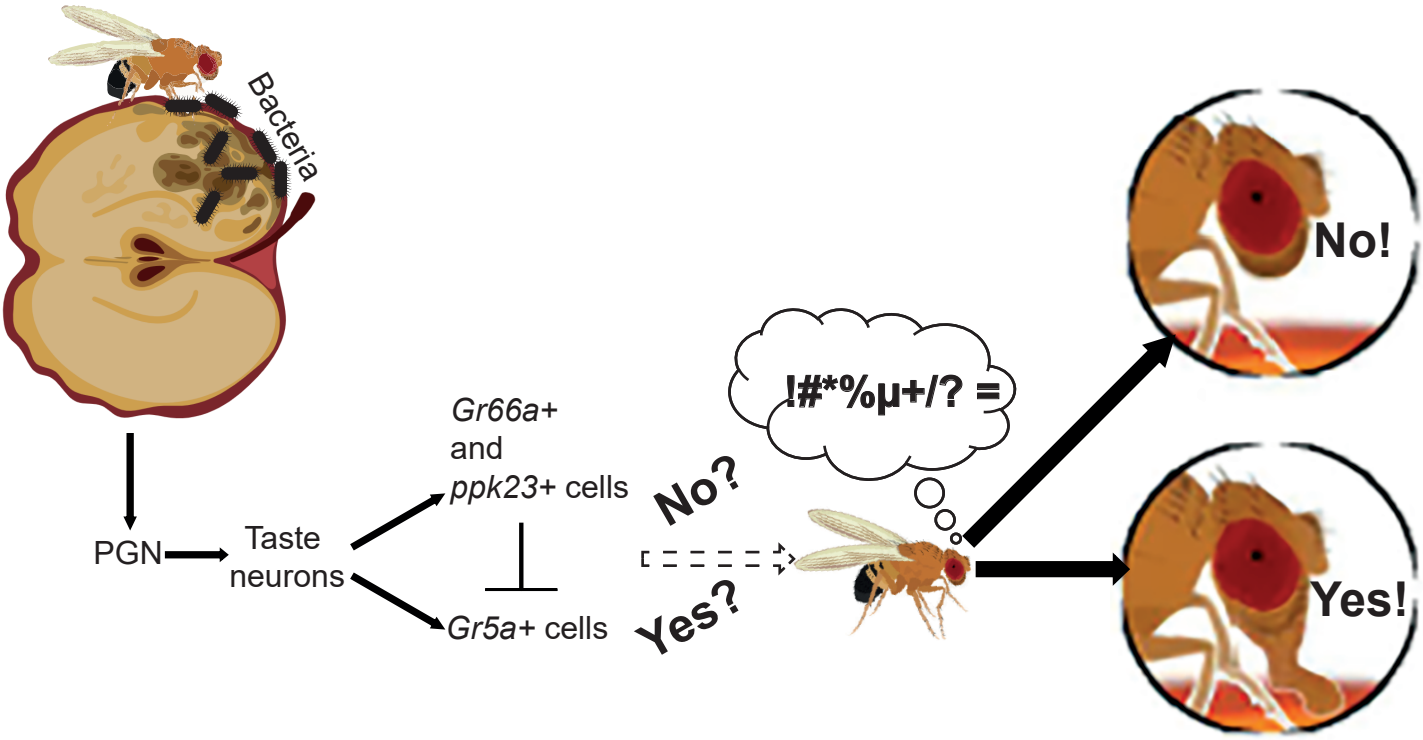
Bacteria
PGN
Taste neurons
Gr66a+ and ppk23+ cells
Gr5a+ cells
No?
Yes?
!#*%µ+/? =
No!
Yes!

Title: Drosophila's sensory responses to bacterial peptidoglycan integrates positive and negative signals

Authors

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Abstract

Interactions between animals, including humans, and surrounding microbes are governed by a delicate balance, crucial for survival. Animals must distinguish and respond adequately to beneficial and harmful microbes to maintain homeostasis. Recent research suggests that bacterial components such as lipopolysaccharide and peptidoglycan (PGN) influence host behavior by modulating neuronal activity. PGN detection by specific neurons can prompt infected female flies to reduce oviposition or trigger avoidance behaviors via gustatory neurons. Using behavioral assays and calcium imaging, we found that PGNs can also act as attractants, activating the sweet taste circuit in a concentration-dependent manner. Our findings demonstrate that flies integrate PGN-derived positive and negative signals to make ad hoc decisions. This dual response underlines the need for *Drosophila* to distinguish between different concentrations of compounds in their environment, integrating sensory data to navigate efficiently in microbe-co-inhabited environments.

Highlights

- Bacterial PGN is attractive to flies
- Gr5a sweet gustatory neurons are activated by PGN
- Adult PER to PGN is not directly influenced by larval life
- Fly gustatory response to PGN integrates both attractive and aversive signals

46 **Introduction**

47 The interactions between animals, including humans, and the microbes surrounding them are
48 governed by a subtle balance. To understand the mechanisms underlying this equilibrium, the
49 insect *Drosophila melanogaster* serves as a powerful model, as microorganisms are essential
50 for the host's development, survival, nutrients, or making certain food sources digestible
51 (Storelli et al., 2011). Some beneficial microbes occupy ecological niches that would otherwise
52 be filled by harmful microorganisms if they were absent (Kim et al., 2010). Conversely, certain
53 pathogenic species must be detected, avoided, or even actively combated by animals
54 (Charroux et al., 2020; Chen et al., 2024; Tleiss et al., 2024). The ability of animals to detect,
55 identify, and respond appropriately to the microbial world is therefore essential for their
56 homeostasis and sustainability.

57 Recent studies have begun to explore the hypothesis that essential bacterial components,
58 such as lipopolysaccharides (LPS) and peptidoglycan (PGN), which are well-characterized
59 ligands for receptors expressed by immune cells, might also modulate host neuronal activity.
60 Such molecular dialogue could allow the host to "sample" its surroundings via its sensory
61 system and adopt behaviors that limit contact with pathogenic microbes or, in case of
62 infection, reduce its impact on the host and its offspring (Kobler et al., 2020; Yanagawa et al.,
63 2017). For instance, PGN detection by a few specific neurons can modulate oviposition
64 behavior in infected females (Kurz et al., 2017; Masuzzo et al., 2022). In this case, the detection
65 of a universal bacterial cell wall component by certain neurons in the brain acts as a molecular
66 alarm, triggering a behavioral change in infected females, reducing their oviposition rate, and
67 potentially conserving energy for the energy-demanding immune response. Other studies
68 demonstrate that bacterial molecular patterns are directly detected by the fly's sensory
69 system. The detection of LPS by bitter taste neurons enables flies to avoid LPS in feeding and

70 oviposition assays (Soldano et al., 2016). We previously reported that by activating the bitter
71 gustatory network, bacterial PGN can suppress the appetitive effect of a sucrose solution,
72 demonstrating that PGN can be perceived as a bitter substance by flies (Montanari et al.,
73 2024).
74 In this study, we used the proboscis extension reflex assay (Shiraiwa and Carlson, 2007) and
75 calcium imaging to assess whether a PGN solution could also activate the sweet taste circuit
76 in flies. Our data reveal that PGN can elicit Gr5a+ neuron-dependent activation within the
77 sweet taste network, demonstrating a dual response of the fly's taste system to PGN. This
78 response is repulsive at low concentrations and attractive at higher concentrations, differing
79 from the dual response to salt or hexanoic acid observed in other gustatory neurons (Jaeger
80 et al., 2018; McDowell et al., 2022; Pradhan et al., 2023; Zhang et al., 2013). This dichotomous
81 response suggests that *Drosophila* shares a need to detect and differentiate between high and
82 low concentrations of specific compounds present in food substrates, engaging different
83 receptors and neuronal cells with distinct coding logic. Furthermore, the detection of bacteria
84 by flies requires integrating inputs from multiple subsets of sensory neurons to generate
85 appropriate responses to bacterial PGN.
86

87 **Results**

88 **Bacterial PGN is Attractive to Flies at High Concentrations**

89 We have previously shown that Diaminopimelic-type peptidoglycan (Dap-type PGN), which
90 forms the cell wall of all Gram-negative bacteria (specifically *Escherichia coli* PGN in our study),
91 activates neurons responsible for aversive behaviors in flies (Montanari et al., 2024).
92 Specifically, Gr66a+ (Wang et al., 2004) and ppk23+ (Jaeger et al., 2018) adult neurons located
93 on the proboscis respond to PGN (Masuzzo et al., 2022; Montanari et al., 2024). Moreover,
94 overexpression of IMD pathway components in Gr66a+ cells alters fly behavior (Masuzzo et
95 al., 2022). Finally, using the Proboscis Extension Reflex (PER) assay, which quantifies gustatory
96 reflexes in immobilized animals (Shiraiwa and Carlson, 2007), we showed that *E. coli* PGN can
97 evoke an aversive response in flies (Montanari et al., 2024).

98 Given the expected presence of bacteria on the natural food source of *Drosophila*
99 *melanogaster*, we wondered whether bacteria might also be perceived as beneficial by the
100 adult fly. To this end, we investigated the potential attractiveness of PGN. To assess whether
101 brief contact with PGN could trigger an attractive response, we focused on the PER (Figure 1A)
102 (Schwarz et al., 2017; Shiraiwa and Carlson, 2007). To accurately assess this response, we
103 tested the molecule without mixing it with anything other than its solvent. When the fly's
104 labellum (analogous to the tongue) is stimulated by a molecule such as sucrose, it immediately
105 extends its proboscis towards the food (Video 1). A given chemical is therefore considered
106 more or less attractive based on its response relative to the reference solvent and a
107 prototypical attractive substance, such as sucrose.

108 To determine if PGN could be attractive to flies, we tested this bacterial molecule alone,
109 diluted in water at various concentrations and compared to sucrose. When the labellum was
110 exposed to 1 mM sucrose, approximately 70% of flies extended their proboscis in an attempt

111 to feed, illustrating the appetitive value of sucrose (Video 1 and Figure 1B). Water alone, the
112 solvent for sucrose and PGN, triggered a PER in 20% of flies, and since the animals were
113 allowed to drink to satiety before the test, we considered this response to water to be neutral,
114 likely due to mechanical stimulation (Video 2 and Figure 1B). We then tested the fly's response
115 to *E. coli* DAP-type PGN. At concentrations of 10 µg/ml (E.c.10) and 100 µg/ml (E.c.100), only
116 20% of flies extended their proboscis, not different from the response to water, suggesting
117 that PGN is not palatable at these concentrations (Figure 1B). However, exposure to PGN at
118 200 µg/ml (*E.c.200*) showed a trend toward increased response, with around 40% of flies
119 performing a PER (Figure 1B). At 500 µg/ml (*E.c.500*), 70% of flies extended their proboscis, a
120 response not different to that seen with 1 mM sucrose (Video 3, Video 4 and Figure 1B). Thus,
121 PGN from *E. coli* at 500 µg/ml is attractive to flies.
122 When DAP-type PGN from the Gram-positive bacterium *Bacillus subtilis* (*B.s.500*) and Lysine-
123 type PGNs from *Staphylococcus aureus* (*S.a.500*), *Micrococcus luteus* (*M.l.500*), and *E. coli* at
124 an OD of 100 (OD100) were used as elicitors, the response was comparable to that of sucrose
125 or *E. coli* PGN at 500 µg/ml (Figure 1B). The attractive effect of *E. coli* PGN at 500 µg/ml
126 (*E.c.500*) was observed in flies from different genetic backgrounds, including *w-*, *yw*, and
127 CantonS strains, as well as in *w-* flies reared on either protein-rich (prot+) or sugar-rich (suc+)
128 media, demonstrating the robustness of the phenotype (Figure 1C and 1D).
129 These findings indicate that, at concentrations of 500µg/mL, both Lysine- and DAP-type PGNs
130 are attractive to the fly's gustatory system.
131
132 **Axenic flies are still capable of triggering the PER response to PGN.**
133 Given that both larvae and adults live in a non-sterile environment, and that we recently
134 reported that larval cohabitation with bacteria is crucial for adults to perceive DAP-type PGN

135 as aversive, we tested whether the palatability of PGN *E.c.500* in adult flies was also
136 dependent on the presence of larval microbiota (Montanari et al., 2024). When flies were
137 reared on antibiotic-containing medium throughout their lifespan (axenic), the PER response
138 to DAP-type PGN *E.c.500* solutions was not different to that observed with sucrose and
139 indistinguishable from the response measured in non-axenic animals (Figure 1E). Thus, unlike
140 our previous findings regarding aversion to DAP-type PGN (Montanari et al., 2024), neither
141 larval cohabitation with bacteria nor adult life in a non-aseptic medium influenced attractive
142 behavior toward high concentrations of DAP-type PGN.
143
144 **PGN Detection by the Gustatory System Involves Gr5a Neurons**
145 To identify which neurons respond to PGN E.c.500, attractive proboscis extension reflexes
146 (PER) were performed in flies where specific neurons were inactivated by overexpressing the
147 Kir2.1 potassium channel (UAS-Kir2.1), which activity impairs the generation of action
148 potentials. The expression of the Gr5a receptor is characteristic of neurons involved in the
149 detection of sweet molecules (Figure 2A) (Dahanukar et al., 2001), and these Gr5a+ neurons
150 play a role in attractive behaviors toward sucrose or threalose. Importantly, inactivation of
151 Gr5a+ neurons using the cell-specific driver Gr5a-Gal4 (Gr5a-Gal4/UAS-Kir2.1) abolished the
152 attractive response to both 1mM sucrose and DAP-type PGN (Figure 2B). Thus, sweet neurons
153 are essential for triggering a PER response to high concentrations of PGN in adult flies.
154 Notably, the PER response of Gr5a-Gal4/UAS-Kir2.1 flies to PGN E.c.500 was significantly lower
155 than the baseline response to water, with less than 10% of flies showing PER (Figure 2B). This
156 suggests that in the absence of sweet neuron activity (Gr5a-Gal4/UAS-Kir2.1), PGN E.c.500 is
157 perceived as aversive by the flies. This finding was consistent with our previous observation
158 that DAP-type PGNs can induce ppk23+ neuron-dependent aversion (Montanari et al., 2024).

159 A possible explanation is that, in the absence of attractive PGN perception (Gr5a-Gal4/UAS-
160 Kir2.1), the aversion to this molecule becomes apparent. These results suggest an integration
161 of positive and negative inputs that influence the fly's decision-making process regarding the
162 taste of different DAP-type PGN concentrations.
163
164 **Gr5a Neurons Are Required in Adults to Detect PGN E.c.500**
165 In a previous study, we showed that Gr66a+ larval neurons are essential for priming the adult
166 behavioral response to DAP-type PGN (Montanari et al., 2024). Despite the fact that Gr5a+
167 neurons do not seem to be present in larvae (Kwon et al., 2011), we wished to test in an
168 unbiased way whether the activity of the larval cells we may target using Gr5a-Gal4 could
169 influence adult attraction to PGN E.c.500. We used the Gal4/Gal80ts binary system, which
170 allows spatial and temporal control of neuron inactivation when coupled with UAS-Kir2.1
171 (Figure 2C). When 1mM sucrose or PGN E.c.500 solutions were used as stimuli, conditional
172 inactivation of Gr5a+ neurons only in adults was as effective as lifelong inactivation of these
173 neurons. In contrast, inactivating Gr5a+ cells only during the larval stage had no impact on the
174 fly's ability to respond to sucrose or PGN E.c.500 (Figure 2D). Therefore, adult PER behavior
175 towards PGN E.c.500 requires functional Gr5a+ neurons in the adult stage.
176
177 **DAP-type PGN Directly Activates Gr5a Sweet Neurons**
178 The observation that Gr5a+ neurons are required for the PER response to PGN stimulation led
179 us to investigate whether these cells could be directly activated by DAP-type PGN. Using
180 *in vivo* GCaMP imaging, we monitored the neuronal activity of Gr5a+ axon projections in the
181 subesophageal zone (SEZ) of the brain of Gr5a-Gal4/UAS-GCaMP6s flies, which were exposed
182 to either water (Video 5) or a drop of PGN (Video 6), followed by 100mM sucrose (Video 7) to

183 assess the viability and identity of neurons. Exposure of the labellum of *Gr5a-Gal4/UAS-*
184 *GCaMP6s* flies to DAP-type PGN at 500µg/ml resulted in an increase in intracellular calcium
185 levels in axonal projections in the SEZ (Figure 3A). These results demonstrate that PGN at high
186 concentrations can directly activate sweet taste neurons.
187
188 **Sweet Neurons Mediating Attraction to PGN E.c.500 Require Gr5a and Gr64 Receptors but**
189 **Are Independent of the IMD Pathway**
190 Having identified the neurons that respond to PGN and are required for the PER, we next
191 investigated the receptors and pathways involved. Previous studies have shown that the Gr5a
192 and Gr64a-f family proteins are receptors expressed in sweet neurons and are involved in
193 detecting sweet molecules such as sucrose and fructose (Dahanukar et al., 2001; Jiao et al.,
194 2008). To identify the receptors involved in PGN sensing, we used mutants lacking both Gr5a
195 and the Gr64a-f family receptors (Gr5a-Gr64a-fCRISPR). The PER response of these mutants
196 to sucrose and PGN E.c.500 was comparable to the response to water (Figure 4A). Since these
197 mutant animals no longer respond to sweet molecules, we used a low salt concentration
198 (50mM NaCl) as a positive attractive control to ensure proper selection of responsive animals.
199 Of note, these mutant animals showed an increasing tendency towards an attraction to water
200 and as anticipated, their response to sucrose was lower compared to wild-type insects and
201 indistinguishable from the water baseline, confirming the role of these receptors. Importantly,
202 the lack of increased PER upon exposure to PGN E.c.500, compared to the baseline water
203 response, indicates that the tested sugar receptors are involved in PGN E.c.500 perception.
204 Regarding the immune response, DAP-type PGN has been shown to be a major inducer of the
205 PGRP/NF-kB-IMD pathway, leading to the production of immune effectors and regulators
206 (Leulier et al., 2003) (Figure 4B). Our previous data indicated that elements of the IMD

207 pathway are required for DAP-type PGN signal transduction in adult bitter taste neurons
208 (Gr66a+ cells) (Masuzzo et al., 2022). To explore the role of the IMD pathway in PGN E.c.500
209 response, we used RNA interference to downregulate components of the IMD pathway. Flies
210 with downregulated PGRP-LC receptor and the IMD transducer FADD specifically in Gr5a+
211 neurons (*Gr5a-Gal4/UAS-pgrp-LC_RNAi* and *Gr5a-Gal4/UAS-Fadd_RNAi*) showed sensitivity
212 to PGN E.c.500 similar to that of control flies, suggesting that contrary to our previous findings
213 regarding Gr66a+ neurons and PGN in adults (Masuzzo et al., 2022), neither PGRP-LC nor FADD
214 is required in Gr5a+ neurons for the PER response to PGN E.c.500 (Figure 4C).
215
216 **Integration of Attractive and Aversive Signals from PGN Exposure Modulates the PER**
217 Our data show that PGN E.c.500 elicits a Gr5a+ neuron-dependent PER response, comparable
218 to that induced by 1mM sucrose. We also demonstrated that DAP-type PGN at concentration
219 of 500µg/ml is capable of activating Gr5a+ neurons (Figure 3A). We previously reported that
220 DAP-type PGN at 200µg/ml antagonizes the PER response to 1mM sucrose by activating adult
221 ppk23+ neurons (Montanari et al., 2024). Taken together, these data suggest that DAP-type
222 PGN can trigger behaviors involving both attractive and aversive neurons. Thus, it is possible
223 that depending on its concentration, *E. coli*-derived DAP-type PGN can either trigger or inhibit
224 a PER via the activation of two independent sets of neurons (Figure 5A).
225 Therefore, when exposed to PGN, opposing signals from different neurons can be integrated
226 by the fly, and the resulting PER will be the outcome of this integration. In this context, the
227 PER to PGN E.c.500 is likely the sum of inputs from Gr5a+ neurons (attraction) as well as from
228 Gr66a+ or ppk23+ neurons (aversion). In the context of a PGN concentration triggering
229 attraction, blocking the input from sweet neurons using Gr5a-Gal4/UAS-Kir2.1 should abolish
230 the attraction and in turn reveal an aversion. Intriguingly, the PER to PGN E.c.500 was lower

231 than the water baseline in Gr5a-Gal4/UAS-Kir2.1 flies indicating a loss of attraction and
232 suggesting an aversion (Figure 2B). This observation was further confirmed by performing the
233 opposite experiment, where blocking the activity of aversive neurons should lead to an
234 increase in attraction. Indeed, while the attractive PER to DAP-type PGN at 100 µg/mL or 200
235 µg/mL in wild-type flies was not statistically different from the solvent control (Figure 1B and
236 5B), blocking the activity of Gr66a+ or ppk23+ neurons (Gr66a-Gal4/UAS-Kir2.1 and ppk23-
237 Gal4/UAS-Kir2.1) resulted in a significantly higher PER to these PGN concentrations compared
238 to the water baseline, concentrations that do not elicit attraction in standard conditions
239 (Figure 5A and 5B).
240 We previously reported that aversion to PGN requires the activity of adult ppk23+ neurons,
241 and that these neurons' ability to respond to PGN is dependent on larval cohabitation with
242 bacteria (Montanari et al., 2024). Based on this, we hypothesized that rearing animals in
243 axenic conditions would impair PGN aversion in adults, making them more attracted to lower
244 PGN concentrations that do not elicit attraction in standard conditions. While the PER to water
245 or 1mM sucrose was not influenced by rearing conditions, axenic animals exposed to DAP-
246 type PGN at 200µg/ml exhibited an increased PER compared to controls, regardless of
247 whether the axenic period lasted throughout their lives or was restricted to the larval stage
248 (Figure 5C). This result supports a model in which the PER to PGN is determined by an
249 integration of attractive and aversive signals from independent neuronal networks.
250
251 **Discussion**
252 Our results demonstrate that at concentrations above 200 µg/ml, both DAP-type and Lys-type
253 PGN, essentially the majority of PGNs, are capable of stimulating sweet neurons through the
254 activation of receptors previously identified as sugar receptors. This activation might be due

255 to the sugar components of PGN, specifically the long polymers of the disaccharides N-
256 acetylmuramic acid and N-acetylglucosamine. Since these sugar components are common to
257 all PGNs, it may explain why both Lys- and DAP-type PGNs are able to trigger attraction, as
258 observed in this study (Vollmer et al., 2008).
259 PGN can be modified by enzymes like amidases and lysozymes, which fragment the polymer
260 into smaller units called muropeptides. Previous studies in insects and mammals have shown
261 that different muropeptides can activate specific receptors with varying degrees of efficacy
262 (Fioriti et al., 2024; Leulier et al., 2003; Tosoni et al., 2019). The minimum structural unit of
263 PGN capable of activating sugar neurons remains to be determined and whether the level of
264 neuronal activation depends on the specific structure of the muropeptides.
265 Interestingly, the activation we observed appears to be independent of the IMD pathway, as
266 the proboscis extension responses (PER) to PGN were similar in both control flies and those
267 RNAi inactivated for certain components of the IMD/NF-kB pathway. This response contrasts
268 with previous findings where PGN, at concentrations near 200 µg/ml, was shown to activate
269 adult bitter Gr66a neurons via part of the IMD pathway, though not involving the Relish
270 transcription factor (Masuzzo et al., 2022). It is also worth noting that the ability to perceive
271 PGN as a bitter molecule has only been observed with DAP-type PGNs, which are typically
272 found in Gram-negative bacteria, making this response specific to a subset of bacterial species,
273 unlike the response observed in our study.
274 In contrast, the sweet neuron response we report here more closely resembles the response
275 observed in ppk23+ neurons, which is also independent of the IMD pathway (Montanari et al.,
276 2024). However, there is a notable difference between the attractive response mediated by
277 Gr5a+ neurons and the aversive response mediated by ppk23+ neurons. The aversive
278 response to PGN requires bacterial priming during the larval phase and is absent in axenic

279 flies, while the Gr5a+ neuron-mediated attraction to PGN occurs even in flies obtained from
280 larva reared in the complete absence of bacteria. These findings suggest that the detection
281 and response to PGN by Gr5a+ neurons in adults are not dependent on prior exposure to
282 bacteria.
283 By measuring PERs in flies with blocked bitter or sweet circuits, we show that the fly's taste
284 system integrates signals from at least three distinct types of neurons—Gr66a+, ppk23+, and
285 Gr5a+—to determine how to respond to PGN. Notably, the behavior requiring these neurons
286 is not triggered by the same concentrations of PGN. Similar dose-dependent behavioral
287 responses have been described for other compounds. For example, NaCl is attractive at 50
288 mM through the Gr5a+ network, but aversive at 250 mM via ppk23+ neurons (Zhang et al.,
289 2013; Jaeger et al., 2018; McDowell et al., 2022). Likewise, hexanoic acid is attractive at low
290 concentrations via Gr5a+ cells, but aversive at higher concentrations through the bitter
291 network (Pradhan et al., 2023). These examples support the idea that different concentrations
292 of the same compound can trigger opposite behaviors, depending on the involved neuronal
293 circuits.
294 Some studies suggest a more complex scenario, where a molecule can be attractive as an odor
295 but aversive as a tastant. A prime example of this is acetic acid, which is attractive when
296 detected by the gustatory system but repulsive when sensed by the olfactory system (Devineni
297 et al., 2019; Joseph et al., 2009). Additionally, systems that integrate opposite inputs from
298 both sweet and bitter signals have been identified, such as the inhibition of sweet neurons by
299 bitter neurons through GABA signaling (Chu et al., 2014) or by the OBP49a protein, which
300 attenuates sweet neuron firing when bitter chemicals are combined (Jeong et al., 2013). These
301 mechanisms help facilitate the avoidance of harmful compounds when sweet molecules are
302 present in mixtures. In our case, the concentration that triggers aversion (200 µg/ml) is lower

303 than the one that triggers attraction (500 µg/ml), suggesting a unique pattern of integration
304 for PGN.
305 While our data go against the intuitive idea that the more you sense PGN, the more you should
306 avoid it, one hypothesis could be that more bacteria equal an advanced decaying fruit with
307 potentially more nutrients. Few bacteria may indicate the presence of a not so advanced
308 decaying fruit. Importantly, we need to consider that the adult aversion to PGN is related to
309 the exposure to pathobionts during the larval life (Montanari et al., 2024). If a larva is only
310 exposed to symbionts, the effect of PGN over adults will be as depicted with the adults of
311 Figures 4B and 4C, with attractions to PGN concentrations as low as 100µg/ml. Thus, the naive
312 adult (Germ-free raised) seems to be primarily tuned toward attraction to lower PGN
313 concentrations, but this can be modulated in the context of a larvae that experienced a stress
314 related to a bacteria like *L. brevis*. Indeed, our findings using the absence of the aversive
315 response (i.e., in Gr66a-Gal4/UAS-Kir2.1 flies) highlight the fact that PGN concentrations at
316 100 µg/ml can trigger attraction. This suggests that sweet neurons are 'naturally' tuned to
317 PGN concentrations lower than those that elicit aversion and lower than those that elicit
318 attraction in 'non-naïve' animals. Taken together, it appears that the integration of bitter
319 signals overrides the attraction, and the PER response toward PGN will necessitate a higher
320 PGN concentration, as if the nutritional value of the environment must outweigh the risk
321 associated with the presence of bacteria, similar to a benefit/risk balance. In a more complex,
322 physiological context where both sweet and bitter molecules are present, the overall influence
323 of microbial molecules like PGN may be balanced by the other chemicals present.
324 Finally, it remains to be explored what these PGN concentrations represent in terms of
325 bacterial populations in natural environments, and whether the physiological state of the
326 bacteria influences their ability to release PGN and if so, in which forms and at what

concentrations. This information would be critical for understanding the role of PGN detection systems in nature.

This task is further complicated by the fact that PGN perception is just one small piece of the sensory information that flies use to navigate and make decisions in an environment teeming with bacteria and other microorganisms. This complex sensory integration is key to the fly's ability to balance the need for food, reproduction, and immunity in an ever-changing microbial landscape.

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References

Charroux, B., Daian, F., Royet, J., 2020. Drosophila Aversive Behavior toward Erwinia carotovora carotovora Is Mediated by Bitter Neurons and Leukokinin. iScience 23, 101152. https://doi.org/10.1016/j.isci.2020.101152

Chen, J., Lin, G., Ma, K., Li, Z., Liégeois, S., Ferrandon, D., 2024. A specific innate immune response silences the virulence of Pseudomonas aeruginosa in a latent infection model in the Drosophila melanogaster host. PLoS Pathog. 20, e1012252. https://doi.org/10.1371/journal.ppat.1012252

Chu, B., Chui, V., Mann, K., Gordon, M.D., 2014. Presynaptic gain control drives sweet and bitter taste integration in Drosophila. Curr. Biol. CB 24, 1978–1984. https://doi.org/10.1016/j.cub.2014.07.020

Dahanukar, A., Foster, K., van der Goes van Naters, W.M., Carlson, J.R., 2001. A Gr receptor is required for response to the sugar trehalose in taste neurons of Drosophila. Nat. Neurosci. 4, 1182–1186. https://doi.org/10.1038/nn765

Devineni, A.V., Sun, B., Zhukovskaya, A., Axel, R., 2019. Acetic acid activates distinct taste pathways in Drosophila to elicit opposing, state-dependent feeding responses. eLife 8, e47677. https://doi.org/10.7554/eLife.47677

Fioriti, F., Rifflet, A., Gomperts Boneca, I., Zugasti, O., Royet, J., 2024. Bacterial peptidoglycan serves as a critical modulator of the gut-immune-brain axis in Drosophila. Brain. Behav. Immun. 119, 878–897. https://doi.org/10.1016/j.bbi.2024.05.009

Jaeger, A.H., Stanley, M., Weiss, Z.F., Musso, P.-Y., Chan, R.C., Zhang, H., Feldman-Kiss, D., Gordon, M.D., 2018. A complex peripheral code for salt taste in Drosophila. eLife 7, e37167. https://doi.org/10.7554/eLife.37167

Jeong, Y.T., Shim, J., Oh, S.R., Yoon, H.I., Kim, C.H., Moon, S.J., Montell, C., 2013. An Odorant Binding Protein required for suppression of sweet taste by bitter chemicals. Neuron 79, 725–737. https://doi.org/10.1016/j.neuron.2013.06.025

Jiao, Y., Moon, S.J., Wang, X., Ren, Q., Montell, C., 2008. Gr64f is required in combination with other gustatory receptors for sugar detection in Drosophila. Curr. Biol. CB 18, 1797–1801. https://doi.org/10.1016/j.cub.2008.10.009

Joseph, R.M., Devineni, A.V., King, I.F.G., Heberlein, U., 2009. Oviposition preference for and positional avoidance of acetic acid provide a model for competing behavioral drives in Drosophila. Proc. Natl. Acad. Sci. U. S. A. 106, 11352–11357. https://doi.org/10.1073/pnas.0901419106

Kim, S.H., Lee, Y., Akitake, B., Woodward, O.M., Guggino, W.B., Montell, C., 2010. Drosophila TRPA1 channel mediates chemical avoidance in gustatory receptor neurons. Proc. Natl. Acad. Sci. U. S. A. 107, 8440–8445. https://doi.org/10.1073/pnas.1001425107

Kobler, J.M., Rodriguez Jimenez, F.J., Petcu, I., Grunwald Kadow, I.C., 2020. Immune Receptor Signaling and the Mushroom Body Mediate Post-ingestion Pathogen Avoidance. Curr. Biol. CB 30, 4693-4709.e3. https://doi.org/10.1016/j.cub.2020.09.022

Kurz, C.L., Charroux, B., Chaduli, D., Viallat-Lieutaud, A., Royet, J., 2017. Peptidoglycan sensing by octopaminergic neurons modulates Drosophila oviposition. eLife 6, e21937. https://doi.org/10.7554/eLife.21937

Kwon, J.Y., Dahanukar, A., Weiss, L.A., Carlson, J.R., 2011. Molecular and cellular organization of the taste system in the Drosophila larva. J. Neurosci. Off. J. Soc. Neurosci. 31, 15300–15309. https://doi.org/10.1523/JNEUROSCI.3363-11.2011

390 Leulier, F., Parquet, C., Pili-Floury, S., Ryu, J.-H., Caroff, M., Lee, W.-J., Mengin-Lecreulx, D.,
391 Lemaitre, B., 2003. The Drosophila immune system detects bacteria through specific
392 peptidoglycan recognition. Nat. Immunol. 4, 478–484. https://doi.org/10.1038/ni922
393 Leulier, F., Vidal, S., Saigo, K., Ueda, R., Lemaitre, B., 2002. Inducible Expression of Double-
394 Stranded RNA Reveals a Role for dFADD in the Regulation of the Antibacterial
395 Response in Drosophila Adults. Curr. Biol. 12, 996–1000.
396 https://doi.org/10.1016/S0960-9822(02)00873-4
397 Masuzzo, A., Manière, G., Grosjean, Y., Kurz, L., Royet, J., 2022. Bacteria-Derived
398 Peptidoglycan Triggers a Noncanonical Nuclear Factor-κB-Dependent Response in
399 Drosophila Gustatory Neurons. J. Neurosci. Off. J. Soc. Neurosci. 42, 7809–7823.
400 https://doi.org/10.1523/JNEUROSCI.2437-21.2022
401 McDowell, S.A.T., Stanley, M., Gordon, M.D., 2022. A molecular mechanism for high salt
402 taste in Drosophila. Curr. Biol. CB 32, 3070-3081.e5.
403 https://doi.org/10.1016/j.cub.2022.06.012
404 Montanari, M., Manière, G., Berthelot-Grosjean, M., Dusabyinema, Y., Gillet, B., Grosjean, Y.,
405 Kurz, C.L., Royet, J., 2024. Larval microbiota primes the Drosophila adult gustatory
406 response. Nat. Commun. 15, 1341. https://doi.org/10.1038/s41467-024-45532-4
407 Pradhan, R.N., Shrestha, B., Lee, Y., 2023. Molecular Basis of Hexanoic Acid Taste in
408 Drosophila melanogaster. Mol. Cells 46, 451–460.
409 https://doi.org/10.14348/molcells.2023.0035
410 Schwarz, O., Bohra, A.A., Liu, X., Reichert, H., VijayRaghavan, K., Pielage, J., 2017. Motor
411 control of Drosophila feeding behavior. eLife 6, e19892.
412 https://doi.org/10.7554/eLife.19892
413 Shiraiwa, T., Carlson, J.R., 2007. Proboscis extension response (PER) assay in Drosophila. J.
414 Vis. Exp. JoVE 193. https://doi.org/10.3791/193
415 Silbering, A.F., Bell, R., Galizia, C.G., Benton, R., 2012. Calcium Imaging of Odor-evoked
416 Responses in the Drosophila Antennal Lobe. JoVE J. Vis. Exp. e2976.
417 https://doi.org/10.3791/2976
418 Soldano, A., Alpizar, Y.A., Boonen, B., Franco, L., López-Requena, A., Liu, G., Mora, N., Yaksi,
419 E., Voets, T., Vennekens, R., Hassan, B.A., Talavera, K., 2016. Gustatory-mediated
420 avoidance of bacterial lipopolysaccharides via TRPA1 activation in Drosophila. eLife 5,
421 e13133. https://doi.org/10.7554/eLife.13133
422 Storelli, G., Defaye, A., Erkosar, B., Hols, P., Royet, J., Leulier, F., 2011. Lactobacillus
423 plantarum promotes Drosophila systemic growth by modulating hormonal signals
424 through TOR-dependent nutrient sensing. Cell Metab. 14, 403–414.
425 https://doi.org/10.1016/j.cmet.2011.07.012
426 Tleiss, F., Montanari, M., Milleville, R., Pierre, O., Royet, J., Osman, D., Gallet, A., Kurz, C.L.,
427 2024. Spatial and temporal coordination of Duox/TrpA1/Dh31 and IMD pathways is
428 required for the efficient elimination of pathogenic bacteria in the intestine of
429 Drosophila larvae. eLife 13, RP98716. https://doi.org/10.7554/eLife.98716
430 Tosoni, G., Conti, M., Diaz Heijtz, R., 2019. Bacterial peptidoglycans as novel signaling
431 molecules from microbiota to brain. Curr. Opin. Pharmacol., • Anti-infectives• New
432 technologies• Neurosciences -CNS diseases and the microbiome 48, 107–113.
433 https://doi.org/10.1016/j.coph.2019.08.003
434 Vollmer, W., Blanot, D., de Pedro, M.A., 2008. Peptidoglycan structure and architecture.
435 FEMS Microbiol. Rev. 32, 149–167. https://doi.org/10.1111/j.1574-
436 6976.2007.00094.x

437 Wang, Z., Singhvi, A., Kong, P., Scott, K., 2004. Taste representations in the Drosophila brain.
438 Cell 117, 981–991. https://doi.org/10.1016/j.cell.2004.06.011
439 Yanagawa, A., Neyen, C., Lemaitre, B., Marion-Poll, F., 2017. The gram-negative sensing
440 receptor PGRP-LC contributes to grooming induction in Drosophila. PloS One 12,
441 e0185370. https://doi.org/10.1371/journal.pone.0185370
442 Zhang, Y.V., Raghuwanshi, R.P., Shen, W.L., Montell, C., 2013. Food-Experience Induced
443 Taste Desensitization Modulated by the Drosophila TRPL Channel. Nat. Neurosci. 16,
444 1468–1476. https://doi.org/10.1038/nn.3513
445

446 **Material and methods**

447 **Fly husbandry**

448 Flies were grown at 25°C on a yeast/cornmeal medium in 12h/12h light/dark cycle-controlled
449 incubators. For 1 L of food, 8.2 g of agar (VWR, cat. #20768.361), 80 g of cornmeal flour
450 (Westhove, Farigel maize H1) and 80 g of yeast extract (VWR, cat. #24979.413) were cooked
451 for 10 min in boiling water. 5.2 g of Methylparaben sodium salt (MERCK, cat. #106756) and 4
452 mL of 99% propionic acid (CARLOERBA, cat. #409553) were added when the food had cooled
453 down (prot+ media).
454 suc+ media: for 1 L of food, 11 g of agar (VWR, cat. #20768.361), 80 g of cornmeal flour
455 (Westhove, Farigel maize H1), 20 g of yeast extract (VWR, cat. #24979.413) and 30g of Sucrose
456 were cooked for 10 min in boiling water. 2.5g of Moldex and 5 mL of 99% propionic acid
457 (CARLOERBA, cat. #409553) were added when the food had cooled down.

458

459 **Fly stocks**

460 The reference strain in this study is noted w^{-} and corresponds to $w^{[1118]}$ (Bloomington #5905).
461 Canton S (Bloomington #64349) and $y^{[1]}$, $w^{[1118]}$ and *Gr5A*$^{-}$; *Gr64a-f*$^{-}$ (gift from Bernard
462 Charroux) and *Gr5a-Gal4* (Bloomington #57992) and *UAS-GCaMP6S* (Bloomington #42746)
463 and *UAS-Kir2.1* (Bloomington #6595) and *Tub-Gal80ts* (Bloomington #7016) and *UAS-GFP*
464 (Bloomington #32195) and *UAS-pgrp-LC_RNAi* (VDRC #101636) and *UAS-Fadd_RNAi* (Leulier
465 et al., 2002) and *ppk23-Gal4* (Bloomington #93026) and *Gr66a-Gal4* (Bloomington #28801).

466

467 **PER assay**

468 All flies used for the test were females between 5 and 7 days old. Unless experimental
469 conditions require it, the flies are kept and staged at 25°C to avoid any temperature changes

470 once they are put into starvation. The day before, the tested flies are starved in an empty tube
471 with water-soaked plug for 24h at 25°C.
472 Eighteen flies are tested in one assay, 6 flies are mounted on one slide and in pairs under each
473 coverslip. To prepare the slide, three pieces of double-sided tape are regularly spaced on a
474 slide. Two spacers are created on the sides of each piece of tape by shaping two thin cylinders
475 of UHT paste. To avoid the use of carbon dioxide flies are anesthetized on ice. Under the
476 microscope, two flies are stuck on their backs, side by side, on same piece of tape so that their
477 wings adhere to the tape. A coverslip is then placed on top of the two flies and pressed onto
478 the UHT paste, blocking their front legs and immobilizing them.
479 Once all slides are prepared, they are transferred to a humid chamber and kept at 25°C for 1.5
480 hours to allow the flies to recover before the assay.
481
482 Flies are tested in pairs, the test is carried out until completion on a pair of flies (under the
483 same coverslip), and then move on to the next pair.
484 Before the test, water is given to each pair of flies to ensure that the flies are not thirsty and
485 do not respond with a PER to the water in which the solutions are prepared. Stimulation with
486 the test solution is always followed by a control stimulation with a sweet solution, to assess
487 the fly's condition and its suitability for the test. During the test small strips of filter paper are
488 soaked in the test solution and used to contact the fly's labellum (three consecutive times per
489 control and test phase). Contact with the fly's proboscis should be as gentle as possible. Ideally
490 the head should not move. A stronger touch may prevent the fly from responding to
491 subsequent stimulation. Based on the protocol needed the test is done following the sequence
492 and the timing in the table below.
493 **Tab.1 Attraction protocol**

Water To satiety	Wait 1 min	**TEST** Repeated 3 times	Wait 1 min	Water one time	Wait 1 min	**CONTROL** Sucrose 500mM Repeated 3 times

All solutions to be tested are prepared the test day and stored at room temperature. The control stimulation at the end of the assay is performed with 500mM sucrose (D(+)-sucrose ≥99.5 %, p.a. Carl Roth GmbH + Co. KG). After each test or control stimulation, a water-soaked strip is used to tap the proboscis and clean it. For the water baseline, a paper stripe soaked in water is used as the test solution.

The response of the fly to each stimulation is recorded and averaged. A fly is considered as responding when a full extension of the proboscis is observed (PER). The duration of the extension is not taken into account. Flies that respond positively (PER) to at least one of the control stimulations are considered for further analysis, the others are discarded. The PER index is calculated as the ratio of flies tested that responded with a PER to the TEST stimulation (number of flies that responded at least 1 time to the test solution and at least 1 time to the control solution) / (total amount of flies that responded at least 1 time to the control solution) and represented as ± 95% CI. In case of stage dependent experiments, flies are shifted from one condition to another upon hatching.

513 **Microscopy**

514 No immunostaining was performed with proboscises. Proboscises of adult females were
515 dissected in PBS, rinsed with PBS, and directly mounted on slides using Vectashield fluorescent
516 mounting medium. The tissues were visualized directly after. Images were captured with a
517 LSM 780 Zeiss confocal microscope (20X air objective was used).

518

519 ***In vivo* calcium imaging**

520 *In vivo* adult calcium imaging experiments were performed on 5-7 day-old starved mated
521 females. Animals were raised on conventional media with males at 25°C. Flies were starved
522 for 20-24 h in a tube containing a filter paper soaked in water prior to experiments. Flies of
523 the appropriate genotype were anesthetized on ice for 1 h. Female flies were suspended by
524 the neck on a plexiglass block (2 x 2 x 2.5 cm), with the proboscis facing the center of the block.
525 Flies were immobilized using an insect pin (0.1 mm diameter) placed on the neck. The ends of
526 the pin were fixed on the block with beeswax (Deiberit 502, Siladent, 1345 209212). The head
527 was then glued on the block with a drop of rosin (Gum rosin, Sigma-Aldrich 1346 -60895-,
528 dissolved in ethanol at 70 %) to avoid any movements. The anterior part of the head was thus
529 oriented towards the objective of the microscope. Flies were placed in a humidified box for 1
530 h to allow the rosin to harden without damaging the living tissues. A plastic coverslip with a
531 hole corresponding to the width of the space between the two eyes was placed on top of the
532 head and fixed on the block with beeswax. The plastic coverslip was sealed on the cuticle with
533 two-component silicon (Kwik-Sil, World Precision Instruments) leaving the proboscis exposed
534 to the air. Ringer's saline (130 mM NaCl, 5 mM KCl, 2 mM MgCl2, 2 mM CaCl2, 36 mM
535 saccharose, 5 mM HEPES, pH 7.3) was placed on the head. The antenna area, air sacs, and the
536 fat body was removed. The esophagus was cut without damaging the brain and taste nerves

537 to allow visual access to the anterior ventral part of the sub-esophageal zone (SEZ). The
538 exposed brain was rinsed twice with Ringer's saline. GCaMP6s fluorescence was viewed with
539 a Leica DM600B microscope under a 40x water objective. Stimulation was performed
540 manually using a pipette with gel loading tip by applying 140 µL of tastant solution diluted in
541 water on the proboscis. The gustatory stimulation was continuous as the tastant is in the drop
542 contacting the proboscis. The recording started before the addition of the tastant and the
543 calcium response could be observed immediately following the contact with the sensilla. For
544 each condition, n=7 to 16. To ensure that GR5a neurons were still alive after application of
545 water or of PGN at 500µg/ml, we used 100mM sucrose as a positive control. Consequently,
546 for the sucrose, n = sum of flies tested with water + flies tested with PGN. Some quantifications
547 with water or PGN were discarded due to fly movements, whereas they could be utilized for
548 the sucrose.
549 GCaMP6s was excited using a Lumencor diode light source at 482 nm ± 25. Emitted light was
550 collected through a 505-530 nm band-pass filter. Images were collected every 500ms using a
551 Hamamatsu/HPF-ORCA Flash 4.0 camera and processed using Leica MM AF 2.2.9. Each
552 experiment consisted of a recording of 70-100 images before stimulation and 160 images after
553 stimulation. Data were analyzed as previously described (Silbering et al., 2012) by using FIJI
554 (https://fiji.sc/).
555
556 **Different PGN used**
557 *E. coli* K12: Invivogen, catalog code #tlrl-kipgn
558 *Staphylococcus aureus :* Invivogen, catalog code #tlrl-kipgn
559 *Micrococcus luteus* : Sigma Aldrich, catalog #53243
560 *Bacillus subtilis*: Sigma Aldrich, catalog #SMB00288

561

562 **Bacterial strains and maintenance**

563 For this study, the following bacterial strain was used: *E. coli* K12 ATCC

564 Grown in LB liquid, in aerobic conditions, 37°C, shaking at 200 rpm.

565

566 **Statistics and data representation**

567 The Prism software was used for statistical analyses.

568 For *in vivo* calcium imaging, the D'Agostino–Pearson test to assay whether the values are

569 distributed normally was applied. As not all the data sets were considered normal, non-

570 parametric statistical analysis such as non-parametric unpaired Mann–Whitney two-tailed

571 tests was used for all the data presented.

572 For PER datasets. As the values obtained from one fly are categorical data with a *Yes* or *No*

573 value, we used the Fisher exact t-test and the 95% confidence interval to test the statistical

574 significance of a possible difference between a test sample and the related control.

575 For PER assays, at least 2 independent experiments were performed. The results from all the

576 experiments were gathered and the total amount of flies tested is indicated in the graph. In

577 addition, we do not show the average response from one experiment representative of the

578 different biological replicates, but an average from all the data generated during the

579 independent experiments in one graph. However, each open circle represents the average

580 PER of 1 experiment.

581

582 **Detailed lines, conditions and statistics for the figure section**

583 All these data are downloadable with the source data file.

584 DOI: 10.6084/m9.figshare.27908889

https://figshare.com/ndownloader/files/50813955

Figure 1

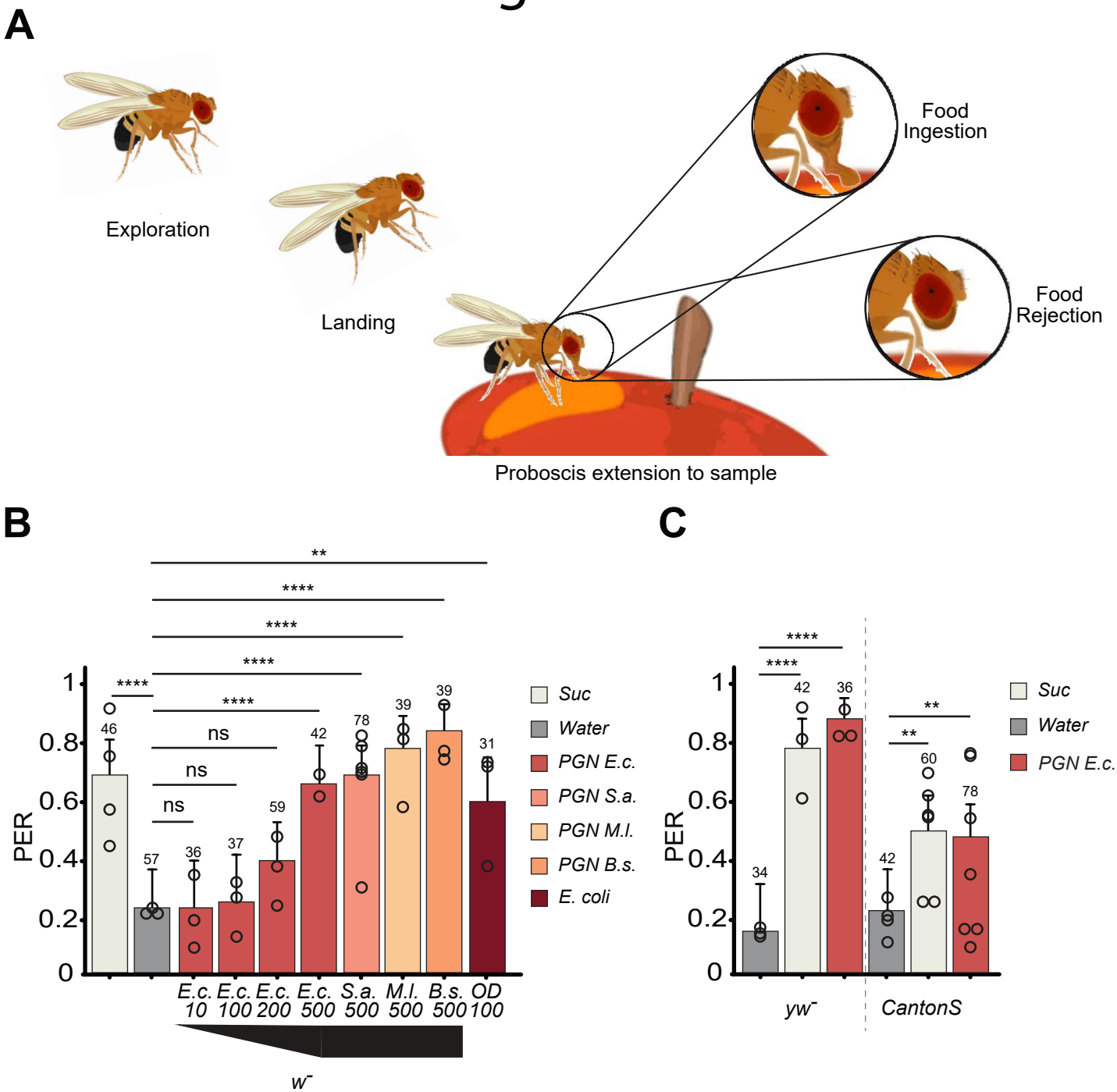

A
Exploration
Landing
Food Ingestion
Food Rejection
Proboscis extension to sample
B
PER
E.c. 10
E.c. 100
E.c. 200
E.c. 500
S.a. 500
M.l. 500
B.s. 500
OD 100
w-
Suc
Water
PGN E.c.
PGN S.a.
PGN M.l.
PGN B.s.
E. coli
ns
C
yw-
CantonS
PGN E.c.500
D
w- media prot+
w- media suc+
E
w- CR
w-GF

Figure 2

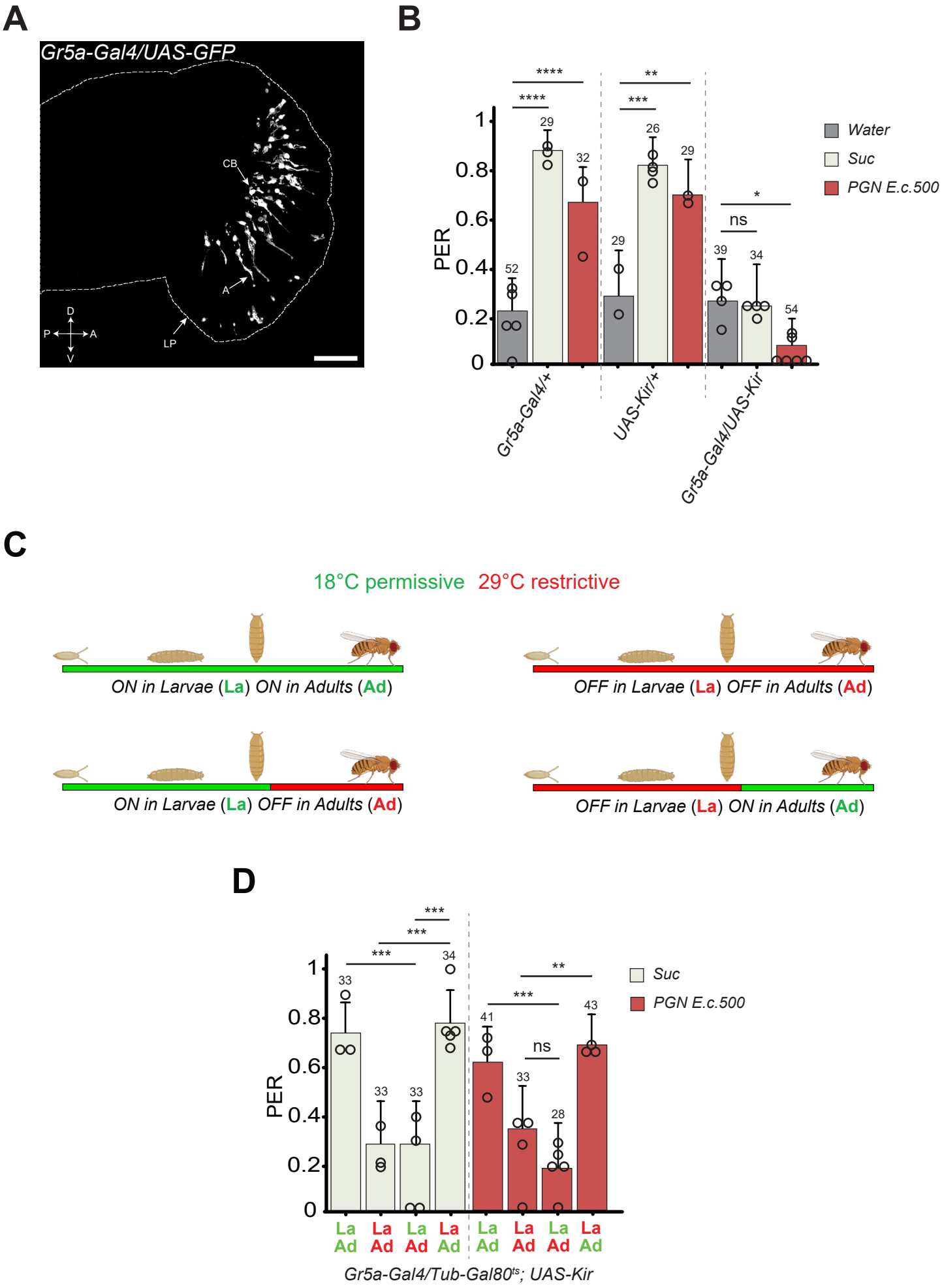
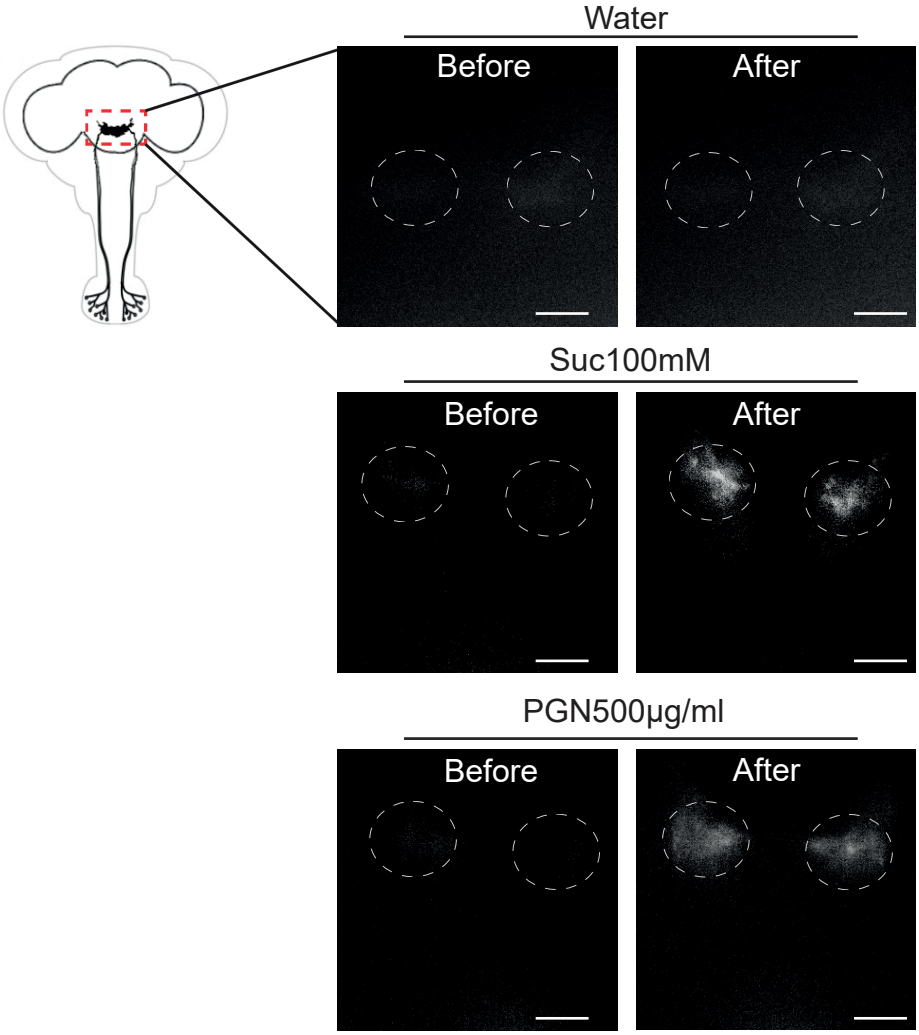

A
Gr5a-Gal4/UAS-GFP
CB
A
LP
D
P
A
V
B
PER
Water
Suc
PGN E.c.500
Gr5a-Gal4/+
UAS-Kir/+
Gr5a-Gal4/UAS-Kir
ns
C
18°C permissive 29°C restrictive
ON in Larvae (La) ON in Adults (Ad)
OFF in Larvae (La) OFF in Adults (Ad)
ON in Larvae (La) OFF in Adults (Ad)
OFF in Larvae (La) ON in Adults (Ad)
D
PER
Suc
PGN E.c.500
ns
La Ad
Gr5a-Gal4/Tub-Gal80ts; UAS-Kir

FIGURE 3

A



B

Gr5a-Gal4/UASGCamp6S

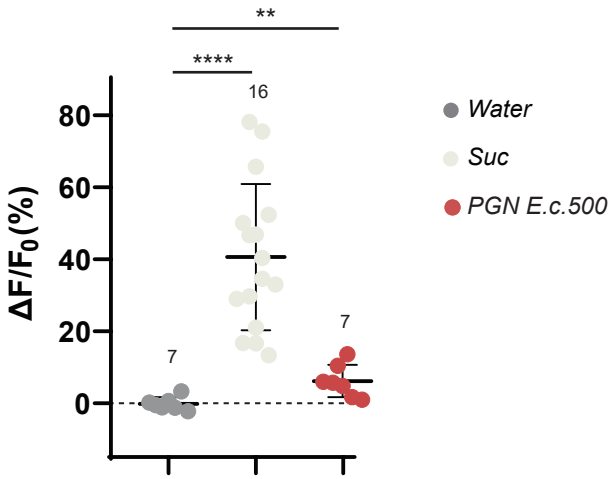
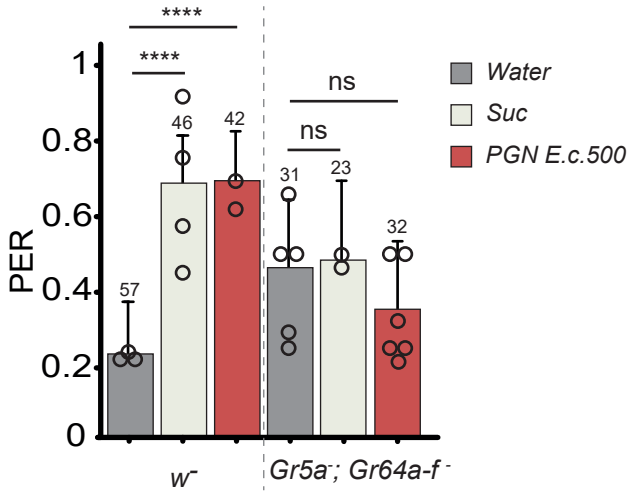
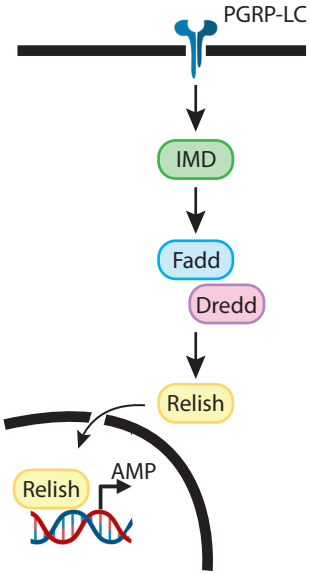


Figure 4

A


****
****
ns
ns
1
0.8
0.6
0.4
0.2
0
PER
57
46
42
31
23
32
Water
Suc
PGN E.c.500
w-
Gr5a-; Gr64a-f-

B


PGRP-LC
IMD
Fadd
Dredd
Relish
Relish
AMP

C

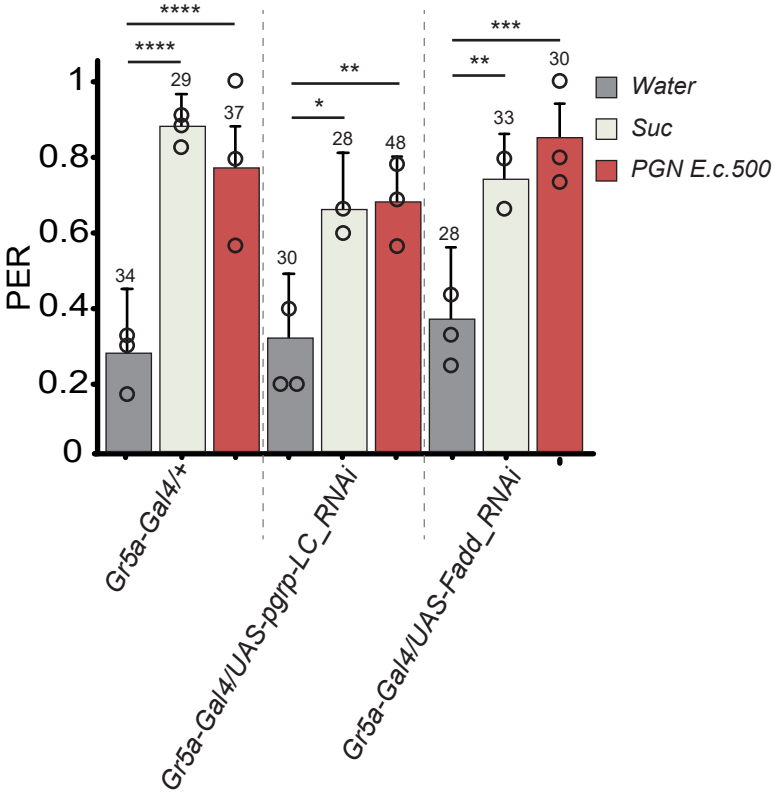

****
****
**
*
***
**
1
0.8
0.6
0.4
0.2
0
PER
34
29
37
30
28
48
28
33
30
Water
Suc
PGN E.c.500
Gr5a-Gal4/+
Gr5a-Gal4/UAS-pgrp-LC_RNAi
Gr5a-Gal4/UAS-Fadd_RNAi

Figure 5

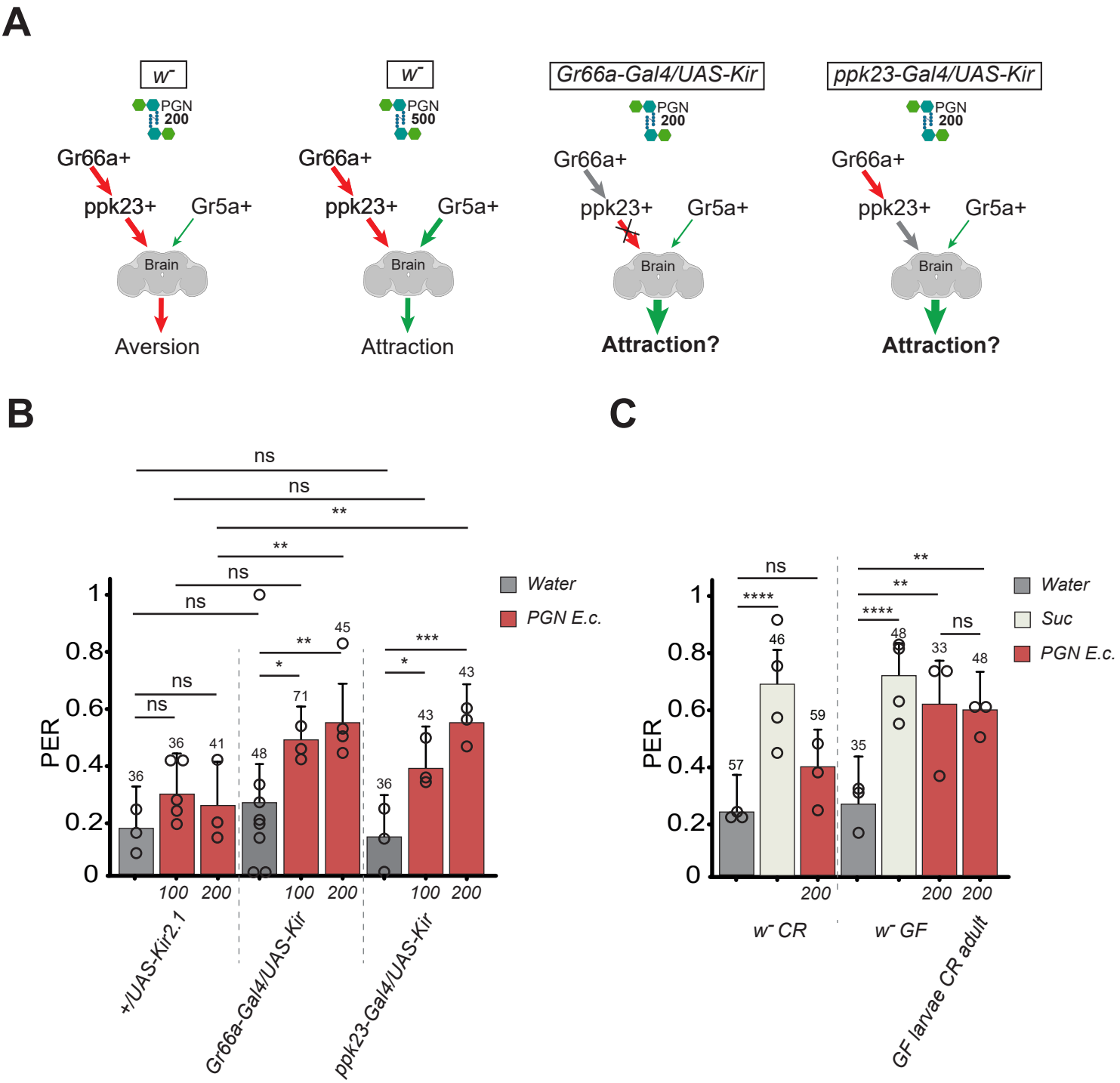

A
w-
PGN 200
Gr66a+
ppk23+
Gr5a+
Brain
Aversion
w-
PGN 500
Gr66a+
ppk23+
Gr5a+
Brain
Attraction
Gr66a-Gal4/UAS-Kir
PGN 200
Gr66a+
ppk23+
Gr5a+
Brain
Attraction?
ppk23-Gal4/UAS-Kir
PGN 200
Gr66a+
ppk23+
Gr5a+
Brain
Attraction?
B
PER
Water
PGN E.c.
+/UAS-Kir2.1
Gr66a-Gal4/UAS-Kir
ppk23-Gal4/UAS-Kir
C
PER
Water
Suc
PGN E.c.
w- CR
w- GF
GF larvae CR adult

Figure Legends

Figure 1 ***Dose-dependent attraction to PGN***

(A) Drosophila feeding behavior is characterized by a sequence of distinct behavioral modules. A starved fly initiates a search for food, and the detection of food odors stops this search. The initial gustatory cues are acquired by sampling food using the proboscis. If the fly encounters a potentially appetizing stimulus, it will begin feeding; if the stimulus is aversive, the fly will retract its proboscis.

(B) PER index of w- flies to control solutions of 1mM sucrose, water, and increasing concentrations of PGN from *E. coli* K12 (E.c.), *S. aureus* (S.a.), *M. luteus* (M.l.), and *B. subtilis* (B.s.), or an *E. coli* culture at OD100. The numbers below the x-axis represent the final PGN concentrations in µg/mL and the OD value for the *E. coli* culture.

(C) Attraction to PGN is independent of genetic background. PER index of yw- and CantonS flies to control solutions of water, 1mM sucrose, and PGN from *E. coli* K12 at 500µg/mL.

(D) Raising medium has no effect on attraction to PGN. PER index of w- flies raised on protein-enriched (prot+) or sucrose-enriched (suc+) media, to control solutions of water, 1mM sucrose, and PGN from *E. coli* K12 at 500µg/mL. Data for w- prot+ flies are the same as in panel 1A.

(E) Germ-free status does not affect attraction to PGN. PER index of w- germ-free (GF) and conventionally raised (CR) flies to control solutions of water, 1mM sucrose, and PGN from *E. coli* K12 at 500µg/mL. Data for w- CR flies are the same as in panel 1A. The PER index is calculated as the percentage of flies that responded with a PER to the stimulation ± 95% confidence interval (CI). A PER value of 1 indicates that 100% of the tested flies extended their proboscis in response to the stimulus, while a value of 0.2 indicates that 20% of the flies responded. The number of flies tested (n) is shown above each bar. For each condition, at least three groups of 10 flies each were tested. "ns" indicates $p>0.05$, * indicates $p<0.05$, ** indicates $p<0.01$, *** indicates $p<0.001$, **** indicates $p<0.0001$, Fisher Exact Test.

Further details are available in the figure section on lines, conditions, and statistics.

Figure 2 Gr5a+ neurons are required for attraction to PGN

(A) Confocal image of the proboscis of Gr5a-Gal4/UAS-GFP flies showing gustatory neurons in the fly proboscis. LP refers to Labellum-Proboscis, CB to cellular body; A denotes axons

emanating from neurons in the proboscis. D, V, P, and A stand for Dorsal, Ventral, Posterior, and Anterior.

(B) Impairing the activity of Gr5a+ neurons via UAS-Kir2.1 eliminates the attraction to PGN. PER index of flies to control solutions of water, 1mM sucrose, and PGN from *E. coli* at 500µg/mL.

(C) Graph showing the life periods during which flies are shifted from 18°C (green) to 29°C (red).

(D) Gr5a+ neurons are functionally required for PGN attraction in adults but are not needed during the larval stage. PER index of flies to 1mM sucrose and PGN from *E. coli* K12 at 500µg/mL. The ubiquitously expressed Tub-Gal80ts inhibits the activity of Gal4 at 18°C and is inactivated at 29°C, allowing the expression of UAS-Kir2.1 and the inhibition of Gr5a+ neuron activity. The PER index is calculated as the percentage of flies responding with a PER to the stimulus ± 95% CI. The number of flies tested (n) is shown above each bar. "ns" indicates $p>0.05$, * indicates $p<0.05$, ** indicates $p<0.01$, *** indicates $p<0.001$, **** indicates $p<0.0001$, Fisher Exact Test.

Further details on conditions and statistics are provided in the figure section.

Figure 3 Real-time calcium imaging with GCaMP6s reflects the *in vivo* neuronal activity of Gr5a+ neurons

(A) Representative images showing the GCaMP6s intensity before and after the addition of control water, 100mM sucrose, or PGN from *E. coli* K12 at 500µg/mL. Scale bar is 10µm.

(B) Averaged fluorescence intensity of negative peaks ± SEM for Gr5a+ neurons in response to water (n=7), 100mM sucrose (n=16), or PGN from *E. coli* K12 at 500µg/mL (n=7). In (A), ** indicates $p<0.01$, **** indicates $p<0.0001$, non-parametric t-test, Mann-Whitney test.

Further details on conditions and statistics can be found in the figure section.

Figure 4 Sugar receptors are involved in PGN attraction

(A) PER index of mutant flies lacking the Gr5a receptor as well as the Gr64a-f family to water, 1mM sucrose, and PGN from *E. coli* K12 at 500µg/mL, compared to w- flies. Data for w- flies are the same as in Fig. 1A.

(B) Diagram of the IMD pathway.

(C) RNAi-mediated inactivation of PGRP-LC (UAS-pgrp-LC RNAi) or Fadd (UAS-Fadd RNAi) in Gr5a+ cells does not affect attraction to PGN. PER index of flies to water, 1mM sucrose, and PGN from *E. coli* K12 at 500µg/mL. For (A) and (C), the PER index is calculated as the percentage of flies responding with a PER to the stimulus ± 95% CI. The number of flies tested (n) is shown above each bar. "ns" indicates p>0.05, * indicates p<0.05, ** indicates p<0.01, *** indicates p<0.001, **** indicates p<0.0001, Fisher Exact Test.
Further details on conditions and statistics are available in the figure section.

Figure 5 Integration of Attractive and Aversive Signals from PGN Exposure Modulates the PER

(A) Schematic illustrating the balance between attraction and aversion to PGN and the effects of genetic manipulations in Gr66a+ and ppk23 neurons on this balance. Our previous work showed that Gr66a+ neurons are not required during adulthood for PGN aversion, but their activity during the larval stage is necessary for ppk23+ neurons to recognize and show aversion to PGN in adulthood (arrow linking Gr66a+ to ppk23+). At a PGN concentration of 500µg/mL, input from Gr5a+ neurons shifts the balance from aversion to attraction. Impairing Gr66a+ or ppk23+ neuron activity (Gr66a-Gal4/UAS-Kir2.1 and ppk23-Gal4/UAS-Kir2.1) abolishes their input, leading to increased attraction to PGN.
(B) Impairing the activity of Gr66a+ or ppk23+ neurons with UAS-Kir2.1 increases attraction to PGN at 200µg/mL. PER index of flies to control solutions of water and PGN from *E. coli* K12 at 100µg/mL or 200µg/mL.
(C) PER index of flies raised as conventional (CR), germ-free (GF), or germ-free only during the larval stage (GF larvae CR adult) to control solutions of water and PGN from *E. coli* K12 at 200µg/mL. Data for w- CR flies are the same as in Fig. 1A. For (B) and (C), the PER index is calculated as the percentage of flies responding with a PER to the stimulus ± 95% CI. The number of flies tested (n) is shown above each bar. "ns" indicates p>0.05, * indicates p<0.05, ** indicates p<0.01, *** indicates p<0.001, **** indicates p<0.0001, Fisher Exact Test.
Further details on conditions and statistics are available in the figure section.

Video 1 PER is triggered following exposure to sucrose 1mM

https://doi.org/10.6084/m9.figshare.27929532.v1

Response following proboscis contact with a stripe of paper soaked in a 1mM solution (water as solvent). 7-days old females starved 15h prior to the assay.

Video 2 PER is not triggered following exposure to water

https://doi.org/10.6084/m9.figshare.27929517.v1

Absence of response following proboscis contact with a stripe of paper soaked in water. 7-days old females starved 15h prior to the assay.

Video 3 PER is triggered following exposure to PGN of *E. coli* at 500µg/ml

https://doi.org/10.6084/m9.figshare.27929547.v1

Response following proboscis contact with a stripe of paper soaked in an *E. coli* PGN solution at 500µg/ml (water as solvent). 7-days old females starved 15h prior to the assay.

Video 4 Comparison of Behavioral Response to Water and E. coli PGN in the Same Fly

https://doi.org/10.6084/m9.figshare.27929553.v1

Using the same fly, this experiment compares the absence of response to a proboscis contact with a paper strip soaked in water, versus a clear response to a paper strip soaked in an *E. coli* PGN solution (500 µg/ml, with water as the solvent). The test was conducted on 7-day-old females, starved for 15 hours prior to the assay.

Video 5 Gr5a+ neurons do not respond to water

https://doi.org/10.6084/m9.figshare.28480388.v1

Real-time calcium imaging in the SEZ of adult females using the calcium indicator GCaMP6s under the control of the Gr5a-Gal4 driver to assess the in vivo neuronal activity of labellar Gr5a+ neurons following exposure to water. The water was added at t+ 2 seconds.

Video 6 Gr5a+ neurons respond to DAP-type peptidoglycan

https://doi.org/10.6084/m9.figshare.28479926.v1

Real-time calcium imaging in the SEZ of adult females using the calcium indicator GCaMP6s under the control of the Gr5a-Gal4 driver to assess the in vivo neuronal activity of labellar

Gr5a+ neurons following exposure to *E. coli* PGN solution at 500µg/mL. The PGN was added at image 100 which corresponds to t+2 seconds.

Video 7 Gr5a+ neurons respond to sucrose

https://doi.org/10.6084/m9.figshare.28480460.v1

Real-time calcium imaging in the SEZ of adult females using the calcium indicator GCaMP6s under the control of the Gr5a-Gal4 driver to assess the in vivo neuronal activity of labellar Gr5a+ neurons following exposure to sucrose 100mM. The sucrose was added at image 80 which corresponds to t+2 seconds.