 3 hours if animals were transferred to 21% O_2 (**Fig. S2***A*, *B*).
124 Selectively expressing NPR-1 215V in RMG interneurons prevented *npr-1* animals from
125 acclimating to 21% O_2 (**Fig. 1***B*), and disrupting the GCY-35 soluble guanylate cyclase,
126 a molecular O_2 sensor in URX required for the URX – RMG circuit to signal 21% O_2
127 (29, 31, 32, 34, 43) had the same effect (**Fig. 1***C*).

128 The NPR-1 215V receptor inhibits RMG peptidergic transmission (36). We
129 speculated that circuitry effects of prior O_2 experience might reflect prolonged
130 differences in RMG peptidergic release. To test this we selectively knocked down the
131 carboxypeptidase E ortholog *egl-21* in RMG using RNAi. Processing of most *C.*

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132 *elegans* neuropeptides depends on EGL-21 (44). RMG-knockdown of *egl-21* prevented
133 *npr-1* animals from acclimating to 21% O_2 (**Fig. 1***D*). These data suggest that
134 neuropeptide release from RMG is required for *C. elegans* acclimated to 21% O_2 to
135 suppress CO_2-evoked arousal.
136
137 **Pheromone valence changes with prior O_2 experience**
138 We studied how prior O_2 experience alters CO_2 responses because of the special
139 relationship between these gases. *C. elegans* has, however, many CO_2-responsive
140 neurons, complicating analysis of how persistent differences in RMG activity alter the
141 CO_2 circuits (39, 45, 46). Several studies have reported differences in the sensory
142 responses of N2 and *npr-1* mutants associated with altered RMG function (36, 37, 47,
143 48). We speculated that at least some of these differences could reflect a diminished
144 capacity of N2 animals to acclimate to 21% O_2 due to reduced neurosecretion from
145 RMG.
146 One such behavior is pheromone preference (37). Select pheromone blends
147 attract *npr-1* hermaphrodites but repel N2 hermaphrodites (37, 49). We replicated
148 these observations using an equimolar 10 nM mix of asc-ωC3 (ascaroside C3), asc-C6-
149 MK (ascaroside C6), and asc-ΔC9 (ascaroside C9) pheromones, (37, 50–52) (**Fig. 2***A*).
150 We then asked if acclimating N2 and *npr-1* hermaphrodites overnight in different O_2
151 environments altered their pheromone response. We assayed animals at 21% O_2.
152 Whereas *npr-1* animals acclimated to 21% O_2 were attracted to the pheromone mix,
153 *npr-1* animals acclimated to 7% O_2 robustly avoided it (**Fig. 2***B*). Acclimating N2
154 animals at different O_2 levels did not alter pheromone avoidance (**Fig. 2***B*),
155 recapitulating our observations with CO_2 (**Fig. 2S***C*) Disrupting *gcy-35* switched the
156 pheromone attraction exhibited by *npr-1* animals acclimated at 21% O_2 into repulsion
157 (**Fig. 2***B*). In summary, reducing the activity of O_2 sensing circuitry for prolonged

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158 periods of time – either via environmental or genetic manipulation – transforms
159 pheromone attraction to pheromone avoidance.
160
161 **O_2 experience changes pheromone responses in ASK neurons**
162 How does prior O_2 experience switch pheromone valence? The altered
163 behavior must reflect some lasting change in the circuitry that couples sensory
164 detection to motor output. The principal neurons driving pheromone attraction are the
165 ASK ciliated head neurons. ASK responds to pheromone with a decrease in Ca^{2+} (the
166 'ON' response) that quickly returns to above baseline when pheromone is removed (the
167 'OFF' response). The pheromone-evoked Ca^{2+} response in ASK is bigger in *npr-1*
168 animals compared to N2 animals, a difference thought to contribute to the opposite
169 pheromone preference of these strains (37). Does prior O_2 experience change the
170 responsiveness of ASK to pheromones? To test this, we measured pheromone-evoked
171 Ca^{2+} responses in ASK using the ratiometric Ca^{2+} indicator YC3.60. Overnight
172 acclimation at 7% O_2 attenuated the ASK pheromone response in *npr-1* animals to
173 levels found in N2 (**Fig. 2***C* and 2*D*). Thus, prior O_2 experience alters ASK pheromone
174 responses, commensurate with a change in behavioural preference..
175
176 **Peptidergic feedback heightens RMG responsiveness to 21% O_2 after sustained**
177 **exposure to 21% O_2**
178 RMG interneurons are connected to both the URX O_2 receptors and the ASK
179 pheromone sensors via gap junctions (wormwiring.org)(16, 37). A simple prediction
180 made by our data is that the response properties of RMG change when *npr-1* animals
181 are acclimated to different O_2 levels, and this alters the properties of ASK. To explore
182 this we compared the RMG Ca^{2+} responses evoked by 21% O_2 in *npr-1* animals
183 acclimated to 21% and 7% O_2. Animals acclimated to 7% O_2 showed significantly

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184 smaller RMG responses than those acclimated to 21% O_2 (**Fig. 3***A* and 3*B*). Persistent
185 exposure to 21% O_2 increases RMG responses to this stimulus.

186 To probe this change in RMG properties we compared the URX Ca^{2+} responses
187 evoked by 21% O_2 in animals acclimated to 21% and 7% O_2. URX drives RMG
188 responses (36). URX responses were smaller in animals acclimated to 7% O_2 (**Fig. 3***C*
189 and 3*D*), suggesting changes in RMG properties partly reflect plasticity in URX. In
190 addition, acclimating *npr-1* animals to 21% O_2 was unable to increase RMG
191 responsiveness to 21% O_2 if we selectively knocked down peptidergic transmission
192 from RMG by RNAi of EGL-21 CPE (**Fig. 3***A* and 3*B***)**. These data suggest there is a
193 positive feedback loop by which tonic peptidergic signalling from RMG in *npr-1* animals
194 kept at 21% O_2 increases RMG responsiveness to 21% O_2. Experience-dependent
195 plasticity in RMG and URX represent neural correlates of acclimation to different O_2
196 environments.

197 Importantly, RNAi knockdown of EGL-21 in RMG altered pheromone responses
198 of *npr-1* animals acclimated to 21% O_2, reducing pheromone-evoked Ca^{2+} responses in
199 ASK to N2-like levels (**Fig. 3***E* and 3*F***)**, and conferring robust pheromone avoidance
200 (**Fig. 3***G*). Thus, peptidergic signalling from RMG mediates multiple effects of
201 acclimation to 21% O_2: an increase tonic Ca^{2+} response to 21% O_2 in RMG, a bigger
202 ASK response to pheromone cues, and decreased *C. elegans* avoidance of
203 pheromone.

204

205 **Communication between neurons in the RMG circuit**

206 The neuroanatomy suggests RMG is gap-junctionally connected to multiple sensory
207 neurons, including ASK, the ADL and ASH nociceptors, the AWB olfactory neurons,
208 and the IL2 chemo/mechanoreceptors (**Fig. 4***A*) (wormwiring.org)(16). Changes in
209 RMG may therefore influence the signalling properties of each of these neurons, and

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210 vice-versa. Previous studies suggest that the O_2-sensing URX neurons cooperate with
211 the nociceptive ADL and ASH neurons, and the ASK pheromone sensors, to promote
212 *C. elegans* aggregation and escape from 21% O_2 (33, 36, 37, 53). However, in the
213 absence of physiological data it is unclear what information RMG neurons receive or
214 transmit, apart from tonic O_2 input from URX (29)(36). We asked if O_2-evoked
215 responses in RMG propagated to ADL and ASK. The wiring diagram suggests ASK
216 and ADL are connected to RMG exclusively via gap junctions. ASK and ADL each
217 showed O_2-evoked Ca^{2+} responses in *npr-1* animals (**Fig. S3***A*–S3*D*). We also imaged
218 RMG responses evoked by the pheromone mix we used to stimulate ASK (**Fig. 4***B*).
219 RMG responded with Ca^{2+} dynamics similar to those observed in ASK (**Fig. 4***B*),
220 suggesting information can flow from ASK to RMG. These results support a hub-and-
221 spoke model in which different sensory inputs are integrated through gap junctions with
222 the RMG hub (37).

223 NPR-1 215V signalling has been proposed to silence the hub-and-spoke circuit
224 (24, 27). One attractive model is that signalling from the neuropeptide receptor closes
225 RMG gap junctions (37, 49). To investigate this, we first compared pheromone-evoked
226 Ca^{2+} responses in RMG in N2 and *npr-1* animals, but did not observe any significant
227 differences (**Fig. 4***C* and 4*D*). We then compared O_2-evoked responses in ASK, and
228 also did not observe differences between the two genotypes (**Fig. S3***A* and S3*B*). By
229 contrast, *npr-1* but not N2 animals displayed a strong O_2-evoked response in ADL
230 neurons, (**Fig. S3***C* and S3*D*); this response, unlike the ADL pheromone response (see
231 below), did not require the TRPV1 ortholog OCR-2 (**Fig. S4***A*–S4*D*). Although other
232 interpretations are possible, a simple model to explain our data is that NPR-1 215V
233 signalling in RMG affects different gap junctions differently, inhibiting RMG – ADL
234 communication but having smaller or no effects on the RMG - ASK connection.
235

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236 **O_2 experience sculpts RMG and ADL pheromone responses**

237 The pheromone attraction mediated by ASK neurons and promoted by RMG
238 signalling is proposed to antagonize pheromone avoidance driven by the ADL neurons
239 in a push-pull mechanism (49). The relative strength of these arms determines the
240 animal's response. We found that acclimating *npr-1* animals to 7% O_2 greatly reduced
241 pheromone-evoked responses in RMG compared to animals kept at 21% O_2 (**Fig. 4***C*
242 and 4*D*). Thus, acclimation to 7% O_2 weakens both the ASK (**Fig. 2***C* and 2*D*) and
243 RMG circuit elements that drive attraction to pheromone.

244 Given the neuroanatomy, and the ability of RMG to influence Ca^{2+} in ADL,
245 changes in pheromone-evoked ASK – RMG responses associated with acclimation to
246 different O_2 levels might alter pheromone-evoked responses in ADL. ADL neurons are
247 activated by the ascaroside C9, and a drop of C9 increases the probability of animals
248 reversing (49). In this behavioral paradigm the fraction of animals reversing provides a
249 measure of the pheromone's repulsiveness, and is significantly higher in N2 than *npr-1*
250 animals at low pheromone concentrations (10 nM). Higher concentrations of C9 elicit
251 strong repulsion irrespective of *npr-1* genotype (49). We confirmed that N2 animals
252 showed enhanced repulsion from 10 nM C9 compared to *npr-1* animals (**Fig. 4***E***)**. We
253 then showed that *npr-1* animals acclimated overnight to 7% O_2 enhanced their
254 avoidance of C9, and behaved indistinguishably from N2 (**Fig. 4***E*). The avoidance
255 index (A.I.) used in this assay (49, 54) is calculated as [(fraction reversing to
256 pheromone) − (fraction reversing to buffer alone)], and any change in the A.I. could
257 reflect an altered response to the buffer rather than to C9. Consistent with enhanced
258 pheromone avoidance, *npr-1* animals reversed more in response to C9 if they were
259 acclimated to 7% O_2 (**Fig. 4***E'*).

260 Pheromone-evoked Ca^{2+} responses in ADL neurons were previously
261 characterized using 100 nM C9, a concentration that elicits strong and comparable

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262 repulsion in N2 and *npr-1* animals (49). By using GCaMP6 var500 we could record
263 ADL responses to 10 nM C9, and assess the impact of previous O_2 experience under
264 conditions similar to those used in behavioral assays. *npr-1* animals acclimated to 7%
265 O_2 showed significantly bigger ADL Ca^{2+} responses compared to siblings acclimated to
266 21% O_2 (**Fig. 4***F* and 4*G***)**. Together our data suggest that acclimation to 7% O_2
267 simultaneously weakens the ASK and RMG circuit elements that drive attraction to
268 pheromone, and strengthens the ADL pheromone response driving repulsion, thereby
269 switching the animal's behavioral choice.
270

271 **DISCUSSION**

272 Unfavorable environments can evoke slow, sustained changes in behavioral priorities
273 that reflect an altered internal state. The neural mechanisms mediating such
274 integrative, experience-dependent plasticity are poorly understood. *C. elegans*
275 persistently attempts to escape 21% O_2 (29), presumably because this O_2
276 concentration signals unfavorable surface exposure (31, 32). We find that the O_2 milieu
277 experienced recently by *C. elegans* changes the way it processes sensory information.
278 Pheromones that attract *C. elegans* acclimated to 21% O_2 repel animals acclimated to
279 7% O_2; 3% CO_2 triggers sustained arousal in animals acclimated to 7% O_2 but has
280 comparatively little effect in animals acclimated to 21% O_2.
281 A memory of previous O_2 experience arises from prolonged differences in the
282 activity of a tonically active circuit. Exposure to 21% O_2 tonically stimulates the URX O_2
283 receptor neurons and their synaptic partners, the RMG interneurons. Sustained
284 stimulation of URX and RMG at 21% O_2 increases their response to 21% O_2. The
285 reprogramming of RMG requires peptidergic signaling competence in this interneuron.
286 Our data suggest a simple model in which over time, sustained peptide release from
287 RMG at 21% O_2 feeds back to alter RMG properties. In animals kept at 7% O_2 peptide

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288 release from RMG is low, disrupting the feedback. In this neural integrator model
289 hysteresis in the build up and decay of peptide signaling accounts for the time delays
290 as animals acclimate to 7% or 21% O_2. We previously showed that neuropeptide
291 expression in RMG is positively coupled to neurosecretion from RMG (Laurent et al.,
292 2015), consistent with a positive feedback loop in this interneuron. Tonic circuit activity
293 is common in brains. We speculate that such circuits will often store information about
294 their activity history, and potentially about the animal's experience, by incorporating
295 peptidergic positive feedback loops.

296 RMG has neuroanatomical gap junctions not only with URX, but also with the
297 ASK and ADL pheromone sensors (16, 37). This arrangement suggests that
298 information can be integrated across the circuit (Macosko et al., 2009), but
299 physiological data was hitherto absent. We show that ASK and ADL show O_2-evoked
300 Ca^{2+} responses, and that acclimating animals to different O_2 levels alters O_2 and / or
301 pheromone-evoked responses in each of the URX, RMG, ASK and ADL neurons.
302 Inhibiting peptidergic transmission from RMG prevents RMG and ASK neurons from
303 changing their pheromone responsive properties in animals acclimated to 21% O_2; it
304 also prevents the experience dependent switch in pheromone valence.

305 Changes in the pheromone-evoked responses of ASK and ADL neurons are
306 consistent with changes in RMG changing communication across the network. For
307 example, in animals acclimated to 21% O_2 pheromone-evoked responses in ASK could
308 inhibit ADL pheromone responses, whereas in animals acclimated at 7% O_2 this
309 communication may be less potent. While this is plausible, we cannot exclude that the
310 intrinsic properties of several neurons in the circuit are altered by O_2 experience.

311 We see parallels between our observations and a *Drosophila* study showing
312 that repeated presentation of an aversive shadow cue leads to a persistent change in
313 behavioral state that scales with the number and frequency of the presentations (55,

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314 56). Our findings are also reminiscent of 'latent modulation' in the feeding network of
315 *Aplysia*, where the history of activation in some circuit elements has a lasting effect on
316 subsequent responses, most likely by changing neuronal excitability through
317 peptidergic modulation (57, 58).

318 Why should *C. elegans* reconfigure its sensory responses according to prior O_2
319 experience? It is tempting to speculate about a behavioral hierarchy (59) that gives
320 priority to escape from 21% O_2, and that dominates over sensory drives that could
321 hinder escape from the surface. Animals at the surface may gradually suppress their
322 aversion to CO_2 to facilitate escape to low O_2/high CO_2 environments. Once the threat
323 of exposure at the surface recedes, strong aversive responses to CO_2 again become
324 adaptive. In a boom-and-bust species like *C. elegans* (18), pheromones may be
325 aversive because they predict an unsustainable population density. However, if
326 escaping the surface is more important than accumulating in a crowded environment,
327 attraction towards pheromones may be transiently adaptive because crowded
328 environments predict reduced O_2. Irrespective of the precise selective advantage(s),
329 our data suggest *C. elegans* can adopt alternate persistent internal states according to
330 the length of time they have been exposed to aversive or preferred O_2 levels. In these
331 states neural circuits process sensory information differently, changing the animal's
332 behavioral priorities.
333

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340 **AUTHOR CONTRIBUTIONS**

341 L.F and M.d.B designed experiments, L.F. performed the experiments, L.F. and M.d.B
342 analysed the data and wrote the manuscript.

343

344 **MATERIALS AND METHODS**

345 **Strains**

346 Strains were grown and maintained under standard conditions with *E. coli* OP50 as
347 food (60). To cultivate animals in specific O_2 environments we used a Coy O_2 control
348 glove box (Coy, Michigan, USA).

349 **Behavioral Assays**

350 Locomotion assays were performed as described previously (45, 46). 20–25 adult
351 hermaphrodites were picked to NGM plates seeded 16–20 h earlier with 20 μL of *E.*
352 *coli* OP50 grown in 2 xTY. To create a behavioral arena with a defined atmosphere we
353 lowered a 1 cm × 1 cm × 200 μm deep polydimethylsiloxane (PDMS) chamber on top
354 of the worms, with inlets connected to a PHD 2000 Infusion syringe pump (Harvard
355 apparatus). We pumped in defined gas mixtures (BOC, UK) that were humidified using
356 a sintered gas bubbler (SciLabware, UK) at a flow rate of 3.0 mL/min. Movies were
357 recorded at 2 frames/s using a Point Gray Grasshopper camera mounted on a Leica
358 M165FC dissecting microscope, and were analysed using custom-written Matlab
359 software (36) to detect omega turns and calculate instantaneous speed.

360 Chemotaxis to pheromones was assayed essentially as described previously (37, 61),
361 using four-quadrant Petri plates (Falcon X plate, Becton Dickinson Labware, USA). For
362 each assay 200 worms were picked to a fresh seeded plate for 2–3 hours, washed
363 three times with chemotaxis buffer, and placed at the centre of a 10 cm assay plate
364 with pheromones in alternating quadrants. Animals were scored after ~ 15 min and a
365 chemotaxis index calculated as (number of animals on pheromone quadrants – number

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366 of animals on buffer quadrants) / (total number of animals). We used an equimolar (10
367 nm) mix of the ascarosides C3, C6, and C9. Assays were repeated on at least 4
368 different days.
369 Acute C9 avoidance was examined in the presence of food, using the drop test (62)
370 and as described by Jang and colleagues (49, 54). Responses were scored as
371 reversals if animals initiated a backward movement within 4 s after stimulation that was
372 equal or longer than half their body length. The fraction reversing is given by (number
373 of animals that make a long reversal) / (number if total animals tested); the effect size
374 or avoidance index was calculated as (fraction reversing to pheromone) – (fraction
375 reversing to buffer alone).

376 **Ca^{2+} Imaging.**

377 Ca^{2+} imaging was performed as described previously (37, 49, 63), using microfluidic
378 devices (MicroKosmos, Ann Arbor, MI) to immobilize animals and either a 1:1:1 ratio of
379 three ascarosides (C3, C6, C9) or C9 alone, at the concentration indicated (10nm –
380 100 nm). For O_2 and CO_2 experiments, worms were glued to agarose pads (2% in M9
381 buffer, 1 mM $CaCl_2$) using Dermabond tissue adhesive, with the nose and tail
382 immersed in M9 buffer (45, 46). All imaging was performed on an inverted microscope
383 (Axiovert; Zeiss) with a 40× C-Apochromat lens (water immersion, N.A. 1.0), and
384 MetaMorph acquisition software (Molecular Devices). Recordings were at two frames/s
385 with a 100 ms exposure time. Photobleaching was minimized using optical density filter
386 2.0 or 1.5. For ratiometric imaging experiments we used an excitation filter (Chroma) to
387 restrict illumination to the cyan channel, and a beam splitter (Optical Insights) to
388 separate the cyan and yellow emission light. Animals were pre-exposed to excitation
389 light for ~ 1 min in all experiments. A custom-written Matlab script was used to analyze
390 image stacks(36).

391 **Molecular Biology and Generation of Transgenic Lines.**

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392 Expression constructs were made using the MultiSite Gateway Three-Fragment Vector
393 Construct Kit (Life Technologies). Promoters used include: *sra-9* (3 kb; ASK), *sre-1* (4
394 kb; ADL), *flp-21* and *ncs-1* (RMG). Promoter fragments were amplified from genomic
395 DNA and cloned in the first position of the Gateway system, genes of interest in the
396 second position, and the *unc-54* 3′ UTR or the SL2::mCherry sequence in the third
397 position. Constructs were injected at 30–55 ng/μL, with a coinjection marker (*unc-*
398 *122::RFP* or *unc-122::GFP*) at 50–60 ng/μL.

399 **Statistical methods**

400 Statistical analyses used Prism 6 (GraphPad) and MATLAB (MathWorks). No statistical
401 method was used to predetermine sample size, which are similar to those generally
402 employed in the field. Exact tests used are indicated in figure legends; imaging and
403 locomotion data were analyzed using a non-parametric Mann-Whitney *U* or Wilcoxon
404 signed-rank test.

405

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554

555 **FIGURE LEGENDS**

556 **Figure 1. Recent O_2 experience regulates CO_2-evoked arousal**

557 (**A**) N2 animals and *npr-1* animals acclimated to 7% O_2 exhibit a robust and
558 persistent increase in speed when CO_2 levels rise to 3%, whereas, *npr-1*
559 animals acclimated to 21% O_2 do not. n = 247–302 animals. **** $p < 0.0001$; ns,
560 not significant; Wilcoxon signed-rank test. In this and subsequent figures solid
561 lines indicate the mean and shaded areas represent the standard error of the
562 mean (S.E.M). Black bars indicate time intervals used for statistical
563 comparisons (boxplots). Assays were performed in the presence of food and
564 background O_2 was kept at 7%. (**B**) Selective expression of NPR-1 215V in
565 RMG, (**C**) knocking out *gcy-35*, or (**D**) RNAi-mediated knockdown of the
566 carboxypeptidase E EGL-21 in RMG, prevent *npr-1* animals acclimated to 21%
567 O_2 from suppressing CO_2-evoked arousal. n = 104–235.

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568 Boxes in this and all subsequent panels show the median (black line) and
569 extend from the 25th to 75th percentiles and whiskers represent 10th to 90th
570 percentiles.
571
572 **Figure 2. Pheromone valence changes with prior O_2 experience**
573 (**A**) Quadrant assay for pheromone preference (after (37)). (**B**) Behavioral
574 responses to an equimolar 10 nM mix of C3, C6 and C9 ascaroside
575 pheromones. *npr-1* animals acclimated to 21% O_2 are attracted to the
576 pheromone whereas siblings acclimated to 7% O_2 robustly avoid it. N2 avoid
577 pheromones irrespective of whether they have been acclimated to 7% or 21%
578 O_2. The soluble guanylate cyclase GCY-35 is required for normal O_2 responses
579 and pheromone attraction in *npr-1* animals acclimated at 21% O_2. ** $p < 0.01$;
580 ns, not significant; One-way ANOVA with Tukey's multiple comparisons test. n =
581 8 assays each. (**C**) Previous O_2 experience sculpts pheromone responses in
582 ASK sensory neurons. Acclimation to 7% O_2 reduces pheromone-evoked Ca^{2+}
583 responses in ASK, consistent with altered behavioral preference. (**D**)
584 Quantification of data shown in (C). Heat maps in this and all subsequent
585 figures show individual Ca^{2+} responses. n = 35–36 animals. **** $p < 0.0001$; ** p
586 < 0.01; *$p < 0.05$ ns, not significant; Mann-Whitney *U* test.
587
588 **Figure 3. Peptidergic feedback regulates RMG properties and pheromone**
589 **preference**

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590 (**A**) Acclimation to 7% O_2, or knockdown of *egl-21*, similarly reduce RMG Ca^{2+}
591 responses evoked by a 21% O_2 stimulus. (**B**) Quantification of data shown in
592 (A). n = 20–21 animals. * $p < 0.05$; ns, not significant; Mann-Whitney *U* test.
593 (**C**) Acclimation to 7% O_2 reduces URX Ca^{2+} responses evoked by a 21% O_2
594 stimulus. (**D**) Quantification of data shown in (C). n = 38–39 animals. * $p < 0.05$;
595 Mann-Whitney *U* test. (**E**) Knockdown of *egl-21* in RMG diminishes pheromone-
596 evoked Ca^{2+} responses in ASK to levels observed in N2. (**F**) Quantification of
597 data shown in (E). n = 20–21 animals. * $p < 0.05$; ** $p < 0.01$; **** $p < 0.0001$; ns,
598 not significant; Mann-Whitney *U* test. (**G**) RNAi knockdown of *egl-21* in RMG
599 prevents *npr-1* animals acclimated to 21% O_2 from being attracted to
600 pheromone. n = 12 assays each. * $p < 0.05$; One-way ANOVA followed by
601 Dunnett's multiple comparisons test.
602

603 **Figure 4. RMG hub neurons respond to pheromones and alter their**
604 **response according to recent O_2 experience**

605 (**A**) Circuit showing connections between RMG interneurons and O_2-sensing,
606 nociceptive, and pheromone-sensing neurons. (**B**) An equimolar (100 nM) mix
607 of C3, C6, and C9 ascarosides inhibit RMG. n = 57 animals. (**C**) RMG shows
608 robust pheromone responses in both *npr-1* and N2 animals. (**D**) Quantification
609 of data shown in (C). n = 35–36 animals. ns, not significant; Mann-Whitney *U*
610 test. (**C**) Acclimation to 7% O_2 alters RMG properties and diminishes both ON-
611 and OFF-responses to pheromone addition and removal. (**D**) Quantification of
612 data shown in (C). n = 36 animals each. *** $p < 0.001$; **** $p < 0.0001$; Mann-
613 Whitney *U* test. (**E–G**) Acclimation to 7% O_2 enhances ADL pheromone

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614 responses and acute pheromone repulsion. *npr-1* animals show decreased
615 avoidance of the C9 ascaroside compared to N2 when grown under standard
616 conditions, but not when acclimated to 7% O_2. Plotted are the avoidance index
617 (**E**) and fraction of animals reversing (**E'**), in response to a drop of diluted C9
618 (10 nM) applied to the nose. n = 260–280 animals each. * p < 0.05; *** p <
619 0.001; **** p < 0.0001; ns, not significant; One-way ANOVA followed by Tukey's
620 multiple comparisons test. (**F**) The Ca^{2+} responses evoked in ADL by 10 nM C9
621 pheromone are larger in *npr-1* animals acclimated to 7% O_2 compared to
622 siblings acclimated at 21% O_2. (**G**) Quantification of data plotted in (F). n = 23–
623 24 animals. ** p < 0.01; Mann-Whitney *U* test. (**H**) Model.

624
625
626 **SUPPLEMENTAL FIGURES**

627 **Figure S1. Acclimation to different O_2 environments reprograms CO_2**
628 **responses in natural *C. elegans* isolates.**

629 (**A–C**) Wild strains modulate their CO_2 response according to recent O_2
630 experience. These strains encode NPR-1 215F, the natural low activity isoform
631 of NPR-1. n = 116–171.

632

633 **Figure S2. Time line and reversibility of acclimation to different O_2 levels.**

634 (**A** and **B**) Mean speed of *npr-1* animals at 3% CO_2, plotted against time
635 exposed to 7% or atmospheric (~ 21%) O_2. (**A**) Acclimation to 7% happens
636 gradually and animals continue to increase their speed over many hours. n =
637 151–171. (**B**) Acclimation is reversed rapidly, and after ≤ 3 hours animals
638 behave like siblings grown at 21% O_2. n = 138–188. Error bars in (**A** and **B**) and

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639 shaded regions in (**A'** and **B'**) represent S.E.M. **** $p < 0.0001$; *** $p < 0.001$;
640 ns, not significant; Kruskal–Wallis ANOVA with Dunn's multiple comparisons
641 test. (**C**) N2 are strongly aroused by a 3% CO_2 stimulus, irrespective of whether
642 they have been acclimated at 21% or 7% O_2. n = 462–518 animals. **** $p <$
643 0.0001; Wilcoxon signed-rank test.
644
645 **Figure S3. ASK and ADL sensory neuron spokes respond to O_2.**
646 (**A**) O_2-evoked Ca^{2+} responses in ASK do not differ between N2 and *npr-1*
647 animals. (**B**) Quantification of data plotted in (A). n = 21–24 animals; Mann-
648 Whitney *U* test. Blue shading indicates a shift from 7% to 21% O_2. (**C**) ADL
649 sensory neurons show robust responses to a 21% O_2 stimulus in *npr-1* but not
650 in N2 animals. (**D**) Quantification of data shown in (C). n = 30 animals each. ****
651 $p < 0.0001$; Mann-Whitney *U* test. Note different Ca^{2+} sensors were used to
652 image ASK (YC3.60) and ADL (GCaMP6).
653
654 **Figure S4. The OCR-2 TRPV channel is required for ADL responses to**
655 **pheromone C9 but not to 21% O_2.**
656 (**A**) OCR-2 is not required for ADL responses to a 21% O_2 stimulus, although
657 Ca^{2+} appears to rise less sharply in mutants. (**B**) Quantification of data plotted in
658 (A). n = 21–22 animals each; Mann-Whitney *U* test. Blue shading indicates a
659 shift from 7% to 21% O_2. (**C**) OCR-2 is required cell autonomously for ADL
660 response to the C9 ascaroside. (**D**) Quantification of data shown in (C). n = 11–
661 12 animals each. **** $p < 0.0001$; Mann-Whitney *U* test. Grey shading indicates
662 stimulation with 10 nM C9.

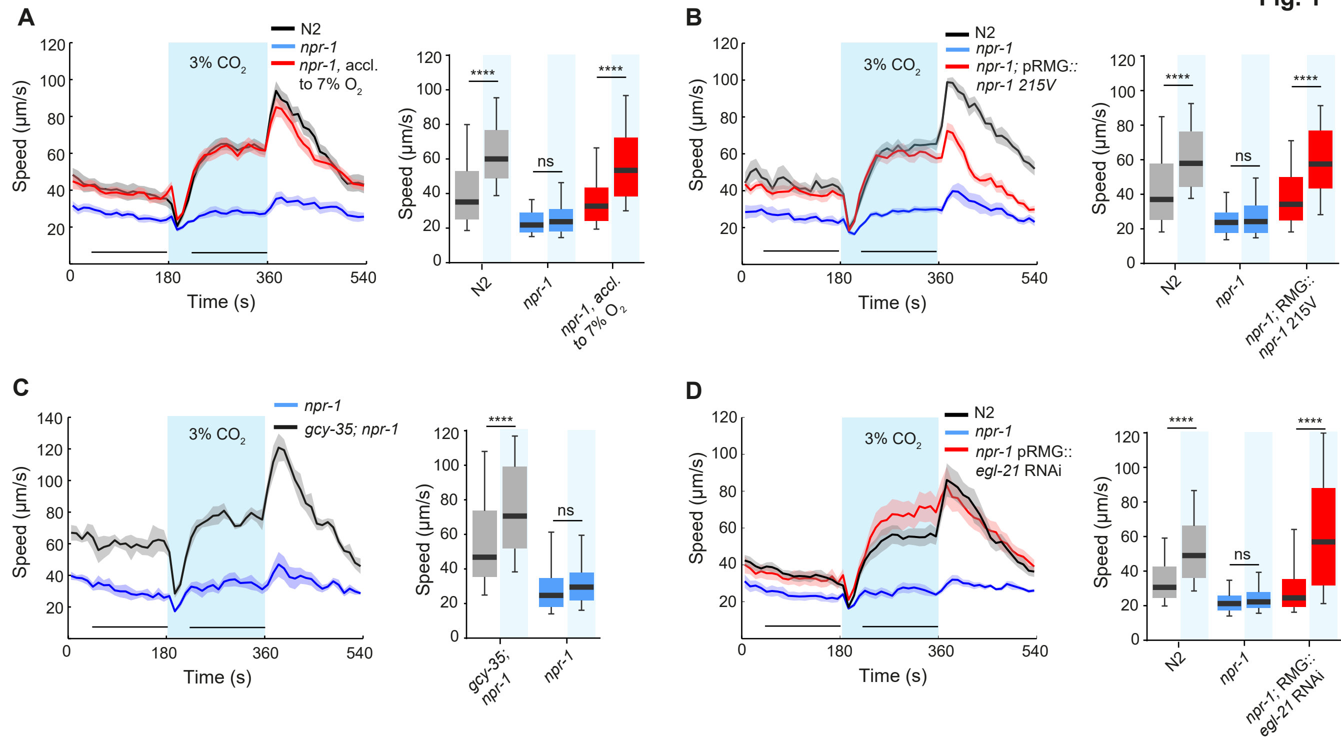
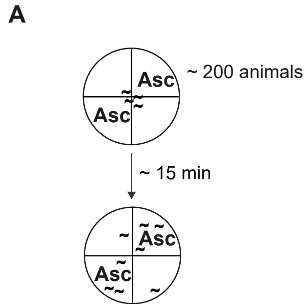
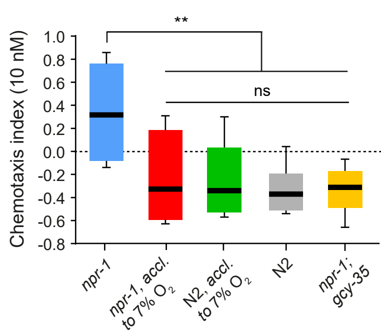
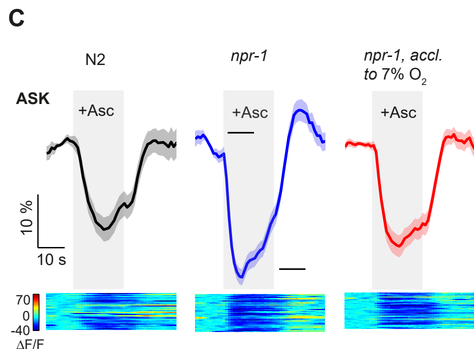


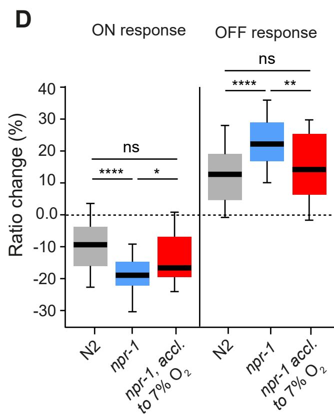
Fig. 1
A
N2
npr-1
npr-1, accl. to 7% O2
3% CO2
Speed (µm/s)
Time (s)
****
ns
****
N2
npr-1
npr-1, accl. to 7% O2
B
N2
npr-1
npr-1; pRMG:: npr-1 215V
3% CO2
Speed (µm/s)
Time (s)
****
ns
****
N2
npr-1
npr-1; RMG:: npr-1 215V
C
npr-1
gcy-35; npr-1
3% CO2
Speed (µm/s)
Time (s)
****
ns
gcy-35; npr-1
npr-1
D
N2
npr-1
npr-1 pRMG:: egl-21 RNAi
3% CO2
Speed (µm/s)
Time (s)
****
ns
****
N2
npr-1
npr-1; RMG:: egl-21 RNAi

Fig. 2









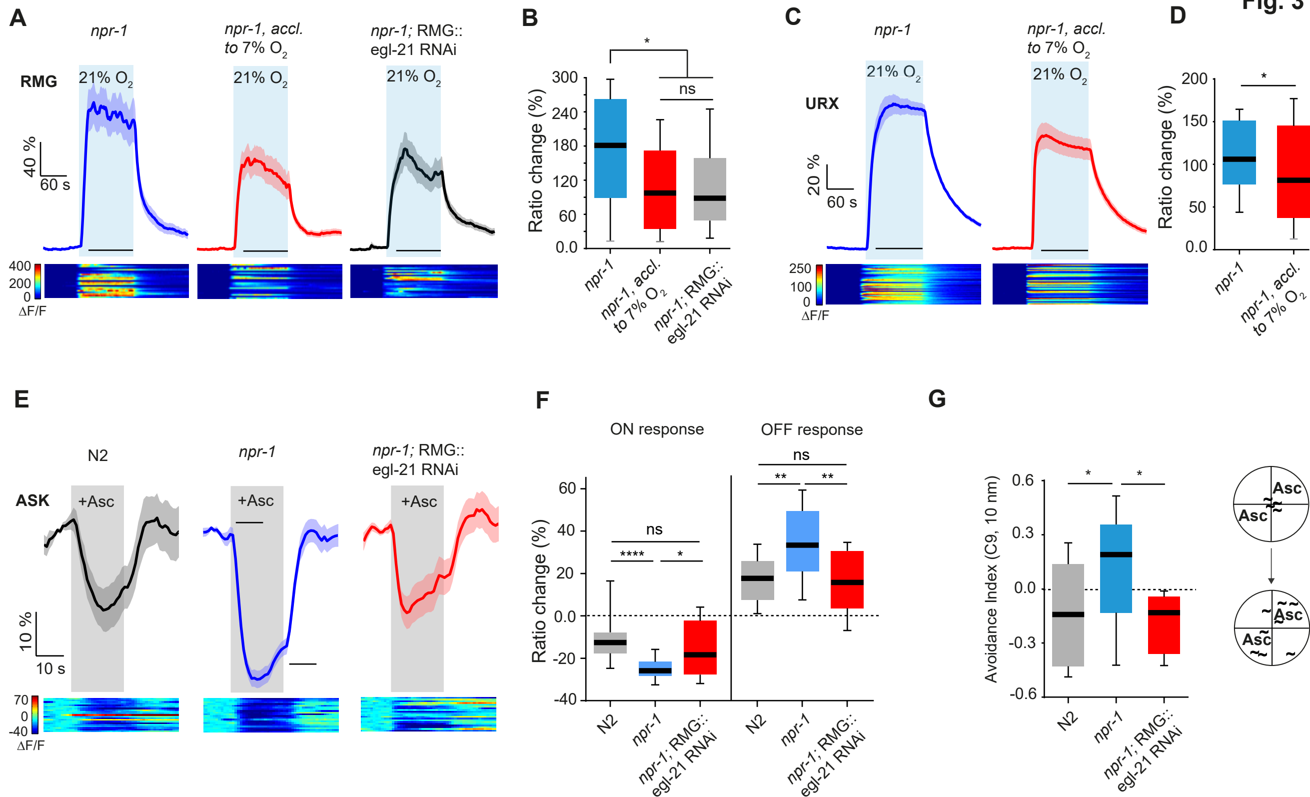
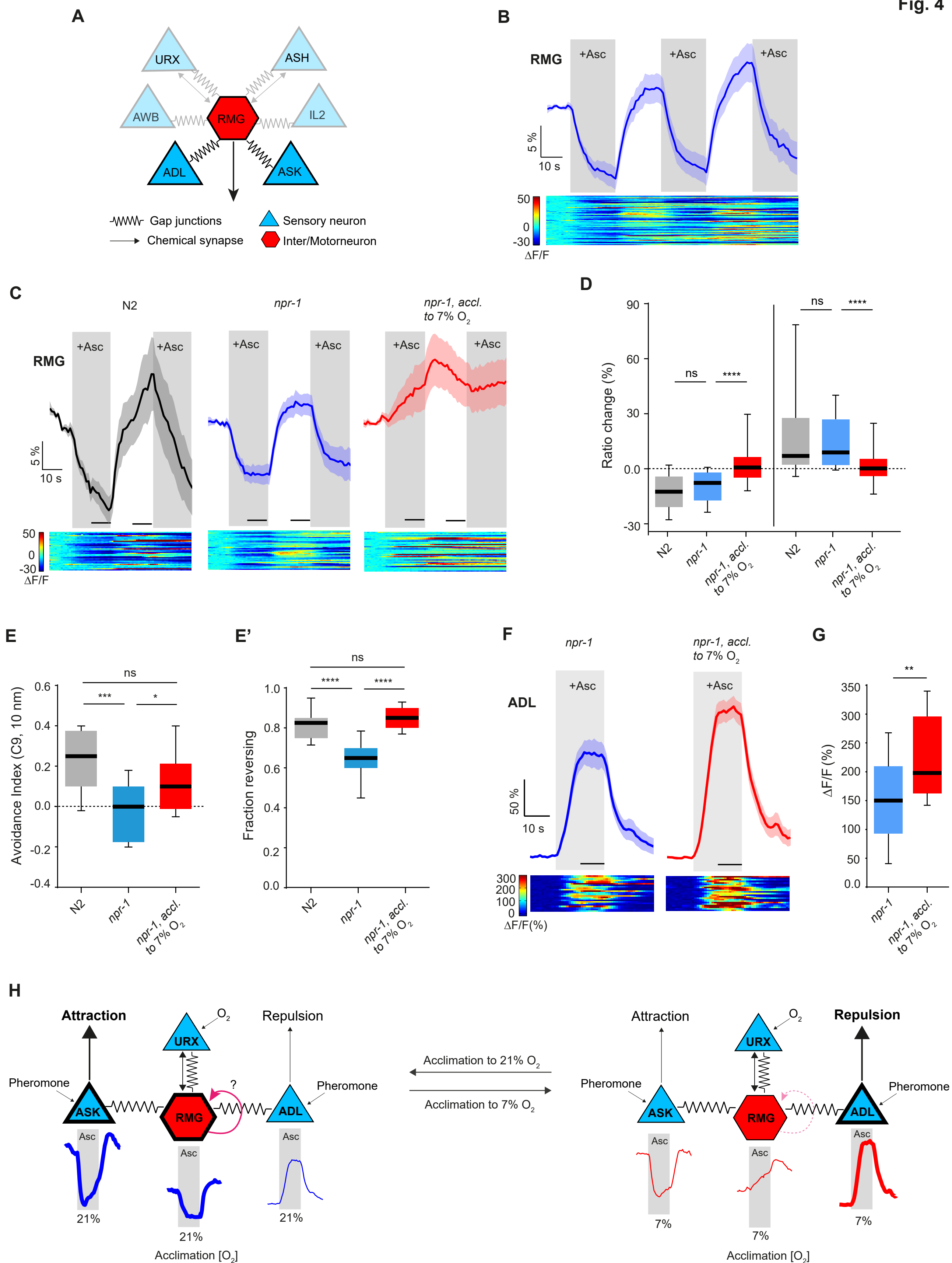


Fig. 3
A
npr-1
npr-1, accl. to 7% O_2
npr-1; RMG:: egl-21 RNAi
RMG
21% O_2
40 %
60 s
400
200
0
ΔF/F
B
Ratio change (%)
300
240
180
120
60
0.0
*
ns
npr-1
npr-1, accl. to 7% O_2
npr-1; RMG:: egl-21 RNAi
C
URX
20 %
60 s
250
100
0
D
200
150
100
50
E
N2
ASK
+Asc
10 %
10 s
70
0
-40
F
ON response
OFF response
60
40
20
-20
-40
****
**
G
Avoidance Index (C9, 10 nm)
0.6
0.3
0.0
-0.3
-0.6
Asc



A
URX
ASH
AWB
RMG
IL2
ADL
ASK
Gap junctions
Chemical synapse
Sensory neuron
Inter/Motorneuron
B
RMG
+Asc
5 %
10 s
50
0
-30
ΔF/F
C
N2
npr-1
npr-1, accl. to 7% O2
D
Ratio change (%)
ns
****
E
Avoidance Index (C9, 10 nm)
ns
***
*
E'
Fraction reversing
F
ADL
50 %
ΔF/F(%)
G
ΔF/F (%)
**
H
Attraction
Repulsion
O2
Pheromone
?
Asc
21%
Acclimation [O2]
Acclimation to 21% O2
Acclimation to 7% O2
7%

Fig. S1

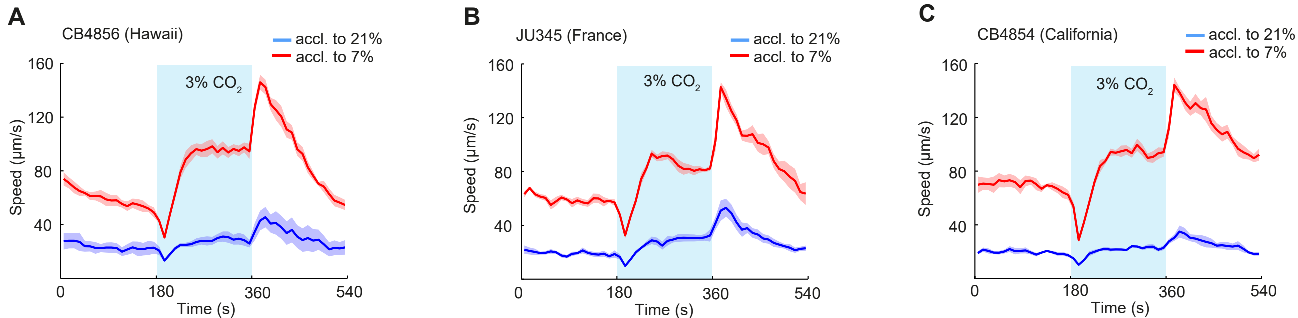
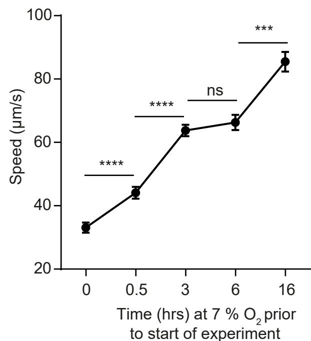
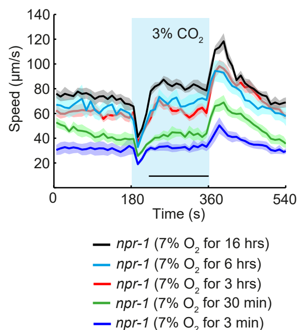


Fig. S2

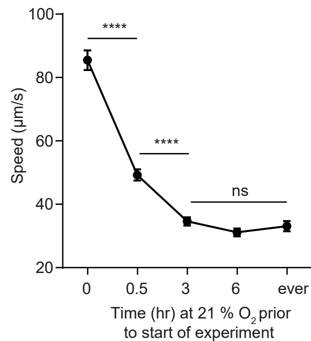
A



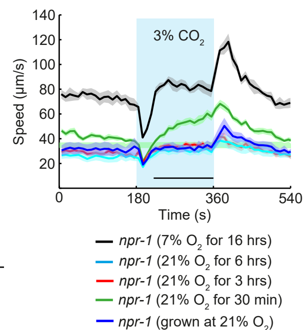
A'



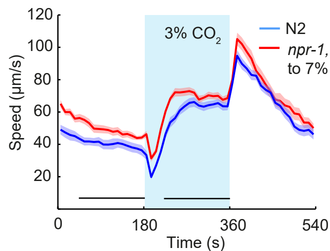
B

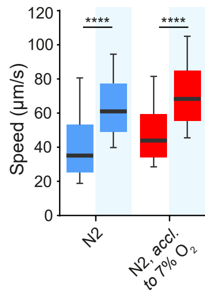


B'



C





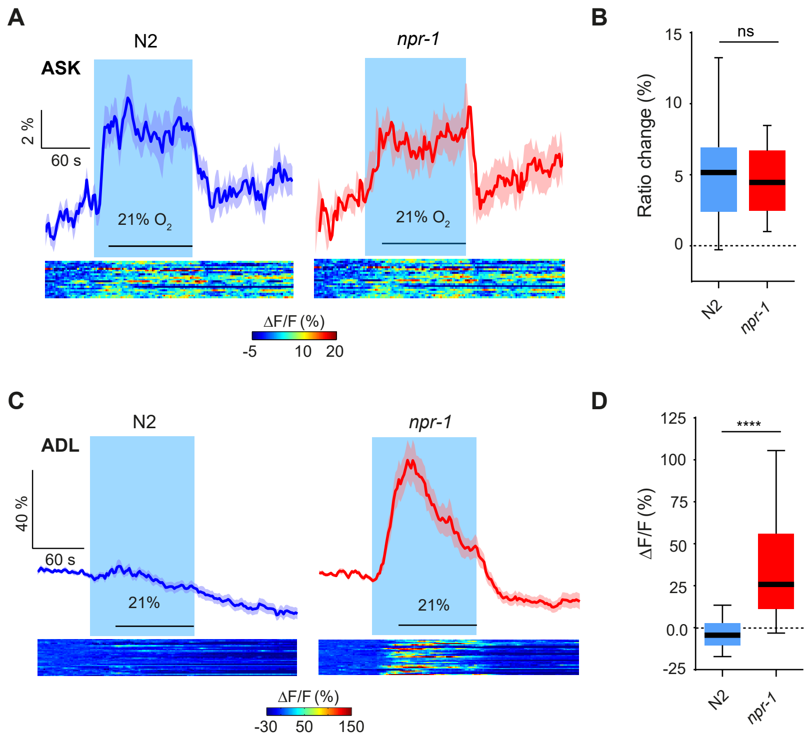


Fig. S3
A
N2
npr-1
ASK
2 %
60 s
21% O_2
21% O_2
ΔF/F (%)
-5
10
20
B
15
10
5
0
ns
Ratio change (%)
N2
npr-1
C
N2
npr-1
ADL
40 %
60 s
21%
21%
ΔF/F (%)
-30
50
150
D
125
100
75
50
25
0.0
-25
****
ΔF/F (%)
N2
npr-1

Fig. S4