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Larval microbiota primes the *Drosophila* adult gustatory response

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Abstract

The survival of animals depends, among other things, on their ability to identify threats in their surrounding environment. Senses such as olfaction, vision and taste play an essential role in sampling their living environment, including microorganisms, some of which are potentially pathogenic. This study focuses on the mechanisms of detection of bacteria by the *Drosophila* gustatory system. We demonstrate that the peptidoglycan (PGN) that forms the cell wall of bacteria triggers an immediate feeding aversive response when detected by the gustatory system of adult flies. Although we identify ppk23+ and Gr66a+ gustatory neurons as necessary to transduce fly response to PGN, we demonstrate that they play very different roles in the process. Time-controlled functional inactivation and *in vivo* calcium imaging demonstrate that while ppk23+ neurons are required in the adult flies to directly transduce PGN signal, Gr66a+ neurons must be functional in larvae to allow future adults to become PGN sensitive. Furthermore, the ability of adult flies to respond to bacterial PGN is lost when they hatch from larvae reared under axenic conditions. Recolonization of axenic larvae, but not adults, with a single bacterial species, *Lactobacillus brevis*, is sufficient to restore the ability of adults to respond to PGN. Our data demonstrate that the genetic and environmental characteristics of the larvae are essential to make the future adults competent to respond to certain sensory stimuli such as PGN.

41

42 **Introduction**

43

44 In nature, animals live in a variety of ecological niches colonized by bacteria, viruses and fungi.

45 As a result, animals interact with, and sometimes even host, these co-inhabitants throughout

46 their development and during adulthood. For the better, as these microbial communities can

47 have a positive impact on various physiological parameters of the host such as fertility, lon-

48 gevity and growth, to name but a few ^{1, 2, 3, 4, 5, 6, 7}. For the worse, as some of these microbes

49 can negatively affect the health and homeostasis of the host ⁸. The ability to detect and re-

50 spond to these potentially harmful threats is an innate process fundamental to animal sur-

51 vival, that is conserved in all species ^{9, 10}. To defend themselves against pathogens, animals

52 have evolved finely tuned cellular and humoral innate immune mechanisms that preserve the

53 physical integrity and health of the host and its offspring ^{11, 12}. Defensive responses triggered

54 upon microbial detection come at a cost to the host and might not always be successful ^{13, 14}

55 ^{15, 16}. Prior detection of potential dangers in the environment and preparations to face them

56 could be complementary to the canonical responses elicited by pathogens within the host and

57 represent a first line of defense ^{17, 18, 19}. Thus, the nervous system's perception of a microbial

58 threat may allow the host to adopt behaviors aimed at reducing the consequences of the in-

59 festation on itself and its offspring. These behaviors can act at the individual or population

60 level. Ants and bees have developed social and behavioral immune mechanisms to defend

61 themselves against infection through grooming ^{20, 21, 22}. Studies in vertebrates and inverte-

62 brates have indicated that multiple sensory systems, including olfaction, hearing and vision,

63 are involved in detecting biological threats ^{23 18, 19, 24}. In *Drosophila*, the subject of this study,

64 it was found that hygienic grooming behaviors can be induced by fungal molecules or bacterial

65 contact chemicals, via different receptors and neural circuits ^{25, 26}. Volatile chemicals, such as

66 geosmin, released by potentially pathogenic fungi can be detected by insects via olfactory re-

67 ceptors and act as repellent, lowering the food intake and modulating the egg-laying rate ²⁷.

68 Hallmarks of bacteria are cell wall components such as peptidoglycan (PGN) or lipopolysac-

69 charide (LPS) that are important ligands for receptors allowing eucaryotes to detect and dif-

70 ferentiate them from other living organisms ²⁸. Interestingly, these receptors are expressed

71 on immune-competent cells as well as on neuronal cells ^{18, 29, 30}. Work from several laborato-

72 rories has shown that bacterial peptidoglycan, an essential component of the bacterial cell wall,

73 mediates many interactions between bacteria and flies ^{31, 32, 33, 34, 35}. Its recognition by PGRP

74 family members activates NF- κ B pathways on immunocompetent cells, leading to the produc-
75 tion of immune effectors and regulators. We have recently shown that the same ligand/re-
76 ceptor interactions maintain a molecular dialogue between bacteria and neurons in the cen-
77 tral and peripheral nervous systems of flies^{36, 37, 38}. We now show that PGN detection by taste
78 neurons triggers an immediate aversive response in adult flies. We identify neurons defined
79 by the expression of the *ppk23* gene (*ppk23+*) as mediators of this bacteria-induced behavior.
80 We also demonstrate that, to be sensitive to PGN, adult flies must hatch from larvae reared
81 in the presence of bacteria and whose *Gr66a+* neuronal circuitry is functional. Thus, larval co-
82 habitation with bacteria is a prerequisite for an adult behavioral response to PGN. This demon-
83 strates that the genetic characteristics of the larvae as well as the environmental conditions
84 in which they live are essential to initiate a sensory response to certain molecules later in the
85 adult stage.
86

87 Results

88

89 Bacterial PGN can trigger PER in *Drosophila*

90 Some Peptidoglycan Recognition Protein family members are expressed in proboscis-hosted
91 taste neurons^{38, 39}. One of them, the membrane receptor PGRP-LC, as well as downstream
92 components of the IMD pathway, are functionally required for transduction of the bacterial
93 PGN signal in these cells^{31, 40, 41}. To test whether this cellular response to a universal bacteria
94 component is associated with a specific fly behavior, we used the proboscis extension re-
95 sponse (PER), which is part of the sensorimotor taste circuit in adult flies⁴². Stimulation of the
96 proboscis by an attractive molecule such as sucrose triggers its immediate and directional ex-
97 tension. When an aversive effect is suspected, the molecule to be tested is combined with a
98 palatable solution such as sucrose. Aversion is measured by the ability of the substance to
99 prevent PER to sucrose. Since our published results³⁸ demonstrated the ability of PGN to stim-
100 ulate bitter neurons, a cell population involved in aversive behaviors⁴³, we tested whether
101 PGN could suppress the PER response to sucrose. Both Diaminopimelic-type PGN which forms
102 the cell wall of all Gram-negative bacteria and of Bacilli (we used here PGN from *Escherichia*
103 *coli*, Ec-PGN), and Lysine-type PGN found in the cell wall of Gram-positive bacteria (we used
104 here PGN from *Staphylococcus aureus*, Sa-PGN) were tested³³. In PER, Dap-PGN was found
105 to be aversive to reference flies (w-) when used at 100 (PGN100) and 200 µg/mL (PGN200)
106 but not at lower concentrations (Fig 1A). These effects were specific to DAP-type PGN as they
107 were not observed with Lys-type PGN (Fig 1A). Similar aversive effects of DAP-type PGN were
108 obtained with two other commonly used reference *Drosophila* strains, yw and canton-S (Fig
109 1B and C), and with flies reared on either sugar or AA-rich medium (Fig S1A and S1B).

110 As with the well-characterized bitter agent caffeine, the ability of PGN to prevent PER was
111 overcome when PGN was mixed with solutions of higher sucrose concentrations (Fig S1C). As
112 the fly legs are taste organs as well⁴⁴, and PER can also be triggered by chemicals coming into
113 contact with tarsi, we tested PGN in this assay. No aversive response to PGN could be detected
114 in leg-triggered PER, either because the tarsal taste neurons are not involved in the detection
115 of this microbial compound, or because the concentration of sucrose required to trigger the
116 PER on legs overcomes the aversion to PGN (Fig S2A and S2B). We, thereafter, focused on PER
117 triggered by direct contact with proboscis. Because taste neurons have been shown to re-
118 spond to acidic solutions^{45 46}, we measured the pH of our different PGNs and they were all

119 neutral. This rules out the possibility that the PER response to PGN is due to its acidity (Fig
120 S2C). Taken together, these data demonstrate that Dap-type PGN is perceived as repulsive
121 while Lys-type PGN is not.

122

123 **PGN-triggered PER aversion involves Gr66a+ and ppk23+ neurons**

124 To identify the type of neurons that respond to PGN, PERs were performed in flies in which
125 specific groups of neurons were inactivated by overexpression of the inward rectifier potas-
126 sium channel Kir2.1. To test whether the bitter network was involved in PER aversion toward
127 Dap-type PGN, neurons expressing the pan-bitter taste receptor Gr66a were inactivated⁴⁷.
128 The ability of Dap-PGN to suppress PER when mixed with sucrose was completely abolished
129 in Gr66a^{Gal4}/UAS-Kir2.1 flies demonstrating that Gr66a+ cells are necessary to transduce PGN
130 signal (Fig 2A). Recent work has demonstrated the existence of Gr66a+/ppk23+ and
131 Gr66a+/ppk23- neuron sub-populations that could co-exist or be housed in different sensilla
132^{48, 49}. These two populations display distinct properties related to salt perception. To assay
133 whether only a subpopulation of neuronal Gr66a+ cells was involved, we used the ppk23^{Gal4}
134 driver. Ppk23^{Gal4}/UAS-Kir2.1 flies no longer perceived PGN as aversive demonstrating that
135 Gr66a+ and ppk23+ neurons are both necessary to mediate PGN signal (Fig 2B).

136

137 **Adult ppk23+/Gr66a- cells respond to PGN**

138 To directly demonstrate that PGN can activate ppk23+ neurons in the adult, we monitored
139 GCaMP signal in the sub esophageal zone (SEZ) of the brain of females following proboscis
140 exposure to PGN. This brain area processes gustatory input from gustatory neurons located in
141 the proboscis. Since adult Gr66a+ neurons can respond to PGN³⁸ and that some labellar
142 Gr66a+ cells are also ppk23+⁴⁸, we used an intersectional genetic strategy to quantify calcium
143 activity modifications only in ppk23+/Gr66a- cells⁴⁸, a neuronal subpopulation sufficient to
144 mediate the PGN-induced PER suppression (Fig S8B). By quantifying live *in vivo* GCaMP fluo-
145 rescence in adult brains of flies whose proboscis has been stimulated with PGN, we confirmed
146 that ppk23+/Gr66a- cells can directly respond to PGN (Fig 2C-2E). Importantly, these cells did
147 not respond to caffeine but did to high salt, a signature expected for ppk23+/Gr66a- cells.

148

149 **Temporal requirement of gustatory neurons for PGN detection during the fly's lifetime**

150 The putative involvement of several neuronal subgroups in PGN detection prompted us to test
151 whether these different neurons are all required at the same time during the fly's life. Indeed,
152 although most studies aimed at dissecting how flies perceive their environment via the taste
153 apparatus are performed at the adult stage, including this one, the bitter neurons exemplified
154 by the Gr66a+ population are present throughout fly development^{50, 51}. To determine when
155 the neurons necessary for the PGN-induced PER suppression are required, we took advantage
156 of the Gal4/Gal80^{ts} binary system that allowed us to control the inactivation of taste neurons
157 in a spatially and temporally controlled manner (Fig 3A). Surprisingly, when assessing aversion
158 to PGN, adult flies in which Gr66a+ neurons were functionally inactivated only before the adult
159 stage could no longer perceive PGN as aversive (Fig 3B). In the parallel experiment, inactiva-
160 tion of Gr66a+ neurons in adult flies only, had no effect. In contrast, perception by the adult
161 flies of quinine and caffeine required Gr66a+ neurons to be functional in the adult but not in
162 larvae (Fig S3A). This demonstrates a specific role for Gr66a+ neurons in the life history of the
163 animal, with their requirement only during the larval stage allowing the future adult to show
164 aversion towards Dap-PGN. Since both Gr66a+ and ppk23+ neurons are required to respond
165 to PGN, we tested whether ppk23+ cells are the ones at play in the adults. The data presented
166 Fig 3B demonstrated that, unlike GR66a+ neurons, ppk23+ neurons are functionally required
167 in the adult, but not during larval life for PGN-triggered aversion. These results reveal an un-
168 expected role for larval Gr66a+ neurons in priming the adult response to PGN. For this adult
169 sensory behavior, ppk23+/Gr66a- neurons are sufficient.

170

171

172 **TRPA1 is functionally required in larval GR66a+ neurons for a response of adult flies to PGN**

173 Using calcium imaging as a readout, we have shown in a previous study that certain compo-
174 nents of the IMD pathway (Fig 4A), including the upstream transmembrane receptor PGRP-
175 LC, are functionally required for the transduction of Dap-PGN signal in adult Gr66a+ neurons
176³⁸. We therefore, tested whether this well-characterized PGN receptor was also required in
177 ppk23+ cells. PER responses to Dap-PGN were not affected by RNAi-mediated inactivation of
178 PGRP-LC in either ppk23+ or Gr66a+ neurons (Fig 4B). These results suggesting that Dap-PGN
179 triggered PER is independent of the IMD pathway were confirmed using RNAi against other
180 components of the pathway (Fig S4A and S4B). Consequently, the IMD pathway is neither re-
181 quired in the Gr66a+ larval neurons nor in the adult ppk23+ cells for the PGN-mediated PER.

182 The Gr66a receptor was also tested for its involvement in PGN-induced PER suppression as it
183 is considered, together with Gr33a, as the main co-receptor for the detection of bitter mole-
184 cules. While Gr66a mutants lost PER aversion for caffeine, they did not for PGN (Fig S4C). In
185 addition to bitter taste receptors, the transient receptor potential TRPA1 channel has been
186 implicated in aversive taste stimulation^{52 53}. Its RNAi-mediated inactivation in larval Gr66a+
187 neurons, but not in ppk23+ neurons, abolished the ability of adult flies to respond to PGN (Fig
188 4C and 4D). These results demonstrate that the expression of TRPA1 receptor in larval Gr66a+
189 neurons is required for the adults that hatch from these larvae to respond to PGN.

190

191 **Axenic flies are not able to trigger PER aversion to PGN**

192 The involvement of multiple temporal and spatial inputs for PGN detection led us to try to
193 identify the nature of the larval-sensed trigger(s) that prepare the adult response to PGN.
194 Since larvae burrow and live in non-aseptic environments, and the larval microbiota has a
195 strong impact on host immunity, physiology and behavior, we tested its necessity for the adult
196 fly response to bacterially-derived PGN. When flies were reared on antibiotic (axenic) me-
197 dium, the number of bacteria by fly was strongly reduced (Fig 5A). Interestingly, while axenic
198 flies were still able to respond to caffeine their response to PGN was completely abolished (Fig
199 5B). Similar results were obtained using different genetic backgrounds demonstrating the uni-
200 versality of the observed effects (Fig S5A and S5B). The PER suppression in response to PGN
201 was also abolished when flies were made axenic by bleaching the eggs (Fig S5C and S5D) elim-
202 inating possible side effects of antibiotics on fly taste. These results demonstrate that while
203 exposure to bacteria is not mandatory for the adult response to caffeine, animals must be
204 exposed to bacteria for the adults to perceive Dap-PGN as aversive. To further confirm the
205 inability of axenic adult flies hatched from germ-free larvae to respond to PGN we used cal-
206 cium imaging. Whereas calcium levels were increased in ppk23+/Gr66a- neurons following
207 PGN exposure to the proboscis of conventionally reared flies, this was no longer the case for
208 axenic flies (Fig 5C-5E). To ensure that aseptic conditions do not, in a non-specific manner,
209 lower the activity potential of neurons, we quantified the salt response that is dependent
210 upon ppk23+ cells. The salt-related activation of these ppk23+/Gr66a- neurons monitored by
211 calcium-imaging was also not affected by germ-free conditions, confirming the specific effect
212 of aseptic conditions upon PGN sensitivity (Fig S5E). These results combined to our temporal
213 genetic inactivation assays suggest that larval Gr66a+ neurons have to be exposed to bacteria

214 for the adult *ppk23+* neurons to respond to PGN. We verified that activation of *Gr66a+* adult
215 neurons by PGN is involved in a process different from the PER by testing axenic and non-
216 axenic animals. As shown in **Fig S5F**, adult *Gr66a+* cells were activated by PGN in both aseptic
217 and non-aseptic conditions. Consequently, in the adult proboscis, while *Gr66a+* cells can re-
218 spond to PGN regardless of the fly's life experience with bacteria, *ppk23+/Gr66a-* neurons can
219 respond to PGN, but non-aseptic conditions are necessary.

220

221 **Larval microbiota is a pre-requisite to implementing the adult gustatory response to PGN**

222 Because flies are in contact with environmental bacteria throughout development and later
223 in adulthood, we sought to identify the temporal window during which the presence of bac-
224 teria impacts the gustatory system's response to PGN. For that purpose, flies were reared on
225 conventional (non-aseptic) medium and transferred to antibiotic-containing medium at differ-
226 ent periods of their life cycle and for different durations (**Fig 6A**). All emerged flies were then
227 tested for their ability to respond to PGN. Whereas flies emerged from non-aseptic larvae
228 reared immediately after hatching on antibiotic medium responded adversely to PGN (**Fig 6B**),
229 those from larvae reared on antibiotic (aseptic) medium lost this ability, despite adults' expo-
230 sure to non-aseptic environment (**Fig 6B**). To further reduce the permissive time window, lar-
231 vae were reared on a non-aseptic medium for only a part of the larval period. The efficacy of
232 the different treatments were monitored using CFU tests (**Fig S6A**). Exposure of larvae to an-
233 tibiotic food for the 72 first hours of development was sufficient to abolish the aversive re-
234 sponse of the adult to PGN (**Fig 6C**). Similarly, rearing germ-free eggs on non-aseptic medium
235 for the first 48 hours of development was sufficient to restore PGN responsiveness in adults
236 (**Fig 6D**). Furthermore, exposure of axenic larval pupae to bacteria (**Fig S6B and S6C**), as well
237 as exposure of germ-free larvae and adults to PGN-containing media (**Fig S7A**) was not suffi-
238 cient for the adult's PGN-induced PER suppression to be re-established. These results indicate
239 that cohabitation of early larvae with bacteria is a prerequisite for the adult taste aversive
240 response to PGN to be established. In addition, these data are consistent with a role for
241 *Gr66a+* taste neurons only during larval life for the adult to respond adversely to PGN in a PER
242 assay.

243

244 **Mono-association of larvae with *Lactobacillus brevis* is sufficient to restore the aversive**
245 **adult response to PGN**

246 Although much simpler than that of mammals, previous work has shown that the *Drosophila*
247 microbiota can host about 20 species mainly belonging to the genus *Acetobacter* or *Lactoba-*
248 *cillus*, depending of the fly's diet ^{54, 55, 56, 57, 58}. To test whether a specific bacterial species could
249 mediate larval priming effects, we sequenced the bacteria present in our laboratory fly colony
250 (Fig 7A). Of the few species identified, *Lactobacillus brevis* was one of the most abundant and
251 caught our attention because it has been shown to affect the behavior of the flies it inhabits
252 ^{59, 60}. Strikingly, PGN-induced PER suppression was restored in axenic adults obtained from
253 larvae mono-associated with *Lactobacillus brevis* (Fig 7B and 7C). When the same experiment
254 was performed with a related species, *Lactobacillus plantarum*, no PER inhibition was ob-
255 served (Fig 7C). It has been previously reported that the presence of *L. brevis* in adult animals
256 can alter the fly behavior by modulating the production of octopamine, a neurotransmitter
257 involved in oviposition, male fighting and locomotor activity ^{60 61, 62}. In our case, exposure of
258 adults to *L. brevis* was not sufficient to restore the PGN PER phenotype and we ruled out the
259 putative involvement of octopamine by demonstrating that the PGN is still perceived as aver-
260 sive by mutants unable to synthesize octopamine (TβH) ⁶³ (Fig S7B).
261 Overall, these results demonstrate that a period of cohabitation between larvae and specific
262 bacterial species, such as *Lactobacillus brevis*, is mandatory for the emerging adult to perceive
263 PGN as an aversive molecule by ppk23+ neurons.

264 265 **Discussion**

266
267 The data presented here demonstrate that flies are competent to perceive and respond to
268 PGN of bacterial origin via ppk23+ neurons exposed to the environment. However, this adult
269 behavior only takes place if the larvae that will give birth to these flies carry functional Gr66a+
270 neurons and are reared in the presence of certain bacterial species such as *Lactobacillus*
271 *brevis*.

272 273 **Are Gr66a+ and ppk23+ subpopulations sufficient to mediate larval priming and adult re-** 274 **sponse to PGN?**

275 The sensilla of the proboscis are the main taste detectors and act as straws sampling the out-
276 side world. Divided into three classes (S, L, I), they each house several cells, including taste
277 neurons whose dendrites are exposed to the outside world via the sensilla opening ⁶⁴. While
278 Gr66a+/ppk23- neurons are hosted in I- and one subset of S-sensilla (called Sb),

279 Gr66a+/ppk23+ neurons are found in the other subset of S-sensilla (called Sa). Gr66a-/ppk23+
280 cells are present in few I-sensilla, almost all S- and all L-sensilla ⁴⁸. Therefore, some sensilla
281 host both Gr66a+/ppk23+ and Gr66a-/ppk23+ cells. Since our data show that Gr66a+ neurons
282 are dispensable and ppk23+ cells essential for PGN-mediated PER, we could conclude that
283 Gr66a-/ppk23+ cells are the only cells involved. However, although Gr66a-/ppk23+ subset can
284 respond specifically to PGN (Fig 2C-2E), flies in which Gr66a-/ppk23+ cells are specifically si-
285 lenced remain able to sense PGN (Fig S8A and S8B). This suggests that two populations are
286 capable of independently triggering the PGN-mediated PER: Gr66a-/ppk23+ and
287 Gr66a+/ppk23+ neurons. The description of new receptors expressed on specific subsets of
288 Gr66a-/ppk23+ neurons such as Ir7c should help to understand the complexity of this taste
289 language ⁶⁵. A recent study related to salt detection illustrates this complexity. While high
290 concentrations of monovalent salt cause aversion via Gr66a+ and Gr66a-/ppk23+ (Gr66a-
291 /Ir7c-/ppk23+) neurons, high concentrations of divalent salt activate only a subset of Gr66a-
292 /Ir7c-/ppk23+ cells⁶⁵. As with adults, the minimal subset of cells required in larvae to prime
293 the adult response to PGN remains partially elusive. Larval Gr66a+ neurons can be divided into
294 Gr32a+ and Gr32a- subpopulations ⁶⁶. Gr32a>Kir2.1 animals respond to PGN like controls, con-
295 firming that adult Gr66a+ neurons, which are all Gr32a+ in flies ⁶⁷, are dispensable for PGN
296 aversion to PER. This also suggests the involvement of the larval Gr66a+/Gr32a- subpopulation
297 (Fig S8C). Silencing other smaller neuronal subgroups using Gr Gal4 drivers ⁶⁶, had no impact
298 on PGN aversion in the adult (Fig S8C). Although our data show that ppk23+ cells are not re-
299 quired during the larval period, the existence of larval Gr66a+/ppk23+ neurons has not been
300 clearly established.

301

302 **What receptor(s) transduce the PGN signal in adult ppk23+ neurons?**

303 The demonstration that adult ppk23+ neurons respond to PGN prompted us to identify the
304 upstream receptor(s). Despite the expected involvement of the classical immune PGN sensor
305 PGRP-LC, its functional inactivation has no impact on aversion towards the microbial com-
306 pound (Fig. 4B). Even elimination of central elements of the signaling pathway, such as Fadd,
307 were without consequences. The lack of involvement of the IMD/NF- κ B pathway in the larval
308 priming phase was also demonstrated for larval Gr66a+ neurons. Our data rather identify
309 TRPA1 as a putative receptor necessary on larval Gr66a+ neurons to sense a ligand related to
310 microbial activity. As for the adult response, the yet to be identified receptor is expected to

311 be expressed on the dendrites of adult ppk23+ neurons, exposed to the sensilla-bathing me-
312 dium and hence to PGN coming from exogenous bacteria. Unfortunately, the physiology of
313 ppk23+ neurons is mostly understood via their role in pheromone perception⁶⁸ and of high
314 salt avoidance^{48,65}. Few surface receptors have been described and include Gr66a, ppk23 and
315 Ir7c. We have shown that Gr66a is not required. An alternative approach to identify the re-
316 ceptor could be to decipher the signaling pathway required for PGN-mediated PER in ppk23+
317 cells. For example, if Gαq is involved, GPCRs would appear as good candidates⁶⁹.

318

319 **How do *Lactobacillus brevis* activate larval priming?**

320 Our results highlight how the genetic characteristics of the larva and the environment in which
321 they live can impact the development of a sensory response in the adult to whom they will
322 give birth. For the priming to take place larvae must co-live with bacteria, while their presence
323 during adulthood is dispensable. If recent reports describe the influence that microbiota has
324 on certain adult fly behaviors^{60,62}, none of them report on the bacterial requirement specifi-
325 cally in larvae. In many studies associating behavioral changes to septic conditions, the neu-
326 rotransmitter octopamine (OA) was shown to play a central role. This does not seem to be the
327 case here since *TβH* mutant flies, which do not produce OA, respond well to PGN. Our data
328 point to changes initiated by the interaction between TRPA1 receptor expressed on larval
329 Gr66a+ neurons and bacteria (at least *Lactobacillus brevis*) present during the first 2-days of
330 the larval life. An important question to address is how such an interaction is taking place.
331 Among many possibilities, one can propose, i) modifications of the media by the bacteria, ii)
332 changes in the gut physiology upon bacteria detection, iii) impact of microbial activity on the
333 host iii) direct effects of some bacterial components. In the latter category, one candidate is
334 the PGN itself which plays key role in the interactions between flies and bacteria. However,
335 although PGN is universally present on all bacteria, only some of them are able to mediate
336 priming as mono association with *L. plantarum* was not sufficient. Furthermore, our unsuc-
337 cessful attempts to rescue adult response by incubating larvae with purified PGN do not sup-
338 port this hypothesis. Intriguingly, while the strain of *Lactobacillus plantarum* that we used,
339 considered as a symbiont, was not able to recapitulate the effect of a septic media, *Lactoba-*
340 *cillus brevis* that is a pathobiont with a distinctive molecular signature involving uracil was
341 sufficient to make competent adults^{70,71}. Further work will be necessary to molecularly dis-
342 sect this interaction.

343

344 **What is missing in adult ppk23+ neurons hatch form axenic larvae?**

345 Our data suggest that some pieces of information acquired during the larval life experience
346 are transmitted to the adult. Interestingly this effect shows some degree of specificity. Indeed,
347 while the ppk23+ cell-dependent aversion to PGN is lost, adult ppk23+ neurons from axenic
348 larvae are not lost or anergic as they remain responsive to salt. In addition, the activation of
349 neurons directly exposed to the environment and the lack of this activity when the larvae were
350 reared in axenic conditions suggests that the competence and the changes occur directly
351 within the ppk23+ neurons and not indirectly via other neurons. However, the situation might
352 be more complex as the *in vivo* imaging is performed in the brain where multiple axons con-
353 verge making the resolution at the neuron-scale difficult. Indeed, a subset of Gr66a-/ppk23+
354 cells at the periphery might be dedicated to PGN-sensing and not especially to salt, and fully
355 incapacitated by axenic rearing while the others only respond to salt, a capacity unaffected by
356 microbial exposure.

357

358 **How can the larval experience influence an adult behavior?**

359 This study raises the question of the mechanisms by which information acquired by larvae in
360 contact with bacteria are specifically transmitted to adults. Since larval Gr66a+ neurons are
361 necessary for this process, it is conceivable that they persist in the adult. However, like most
362 taste neurons that are either lost or transformed during metamorphosis, larval Gr66a+ neu-
363 rons do not persist into adulthood, except for a subset that becomes adult pharyngeal taste
364 cells^{72 73 74 75}. However, since PER is performed by sensory touch without ingestion and adult
365 Gr66a+ cells are dispensable for the phenotype, this is unlikely to be the case. Another possi-
366 bility would be that some larval Gr66a+ cells become adult ppk23+ neurons. The activation
367 state of the larval GR66a+ neurons will then have a direct impact on that of the adult ppk23+
368 neurons.

369 A long-term memory or a memory maintained throughout metamorphosis or pre-imaginal
370 conditioning could be considered. Definitive proofs of such mechanisms are still lacking since
371 chemicals used to condition and train the larvae could remain in trace form in the media or
372 on the resulting pupae and, in term, influence the newborn flies^{76, 77}. These caveats are not
373 valid here since the spatial and temporal genetic inactivation of neurons and the silencing of
374 TRPA1 unambiguously demonstrate the role of these cells specifically and only during larval

375 life. In addition, the effect of time-controlled bacterial exposure and the lack of response to
376 PGN in adults exposed for five days or from pupae to septic media support the idea that larvae
377 must be reared in non-axenic conditions to produce PGN responsive adults. Bacteria-larvae
378 interactions taking place during the priming period could activate some kind of memory. Such
379 hypothesis is not supported by a recent study that tracked the fate of neurons involved in
380 memory finding no anatomical substrate for a memory trace persisting from larva to adults ⁷⁵.
381 Conditions altering memory consolidation in general will be tested in the future ⁷⁸.
382 Epigenetic modifications such as histone mark alterations could be affected by the presence
383 of bacteria in the larval gut. Previous reports indicate that defects associated with histone
384 demethylase kdm5 inactivation can be partially rescued by modulating the gut microbiota ei-
385 ther by supplementation with *L. plantarum* or by antibiotic treatment ⁷⁹. If epigenetic modifi-
386 cations are involved, they will have to be limited since the changes we detected in adult taste
387 are not generic for all tastants. However, such a possibility should not be ruled out as it has
388 already been shown that animal exposure to microbe can influence behavior and vertical
389 transmission of the phenotype via epigenetic modifications ^{80, 81}.

390

391 **Multiple inputs of bacterially-derived PGN on taste neurons**

392 Using calcium imaging, we previously demonstrated that PGN exposure impacts neuronal ac-
393 tivities in an IMD/NF- κ B pathway dependent manner ³⁸. Both brain octopaminergic neurons
394 and adult Gr66a+ taste neurons present IMD pathway dependent calcium modulation follow-
395 ing PGN exposure. In the current report we demonstrate that Gr66a+ cells are dispensable for
396 the behavior in the adult stage. Different elements support the fact that the proboscis Gr66a+
397 cells activation we previously described is linked to a phenotype yet to be fully uncovered.
398 First, when the Gr66a+ are silenced specifically during the adult stage, while the animals no
399 longer respond to caffeine, we still observe the PGN-mediated PER. Second, when the animals
400 no longer react to the PGN for they have been raised in axenic conditions, the Gr66a+ neurons
401 still respond to the presence of PGN. Finally, while the IMD pathway was essential for the
402 increased calcium concentration in Gr66a+ neurons following PGN exposure, this signaling
403 cascade is not required for the PGN-mediated PER. In agreement, we've identified that an-
404 other set of neurons present in the proboscis is necessary and sufficient for the PGN-mediated
405 PER, the ppk23+ neurons. Further work will be needed to understand how flies integrate these

406 responses to PGN and to other bacterial components such as lipopolysaccharide to respond
407 adequately to the presence of bacteria in their immediate surrounding.
408

409

410 **Author contributions**

411 M.M., G.M., L.K, Y.G. and J.R. conceived the experiments. M.M., G.M., M.B.G., I.D., G.G., L.K.
412 performed the experiments. M.M., L.K. and J.R wrote the manuscript. Y.G. and J.R. secured
413 funding.

414 **Competing interests**

415 The authors have declared that no competing interests exist.

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424 Development), and the European Council (ERC starting grant, GliSFCo-311403)

425

426 Figures legends

427 **Fig. 1**

428 Dose-dependent PGN inhibition of PER. **(A)** PER index of w- flies to control solutions of sucrose
429 1mM and sucrose 1mM + caffeine 10mM and to sucrose 1mM + increasing concentrations of
430 PGN from *E. coli* K12 (Ec) and *S. aureus* (SA). The numbers below the x-axis correspond to the
431 PGN final concentrations in µg/mL. **(B and C)** Aversion to PGN is independent of genetic back-
432 ground. PER index of yw- **(B)** and CantonS **(C)** flies to control solutions of sucrose 1mM and
433 sucrose 1mM + caffeine 10mM and to sucrose 1mM + PGN from *E. coli* K12 at 200µg/mL. The
434 PER index is calculated as the percentage of flies tested that responded with a PER to the
435 stimulation ± 95% confidence interval (CI). A PER value of 1 means that 100% of the tested
436 flies extended their proboscis following contact with the mixture, a value of 0,2 means that
437 20% of the animals extended their proboscis. The number of tested flies (n) is indicated on top
438 of each bar. ns indicates p>0.05, * indicates p<0.05, ** indicates p<0.01, *** indicates

439 $p < 0.001$, **** indicates $p < 0.0001$ Fisher Exact Test. Further details can be found in the de-
440 tailed lines, conditions and, statistics for the figure section.

441

442 **Fig. 2**

443 Impairing the activity of Gr66a+ (**A**) or ppk23+ (**B**) neurons via UAS-Kir2.1 abrogates the aver-
444 sion to PGN. PER index of flies to control solutions of sucrose 1mM and sucrose 1mM + caf-
445 feine 10mM and to sucrose 1mM + PGN from *E. coli* K12 at 200 μ g/mL. (**C-E**) Gr66a-/ppk23+
446 neurons respond to stimulation with PGN on the labellum. Real-time calcium imaging using
447 the calcium indicator GCaMP6s to reflect the *in vivo* neuronal activity of Gr66a-/ppk23+ neu-
448 rons (Gr66a^{LexA};LexAopGal80;ppk23^{Gal4}/UAS-GCaMP6s) in adult brains of flies whose probos-
449 cis has been stimulated with PGN. The expression of LexAop-Gal80 antagonizes the activity of
450 Gal4, thus preventing the expression of GCaMP6S in Gr66a+/ppk23+ neurons. (**C**) Representa-
451 tive images showing the GCaMP6s intensity before and after addition of either the control
452 water or the peptidoglycan. (**D**) Averaged \pm SEM time course of the GCaMP6s intensity varia-
453 tions ($\Delta F/F_0$ %) for Gr66a-/ppk23+ neurons. The addition of water (n=7 flies) or peptidoglycan
454 at 200 μ g/mL (n=8 flies) at a specific time is indicated by the arrow. (**E**) Averaged fluorescence
455 intensity of negative peaks \pm SEM for control (n=7) and peptidoglycan-treated flies (n=8). For
456 (**A**) and (**B**), PER index is calculated as the percentage of flies tested that responded with a PER
457 to the stimulation \pm 95% CI. The number of tested flies (n) is indicated on top of each bar. For
458 each condition, at least 3 groups with a minimum of 10 flies per group were used. ns indicates
459 $p > 0.05$, * indicates $p < 0.05$, ** indicates $p < 0.01$, *** indicates $p < 0.001$, **** indicates
460 $p < 0.0001$ Fisher Exact Test. In (**E**) ** indicates $p < 0.01$ non-parametric t-test, Mann-Whitney
461 test. Further details can be found in the detailed lines, conditions and, statistics for the figure
462 section.

463

464 **Fig. 3**

465 (**A**) Graphical representation of the life periods during which flies are shifted from 18°C (green)
466 to 29°C (blue). (**B**) Gr66a+ neurons are functionally required in the larval stage for PGN-trig-
467 gered aversion, but are dispensable during adult life. In opposition to ppk23+ neurons which
468 are required in the adult but not during the larval stages. PER index of flies to sucrose 1mM +
469 PGN from *E. coli* K12 at 200 μ g/mL. The ubiquitously expressed Tub-Gal80^{ts}, that inhibits the
470 activity of Gal4, is temperature sensitive: it's active at 18°C and inactivated at 29°C, allowing

471 the expression of UASKir2.1 and the consequent impairment of Gr66a+ or ppk23+ neurons
472 activity. For **(B)**, PER index is calculated as the percentage of flies tested that responded with
473 a PER to the stimulation \pm 95% CI. The number of tested flies (n) is indicated on top of each
474 bar. ns indicates $p > 0.05$, * indicates $p < 0.05$, ** indicates $p < 0.01$, *** indicates $p < 0.001$, ****
475 indicates $p < 0.0001$ Fisher Exact Test. Further details can be found in the detailed lines, condi-
476 tions and, statistics for the figure section.

477

478 **Fig. 4**

479 **(A)** Graphical representation of the IMD pathway. **(B)** RNAi-mediated PGRP-LC (UAS-pgrp-LC
480 RNAi) inactivation in the Gr66a+ or ppk23+ cells does not affect PGN-triggered aversion. **(C)**
481 RNAi-mediated TRPA1 (UAS-TRPA1 RNAi) inactivation in the Gr66a+ cells abrogates PGN-in-
482 duced aversion, while its inactivation in ppk23+ cells has no effect on the aversion phenotype.
483 **(D)** The nociceptive receptor TRPA1 is required in the larval stages for PGN-induced aversion,
484 while its RNAi dependent inactivation in the adult stage has no effect on PGN-triggered aver-
485 sion. The ubiquitously expressed Tub-Gal80^{ts}, that inhibits the activity of Gal4, is temperature
486 sensitive: it's active at 18°C and inactivated at 29°C, allowing the expression of TRPA1-RNAi
487 and the consequent inactivation of TRPA1 in Gr66a+ cells. For **(B)**, **(C)** and **(D)** PER index of flies
488 to control solutions of sucrose 1mM and sucrose 1mM + caffeine 10mM and to sucrose 1mM
489 + PGN from *E. coli* K12 at 200µg/mL. PER index is calculated as the percentage of flies tested
490 that responded with a PER to the stimulation \pm 95% CI. The number of tested flies (n) is indi-
491 cated on top of each bar. ns indicates $p > 0.05$, * indicates $p < 0.05$, ** indicates $p < 0.01$, ***
492 indicates $p < 0.001$, **** indicates $p < 0.0001$ Fisher Exact Test. Further details can be found in
493 the detailed lines, conditions and, statistics for the figure section.

494

495 **Fig.5**

496 **(A)** CFU/fly comparing conventionally raised flies to those raised on antibiotic media. Antibio-
497 tic treatment is effective in reducing the fly bacterial load. **(B)** Germ-free flies do not show
498 PGN-induced PER suppression. PER index of w- Germ-free (GF) and conventionally raised (CR)
499 flies to control solutions of sucrose 1mM and sucrose 1mM + caffeine 10mM and to sucrose
500 1mM + PGN from *E. coli* K12 at 200µg/mL. The data showed for CR flies are the same as in
501 figure 1A. For **(B)**, PER index is calculated as the percentage of flies tested that responded with
502 a PER to the stimulation \pm 95% CI. The number of tested flies (n) is indicated on top of each

503 bar. For each condition, at least 3 groups with a minimum of 10 flies per group were used. ns
504 indicates $p > 0.05$, * indicates $p < 0.05$, ** indicates $p < 0.01$, *** indicates $p < 0.001$, **** indi-
505 cates $p < 0.0001$ Fisher Exact Test. (C-E) Real-time calcium imaging using the calcium indicator
506 GCaMP6s to reflect the *in vivo* neuronal activity of Gr66a-/ppk23+ neurons (Gr66a^{LexA};
507 LexAopGal80;ppk23^{Gal4}/UASGCaMP6s) in adult brains of flies whose proboscis has been stim-
508 ulated with PGN. The expression of LexAop-Gal80 antagonizes the activity of Gal4, thus pre-
509 venting the expression of GCaMP6S in Gr66a+/ppk23+ neurons. (C); Representative images
510 showing the GCaMP6s intensity before and after addition of either the control water or the
511 peptidoglycan. (D); Averaged \pm SEM time course of the GCaMP6s intensity variations
512 ($\Delta F/F_0$ %) for Gr66a-/ppk23+ neurons. The addition of water (n= flies) or peptidoglycan (n=
513 flies) at a specific time is indicated by the arrow. (E) Averaged fluorescence intensity of posi-
514 tive peaks \pm SEM for control (n= 8) and peptidoglycan-treated flies (n= 8) In (E), * indicates
515 $p < 0.05$ non-parametric t-test, Mann-Whitney test. Further details can be found in the detailed
516 lines, conditions and, statistics for the figure section.

517

518 Fig.6

519 (A) Graphical representation of the protocol to contaminate Germ-free larvae at different
520 stage of the larvae development. Two ovipositions of yw⁻ flies on standard medium and w⁻
521 flies on antibiotic-enriched medium started simultaneously. After 4h the embryos of the w⁻
522 flies are sterilized using bleach and transferred onto antibiotic-enriched media. Upon reaching
523 the desired stage of development the w⁻ germ-free larvae are transferred to the same tube in
524 which the yw⁻ larvae are growing. Once the adult stage is reached, flies are separated accord-
525 ing to body color and their PER response assayed. (B) Co-habitation with bacteria only during
526 the larval stage is sufficient to trigger PGN-induced PER suppression. PER index of w- flies,
527 shifted from Germ-free to conventional raising conditions (and the reverse) upon pupation,
528 to control solutions of sucrose 1mM + PGN from *E. coli* K12 at 200 μ g/mL. The data showed for
529 conventionally raised (CR) and Germ-free (GF) flies are the same as in figure 1A and 5B. (C)
530 PER index of w- Germ-free animals, shifted to conventional raising conditions at different
531 stages of their larval development (as in fig. 6A), to solutions of sucrose 1mM + PGN from *E.*
532 *coli* K12 at 200 μ g/mL. The graphic represents how many days after the egg laying (AEL) larvae
533 were transferred to yw⁻ used media, and their response to stimulation with PGN. (D) PER index
534 of w- Germ-free animals, transferred for short periods of time of their development under

535 conventional raising conditions, to solutions of sucrose 1mM + PGN from *E. coli* K12 at
536 200µg/mL. The graph represents the timing of the shift from one condition to another. For
537 (B), (C) and (D), PER index is calculated as the percentage of flies tested that responded with
538 a PER to the stimulation ± 95% CI. The number of tested flies (n) is indicated on top of each
539 bar. For each condition, at least 3 groups with a minimum of 10 flies per group were used. ns
540 indicates p>0.05, * indicates p<0.05, ** indicates p<0.01, *** indicates p<0.001, **** indi-
541 cates p<0.0001 Fisher Exact Test. Further details can be found in the detailed lines, conditions
542 and, statistics for the figure section.

543

544 **Fig.7**

545 (A) Lactobacilli are among the most abundant genera in our conventional raising conditions.
546 Graphical representation of the bacterial genera present in our conventional raising condi-
547 tions according to 16S sequencing. (B) Graphical representation of the protocol for the mono-
548 association of germ-free flies with a bacteria. After oviposition the embryos are sterilized with
549 bleach and transferred under sterile conditions into a fresh sterile tube then 200µL of bacterial
550 culture at OD 1 is added. Adults emerging from pupae are then transferred on antibiotic-en-
551 riched media. (C) Mono association of Germ-free flies with *L. brevis*, but not *L. plantarum*, is
552 sufficient to trigger the PGN-induced PER suppression. PER index of w- flies mono-associated
553 with *L. brevis*, *L. plantarum* or exposed to their culture media (MRS) to sucrose 1mM and su-
554 crose 1mM + caffeine 10mM and to sucrose 1mM + PGN from *E. coli* K12 at 200µg/mL. For (C)
555 PER index is calculated as the percentage of flies tested that responded with a PER to the
556 stimulation ± 95% CI. The number of tested flies (n) is indicated on top of each bar. For each
557 condition, at least 3 groups with a minimum of 10 flies per group were used. ns indicates
558 p>0.05, * indicates p<0.05, ** indicates p<0.01, *** indicates p<0.001, **** indicates
559 p<0.0001 Fisher Exact Test. Further details can be found in the detailed lines, conditions and,
560 statistics for the figure section.

561

562 **Fig.S1**

563 (A) and (B), raising medium has no effect on PGN-induced PER suppression. PER index of w-
564 (A) and CantonS (B) flies, raised on sucrose (suc+) or amino acids (prot+) enriched media, to
565 control solutions of sucrose 1mM and to sucrose 1mM + PGN from *E. coli* K12 at 200µg/mL.
566 The data for w- raised on media prot+ are the same as figure 1A, and those for CantonS raised

567 on media prot+ are the same as figure 1C. **(C)** Sucrose concentrations above 5mM mask the
568 aversive effect of PGN and caffeine. PER index of w- flies to increasing concentrations of su-
569 crose, caffeine10mM + increasing concentration of sucrose and PGN from *E. coli* K12 at
570 200µg/mL + increasing concentrations of sucrose. For **(A)**, **(B)** and **(C)** PER index is calculated
571 as the percentage of flies tested that responded with a PER to the stimulation ± 95% CI. The
572 number of tested flies (n) is indicated on top of each bar. For each condition, at least 3 groups
573 with a minimum of 10 flies per group were used. ns indicates p>0.05, * indicates p<0.05, **
574 indicates p<0.01, *** indicates p<0.001, **** indicates p<0.0001 Fisher Exact Test. Further
575 details can be found in the detailed lines, conditions and, statistics for the figure section.

576

577 **Fig.S2**

578 **(A)** PER index of w- flies upon stimulation of the front leg with increasing concentrations of
579 sucrose. The numbers below the x-axis correspond to the sucrose final concentrations in mM.
580 As 1mM sucrose does not trigger a PER robust and reproducible enough to qualify the animal
581 as able to respond, this concentration was not used. **(B)** PER index of w- flies upon stimulation
582 of the front leg with PGN from *E. coli* K12 at 200µg/mL + increasing concentrations of sucrose.
583 The numbers below the x-axis correspond to the sucrose final concentrations in mM, once is
584 mixed with PGN. **(C)** PGN solutions from *E. coli* K12 (Ec) and *S. aureus* (SA) at 200µg/mL are
585 neutral. The pH of the two solutions was measured with pH test strips. For **(A)** and **(B)** PER
586 index is calculated as the percentage of flies tested that responded with a PER to the stimula-
587 tion ± 95% CI. The number of tested flies (n) is indicated on top of each bar. For each condition,
588 at least 3 groups with a minimum of 10 flies per group were used. ns indicates p>0.05, * indi-
589 cates p<0.05, ** indicates p<0.01, *** indicates p<0.001, **** indicates p<0.0001 Fisher Exact
590 Test. Further details can be found in the detailed lines, conditions and, statistics for the figure
591 section.

592

593 **Fig.S3**

594 **(A)** Perception by the adult flies of sucrose 1mM + quinine 10mM and sucrose 1mM + caffeine
595 10mM required Gr66a+ neurons to be functional in the adult but not in larvae. The ubiqui-
596 tously expressed Tub-Gal80^{ts}, that inhibits the activity of Gal4, is temperature sensitive: it's
597 active at 18°C and inactivated at 29°C, allowing the expression of UASKir2.1 and the conse-
598 quent impairment of Gr66a+ or ppk23+ neuron activity. The PER index is calculated as the

599 percentage of flies tested that responded with a PER to the stimulation \pm 95% CI. The number
600 of tested flies (n) is indicated on top of each bar. ns indicates $p>0.05$, * indicates $p<0.05$, **
601 indicates $p<0.01$, *** indicates $p<0.001$, **** indicates $p<0.0001$ Fisher Exact Test. Further
602 details can be found in the detailed lines, conditions and, statistics for the figure section

603

604

605 **Fig.S4**

606 **(A)** and **(B)** RNAi-mediated inactivation of different elements of the IMD pathway has no effect
607 on the PGN induced PER suppression. **(A)** PER index of flies in which different elements of the
608 IMD pathway are inactivated via RNAi in Gr66a+ neurons, to control solutions of sucrose and
609 sucrose 1mM + PGN from *E. coli* K12 at 200 μ g/mL. **(B)** PER index of flies upon RNAi-mediated
610 Fadd (UAS-Fadd RNAi) inactivation in ppk23+ cells, to control solutions of sucrose and sucrose
611 + PGN from *E. coli* K12 at 200 μ g/mL. **(C)** The Gr66a receptor is not involved in PGN induced
612 PER suppression, Gr66a mutants lose their aversion to caffeine but retain their aversion to
613 PGN. PER index of fly mutant for the Gr66a receptor to control solutions of sucrose 1mM and
614 sucrose 1mM + caffeine and to sucrose + PGN from *E. coli* K12 at 200 μ g/mL. For **(A)**, **(B)** and
615 **(C)** PER index is calculated as the percentage of flies tested that responded with a PER to the
616 stimulation \pm 95% CI. The number of tested flies (n) is indicated on top of each bar. For each
617 condition, at least 3 groups with a minimum of 10 flies per group were used. ns indicates
618 $p>0.05$, * indicates $p<0.05$, ** indicates $p<0.01$, *** indicates $p<0.001$, **** indicates
619 $p<0.0001$ Fisher Exact Test. Further details can be found in the detailed lines, conditions and,
620 statistics for the figure section.

621

622 **Fig.S5**

623 **(A)** and **(B)** Loss of aversion to PGN in axenic conditions is independent of genetic background.
624 PER index of yw- **(A)** and CantonS **(B)** axenic flies to control solutions of sucrose 1mM and
625 sucrose 1mM + caffeine 10mM and to sucrose 1mM + PGN from *E. coli* K12 at 200 μ g/mL. **(C)**
626 and **(D)** the antibiotic treatment has no effect on the fly taste for PGN. **(C)** Bacterial load
627 (CFU/fly) of w- flies reared under conventional conditions, germ-free with antibiotic treatment
628 or sterilized by embryo bleaching. The data for conventionally raised and germ-free flies are
629 the same as fig. 5A. **(D)** Flies emerged from bleached embryos retain the PGN induced PER
630 suppression, PER index of w- flies sterilized by embryos bleaching to control solutions of

631 sucrose 1mM and sucrose 1mM +caffeine 10mM and to sucrose 1mM + PGN from *E. coli* K12
632 at 200 μ g/mL. (E) Real-time calcium imaging using the calcium indicator GCaMP6s to reflect
633 the *in vivo* neuronal activity of Gr66a+ neurons (Gr66a^{Gal4}, UAS-GCaMP6s) in adult brains of
634 conventionally raised and germ-free flies whose proboscis has been stimulated with PGN. (E)
635 Averaged fluorescence intensity of positive peaks \pm SEM for conventionally raised (n= 22 flies)
636 and germ-free flies (n= 14 flies) in response to caffeine 10mM and peptidoglycan from *E. coli*
637 K12 at 200 μ g/mL. (F) Averaged fluorescence intensity of positive peaks \pm SEM for convention-
638 ally raised (n= 8) and germ-free flies (n=8) in response to Sodium chloride 250mM. For (A), (B)
639 and (D) PER index is calculated as the percentage of flies tested that responded with a PER to
640 the stimulation \pm 95% CI. The number of tested flies (n) is indicated on top of each bar. For
641 each condition, at least 3 groups with a minimum of 10 flies per group were used. ns indicates
642 p>0.05, * indicates p<0.05, ** indicates p<0.01, *** indicates p<0.001, **** indicates
643 p<0.0001 Fisher Exact Test. In (E) and (F), ns indicates p>0.05, ** indicates p<0.001, **** in-
644 dicates p<0.00001 non-parametric t-test, Mann-Whitney test. Further details can be found in
645 the detailed lines, conditions and, statistics for the figure section.

646

647 **Fig.S6**

648 (A) Bacterial load (CFU/fly) of w- flies reared under conventional conditions, germ-free or
649 shifted between the two upon pupation. The data for conventionally raised and germ-free
650 flies are the same as fig. 5A. (B) Graphical representation of the protocol for pupae contami-
651 nation. yw- flies are reared under conventional conditions, after a few days the fly tube is filled
652 with Luria Bertani's liquid medium and used as an inoculum to start a bacterial culture. The
653 culture is then diluted to OD1 and used to soak filter paper placed over the fly culture medium
654 in a new tube. The plug containing the germ-free pupae is dipped into the OD1 bacterial solu-
655 tion and used to close the previously prepared contaminated tube. (C) Contamination from
656 the pupal stage is not sufficient to elicit PGN induced PER suppression. PER index of flies, that
657 developed as germ-free larvae and were contaminated at the pupal stage, to sucrose 1mM +
658 PGN from *E. coli* K12 at 200 μ g/mL. The data for flies raised in conventional conditions, germ
659 free and for those switched between the two are the same as in Fig.6B. For (C) PER index is
660 calculated as the percentage of flies tested that responded with a PER to the stimulation \pm
661 95% CI. The number of tested flies (n) is indicated on top of each bar. For each condition, at
662 least 3 groups with a minimum of 10 flies per group were used. ns indicates p>0.05, * indicates

663 p<0.05, ** indicates p<0.01, *** indicates p<0.001, **** indicates p<0.0001 Fisher Exact Test.
664 Further details can be found in the detailed lines, conditions and, statistics for the figure sec-
665 tion;

666

667 **Fig.S7**

668 Exposing germ-free larvae and adults to PGN-containing media it is not sufficient to trigger
669 PGN induced PER suppression. **(A)** PER index of germ-free flies exposed to PGN as a larva or
670 as adult, to sucrose 1mM + PGN from *E. coli* K12 at 200µg/mL. The data for germ-free flies
671 (GF) are the same as in Fig.5B. **(B)** Octopamine is not involved in PGN induced PER suppression.
672 PER index of TβH mutant flies unable to synthesize octopamine, to control sucrose solution
673 and to sucrose 1mM + PGN from *E. coli* K12 at 200µg/mL. For **(A)** and **(B)** PER index is calcu-
674 lated as the percentage of flies tested that responded with a PER to the stimulation ± 95% CI.
675 The number of tested flies (n) is indicated on top of each bar. For each condition, at least 3
676 groups with a minimum of 10 flies per group were used. ns indicates p>0.05, * indicates
677 p<0.05, ** indicates p<0.01, *** indicates p<0.001, **** indicates p<0.0001 Fisher Exact Test.
678 Further details can be found in the detailed lines, conditions and, statistics for the figure sec-
679 tion.

680 **Fig.S8**

681 **(A)** Flies in which Gr66a-/ppk23+ cells are silenced remains able to sense PGN. PER index of
682 flies to control solutions of sucrose and sucrose 1mM + PGN from *E. coli* K12 at 200µg/mL. The
683 expression of LexAop-Gal80 antagonizes the activity of Gal4, thus preventing the expression
684 of Kir2.1 in Gr66a+ neurons. The scheme represents in red the neurons inactivated by Kir2.1
685 and in green the active ones. 'ppk23Glut' indicates *ppk23^{Gal4}* with *Gr66a^{LexA}* and *LexAop-*
686 *Gal80*; and '>' denotes Gal4 driving *UAS-Kir2.1* with temporal restriction by *tub-Gal80^{ts}*. **(B)**
687 The scheme represents the result of driving the expression of Kir2.1 in Gr66a+ or ppk23+ neu-
688 rons, in red the neurons inactivated by Kir2.1 and in green the active ones. '>' denotes Gal4
689 driving *UAS-Kir2.1*. **(C)** Silencing of smaller subgroup of the Gr66a+ neurons population has no
690 effect on the PGN-induced PER suppression. PER index of flies in which part of Gr66a+ neurons
691 are inactivated via different Gr drivers guiding the expression of Kir2.1, to solutions of sucrose
692 1mM + PGN from *E. coli* K12 at 200µg/mL. For **(A)** and **(C)** PER index is calculated as the per-
693 centage of flies tested that responded with a PER to the stimulation ± 95% CI. The number of
694 tested flies (n) is indicated on top of each bar. For each condition, at least 3 groups with a

695 minimum of 10 flies per group were used. ns indicates $p > 0.05$, * indicates $p < 0.05$, ** indicates
696 $p < 0.01$, *** indicates $p < 0.001$, **** indicates $p < 0.0001$ Fisher Exact Test. Further details can
697 be found in the detailed lines, conditions and, statistics for the figure section.

698

699 **Material and methods**

700 **Fly husbandry**

701 Flies were grown at 25°C on a yeast/cornmeal medium in 12h/12h light/dark cycle-controlled
702 incubators. For 1 L of food, 8.2 g of agar (VWR, cat. #20768.361), 80 g of cornmeal flour
703 (Westhove, Farigel maize H1) and 80 g of yeast extract (VWR, cat. #24979.413) were cooked
704 for 10 min in boiling water. 5.2 g of Methylparaben sodium salt (MERCK, cat. #106756) and 4
705 mL of 99% propionic acid (CARLOERBA, cat. #409553) were added when the food had cooled
706 down.

707 Schnorrer food recipe. For 1 L of food, 11 g of agar (VWR, cat. #20768.361), 80 g of cornmeal
708 flour (Westhove, Farigel maize H1), 20 g of yeast extract (VWR, cat. #24979.413) and 30g of
709 Sucrose were cooked for 10 min in boiling water. 2.5g of Moldex and 5 mL of 99% propionic
710 acid (CARLOERBA, cat. #409553) were added when the food had cooled down.

711

712 **PER assay**

713 All flies used for the test were females between 5 and 7 days old. Unless experimental condi-
714 tions require it, the flies are kept and staged at 25°C to avoid any temperature changes once
715 they are put into starvation. The day before, the tested flies are starved in an empty tube with
716 water-soaked plug for 24h at 25°C.

717 Eighteen flies are tested in one assay, 6 flies are mounted on one slide and in pairs under each
718 coverslip. To prepare the slide, three pieces of double-sided tape are regularly spaced on a
719 slide. Two spacers are created on the sides of each piece of tape by shaping two thin cylinders
720 of UHT paste. To avoid the use of carbon dioxide flies are anesthetized on ice. Under the mi-
721 croscope, two flies are stuck on their backs, side by side, on same piece of tape so that their
722 wings adhere to the tape. A coverslip is then placed on top of the two flies and pressed onto
723 the UHT paste, blocking their front legs and immobilizing them.

724 Once all slides are prepared, they are transferred to a humid chamber and kept at 25°C for 1.5
725 hours to allow the flies to recover before the assay.

726

727 Flies are tested in pairs, the test is carried out until completion on a pair of flies (under the
728 same coverslip), and then move on to the next pair.

729 Before the test, water is given to each pair of flies to ensure that the flies are not thirsty and
730 do not respond with a PER to the water in which the solutions are prepared. Stimulation with
731 the test solution is always preceded and followed by a control stimulation with a sweet solu-
732 tion, to assess the fly's condition and its suitability for the test. During the test small strips of
733 filter paper are soaked in the test solution and used to contact the fly's labellum (three con-
734 secutive times per control and test phase). Contact with the fly's proboscis should be as gentle
735 as possible. Ideally the head should not move. A stronger touch may prevent the fly from re-
736 sponding to subsequent stimulation. Based on the protocol needed the test is done following
737 the sequence and the timing in the table below.

738 **Tab.1 Aversion protocol**

| | | | | | | | | | | |
|-------------------------|---------------|---|---------------|----------------------|---------------|-----------------------------|---------------|----------------------|---------------|---|
| Water To sa- tety | Wait 1 min | CON- TROL Sucrose 1mM Repeated 3 times | Wait 1 min | Water one time | Wait 1 min | TEST Repeated 3 times | Wait 1 min | Water one time | Wait 1 min | CON- TROL Sucrose 1mM Repeated 3 times |
|-------------------------|---------------|---|---------------|----------------------|---------------|-----------------------------|---------------|----------------------|---------------|---|

739

740

741

742 All solutions to be tested are prepared the test day and stored at room temperature. In the
743 aversion protocol, the control stimulation is performed with 1mM sucrose (D(+)-sucrose ≥ 99.5
744 %, p.a. Carl Roth GmbH + Co. KG). This concentration is sufficient to elicit a PER but is not so
745 high as to influence the response to the subsequent test stimulation. After each control or test
746 stimulation, a water-soaked strip is used to tap the proboscis and clean it.

747 The response of the fly to each stimulation is recorded and averaged. Flies that respond posi-
748 tively (PER) to at least one of the control stimulations are considered for further analysis, the
749 others are discarded. The PER index is calculated as the percentage of flies tested that re-
750 sponded with a PER to the TEST stimulation and represented as $\pm 95\%$ CI.

751

752 ***In vivo* calcium imaging**

753 *In vivo* calcium imaging experiments were performed on 5-7 day-old starved mated females.
754 Animals were raised on conventional media with males at 25°C. Flies were starved for 20-24
755 h in a tube containing a filter soaked in water prior any experiments. Flies of the appropriate
756 genotype were anesthetized on ice for 1 h. Female flies were suspended by the neck on a
757 plexiglass block (2 x 2 x 2.5 cm), with the proboscis facing the center of the block. Flies were
758 immobilized using an insect pin (0.1 mm diameter) placed on the neck. The ends of the pin
759 were fixed on the block with beeswax (Deiberit 502, Siladent, 1345 209212). The head was
760 then glued on the block with a drop of rosin (Gum rosin, Sigma-Aldrich 1346 -60895-, dissolved
761 in ethanol at 70 %) to avoid any movements. The anterior part of the head was thus oriented
762 towards the objective of the microscope. Flies were placed in a humidified box for 1 h to allow
763 the rosin to harden without damaging the living tissues. A plastic coverslip with a hole corre-
764 sponding to the width of the space between the two eyes was placed on top of the head and
765 fixed on the block with beeswax. The plastic coverslip was sealed on the cuticle with two-
766 component silicon (Kwik-Sil, World Precision Instruments) leaving the proboscis exposed to
767 the air. Ringer's saline (130 mM NaCl, 5 mM KCl, 2 mM MgCl₂, 2 mM CaCl₂, 36 mM saccharose,
768 5 mM HEPES, pH 7.3) was placed on the head. The antenna area, air sacs, and the fat body
769 were removed. The gut was cut without damaging the brain and taste nerves to allow visual
770 access to the anterior ventral part of the sub-esophageal zone (SEZ). The exposed brain was
771 rinsed twice with Ringer's saline. GCaMP6s fluorescence was viewed with a Leica DM600B
772 microscope under a 40x water objective. GCaMP6s was excited using a Lumencor diode light
773 source at 482 nm ± 25. Emitted light was collected through a 505-530 nm band-pass filter.
774 Images were collected every 500ms using a Hamamatsu/HPF-ORCA Flash 4.0 camera and pro-
775 cessed using Leica MM AF 2.2.9. Stimulation was performed by applying 140 µL of tastant
776 solution diluted in water on the proboscis. A minimum of 2 independent experiments with a
777 total n for each condition ranging from 7 to 9 were performed. Each experiment consisted of
778 a recording of 70-100 images before stimulation and 160 images after stimulation. Data were
779 analyzed as previously described (ref) by using FIJI (<https://fiji.sc/>).

780

781 **Bacterial strains and maintenance**

782 For this study, the following bacterial strains were used: *L. brevis* (Leulier's Lab) and *L. planta-*
783 *rum* (Gallet's lab). All the strains were grown in 49mL MRS liquid media (MRS Broth Fluka
784 analytical 69966) static at 37 °C in sealed 50mL tubes for anaerobic conditions. To concentrate

785 bacteria and reach the requested OD, 250 mL overnight cultures were centrifuged 15 min at
786 2250 rcf, OD at 600nm was measured and bacteria were diluted in MRS medium to the desired
787 concentration.

788

789 **Flies bacteria load**

790 The flies are anesthetized on ice, ten females for each experimental condition are pulled in
791 1.5 mL microcentrifuge tubes. Under sterile conditions, 600 μ L of Luria-Bertani liquid media
792 (LB) is added to each tube and a sterile pestle is used to homogenize the flies. The fly homog-
793 enate is then serially diluted in LB medium and plated in triplicate on LB plates. Plates are kept
794 overnight at 37°C to facilitate bacterial growth. After two or three days of growth, the average
795 number of colonies per dilution is calculated and the bacterial count of the fly is determined
796 as follows:

797 $CFU/mL = (1000\mu L/volume\ plated) * average\ n\ of\ colonies$

798 $CFU/mL\ at\ the\ origin = CFU/mL * dilution\ factor$

799 $CFU/fly = (CFU/mL\ at\ the\ origin * volume\ in\ which\ you\ homogenized\ flies)/n\ of\ flies\ homog-$
800 $nized.$

801

802 **Embryo sterilization**

803 Three petri dishes are filled with 2.6% bleach, 70% ethanol (Ethanol 96° RPE Carlo Erba Ref
804 414638) and autoclaved purified distilled water respectively. The embryos are collected by
805 filling the tube in which oviposition occurred with PBS and using a small brush to gently detach
806 them from the flies' food. A 40 μ m cell strainer is used to collect the embryos. The cell strainer
807 with the embryos is then dipped into: bleach 2.6% for 5 minutes, ethanol 70% for 1 minute,
808 purified water for 1 minute, ethanol 70% for 1 minute, purified water for 1 minute. The brush
809 used to collect the embryos is sterilized in 2.6% bleach for ten minutes, rinsed and then used
810 to transfer the sterile embryos onto the desired media.

811

812 **Larvae contamination**

813 An oviposition of w- germ free flies is set up on an apple agar plate at 25°C for 4h. Simultane-
814 ously an oviposition of yw- flies is set up in a regular fly tube. After 4h the w- embryos are
815 sterilized (see protocol above) and transferred on antibiotic enriched media. Once they reach
816 the desired developmental stage w- larvae are filtered out of the antibiotic enriched media

817 and rinsed off with PBS. They are then transferred in the tube in which yw- larvae are growing.
818 In this way w- larvae are exposed to the same bacteria as yw- flies but starting at a defined
819 developmental stage. Once the adults emerge from the pupae, they are distinguished on the
820 basis of body color. yw- flies were exposed to the bacteria throughout their lives, whereas w-
821 flies were only exposed from the moment of transfer.

822

823 **Pupae contamination**

824 yw- flies are raised in a regular tube, after a few days the flies are removed and the tube is
825 filled with Luria Bertani liquid media (LB) and incubated at 37°C. The day after the OD is meas-
826 ured and the culture is diluted to OD1. 200µL of bacterial culture are added on filter paper on
827 top of the fly media. Germ-free fly pupae attached to the cotton plug are soaked in the bac-
828 terial suspension and this plug is used to close the previously contaminated tube. This ensures
829 that flies are exposed to the bacteria as soon as they leave the pupal case.

830

831 **Exposure of adult flies to PGN**

832 As soon as they emerge from the pupal case, the germ-free adult flies are transferred to a test
833 tube containing filter paper soaked in a Sucrose1mM+PGN200µg/mL solution and placed on
834 top of the fly medium. Flies are then flipped into tubes prepared in the same way, every day
835 for five days and then used for the PER test.

836

837 **Exposure of larvae to PGN**

838 An oviposition of w- germ free flies is set up on antibiotic culture medium, in parallel with
839 another oviposition of yw- flies set up on antibiotic culture medium. Once the fly media has
840 been softened by the yw- larvae 500µl of PGN PGN200µg/mL solution are added on top of it
841 together with 48h old w- germ- free larvae. Once emerged from the pupal case adult flies are
842 sorted based on body color and staged for the PER assay on antibiotic enriched fly food.

843

844 **Microbiota sequencing**

845 Five replicates of 20 larvae were considered for 16S metagenomic analysis. DNAs were ex-
846 tracted using DNeasy PowerSoil kit (Qiagen) by grinding the larvae in the C1 buffer of this kit
847 with a Precellys (VWR).

848 The Oxford Nanopore Technologies 16S Barcoding Kit (SQK16S-024) was used to amplify and
849 sequence the full-length 16S ribosomal RNA. Starting with 20-25ng of extracted DNA, 45 cycles
850 of PCR amplification were performed using New England Biolabs LongAmp Hot Start Taq 2X
851 Master Mix and ONT 16S barcoded primers (27F and 1492R). After purification, the amplicons
852 were quantified and qualified to be mixed equimolarly. Sequencing was performed on Mk1C
853 (MinKNOW 21.10.8) using an R9 flongle and the run initiated with the high-accuracy base-
854 calling model (Guppy 5.0.17). The run generated about 429000 reads of which more than
855 450,000 had a QC > 9. The reads were analysed with the dedicated EPI2ME 16S pipeline
856 (v2022.01.07) using the following parameters: minimum score > Q10, minimum length 1000,
857 maximum length 2200, minimum coverage 50%, 5 max target sequence and two different
858 BLAST identity thresholds were tested (85 or 90%). This version used 22,162 sequences of
859 reference coming from the microbial 16S rRNA NCBI RefSeq database.

860

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FIGURE 1

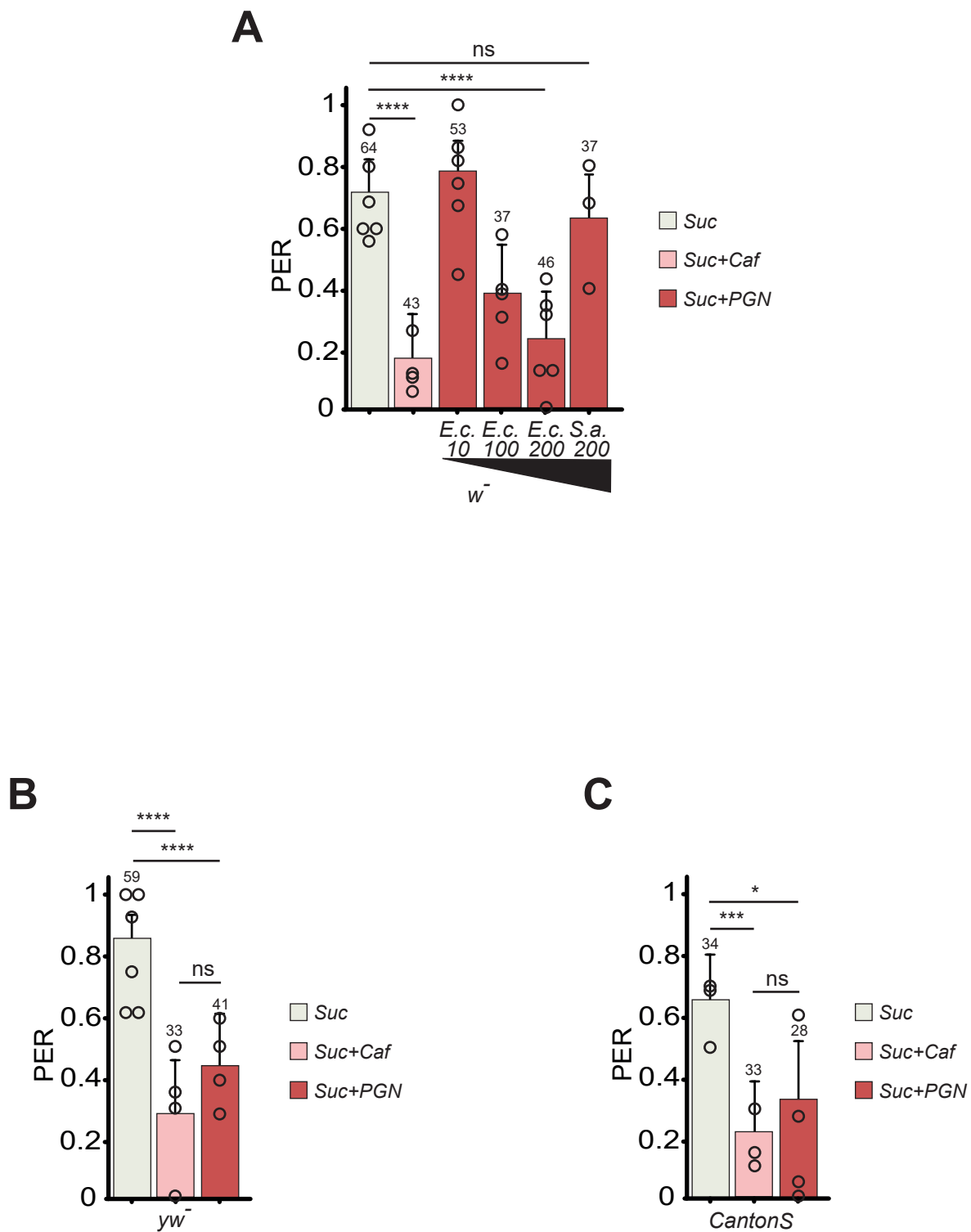


FIGURE 2

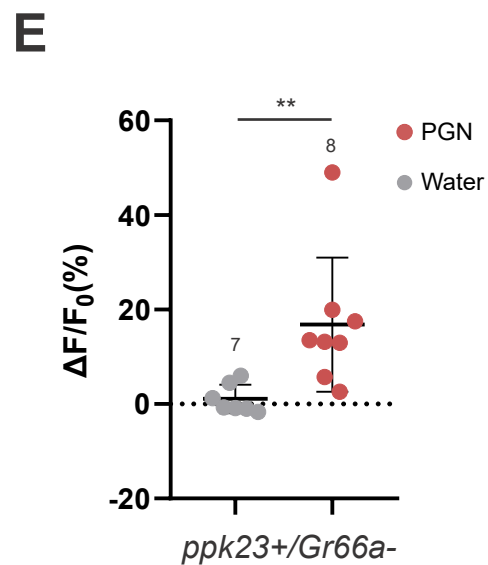
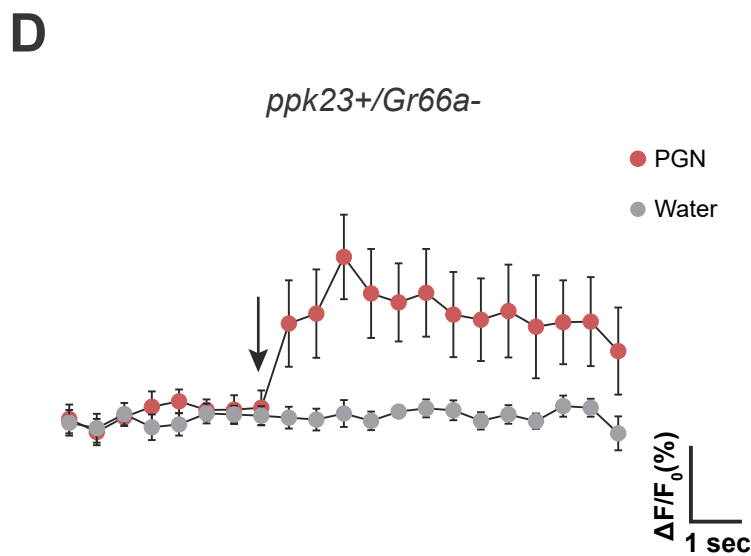
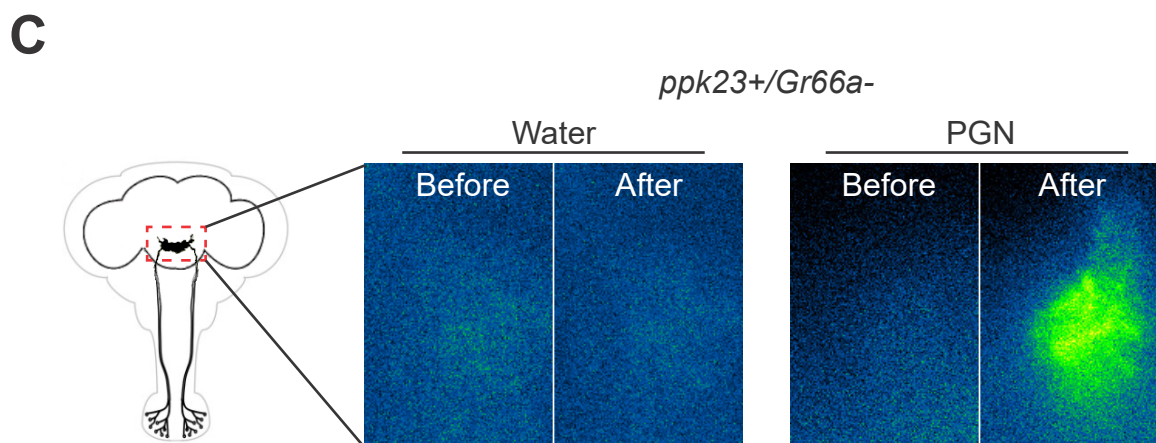
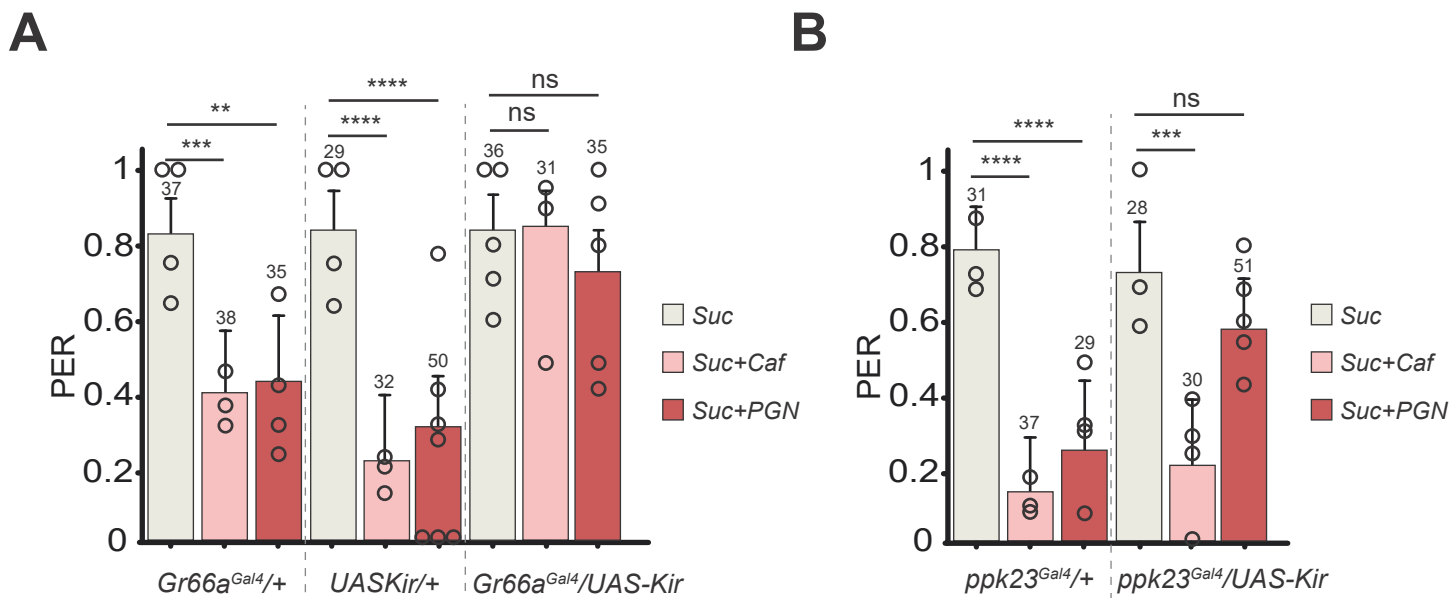
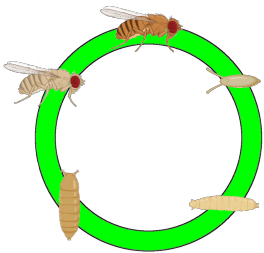


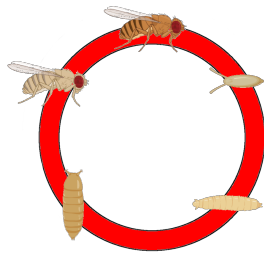
FIGURE 3

A

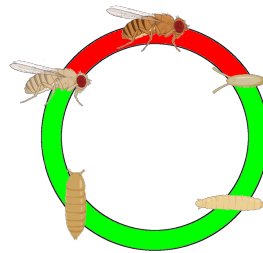
18°C permissive 29°C restrictive



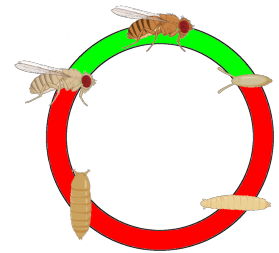
ON in Larvae (La)
ON in Adults (Ad)



OFF in Larvae (La)
OFF in Adults (Ad)



ON in Larvae (La)
OFF in Adults (Ad)



OFF in Larvae (La)
ON in Adults (Ad)

B

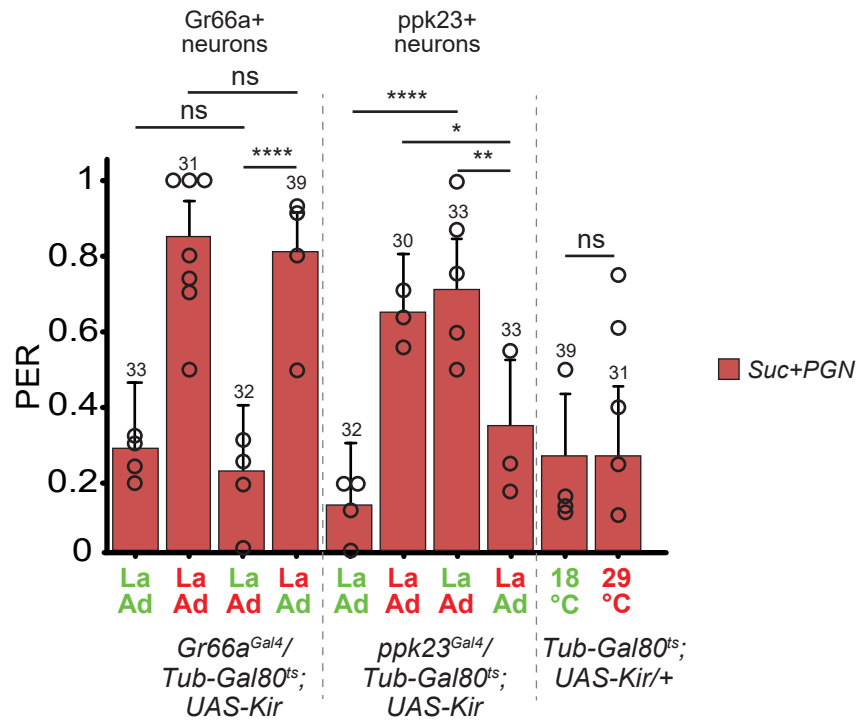
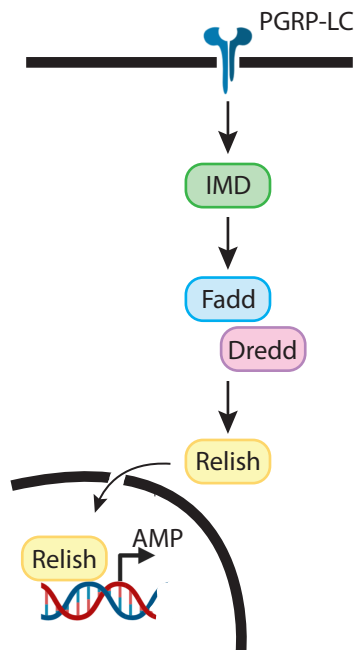
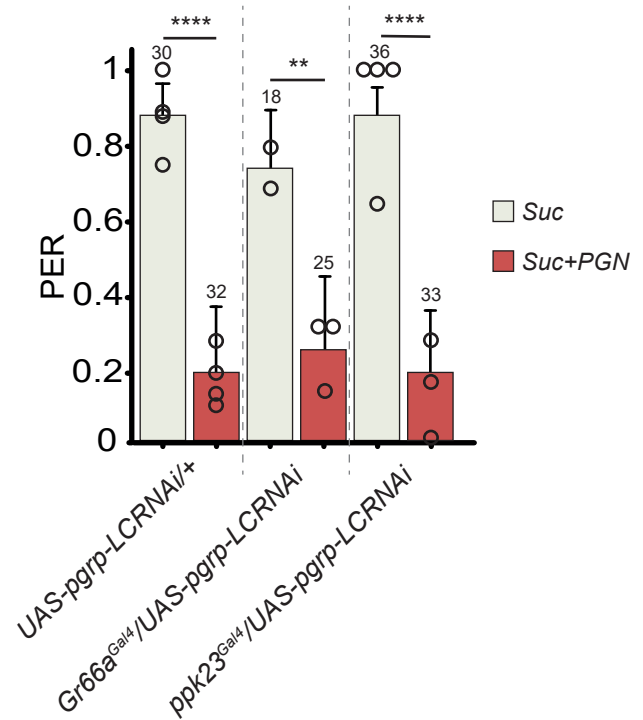


FIGURE 4

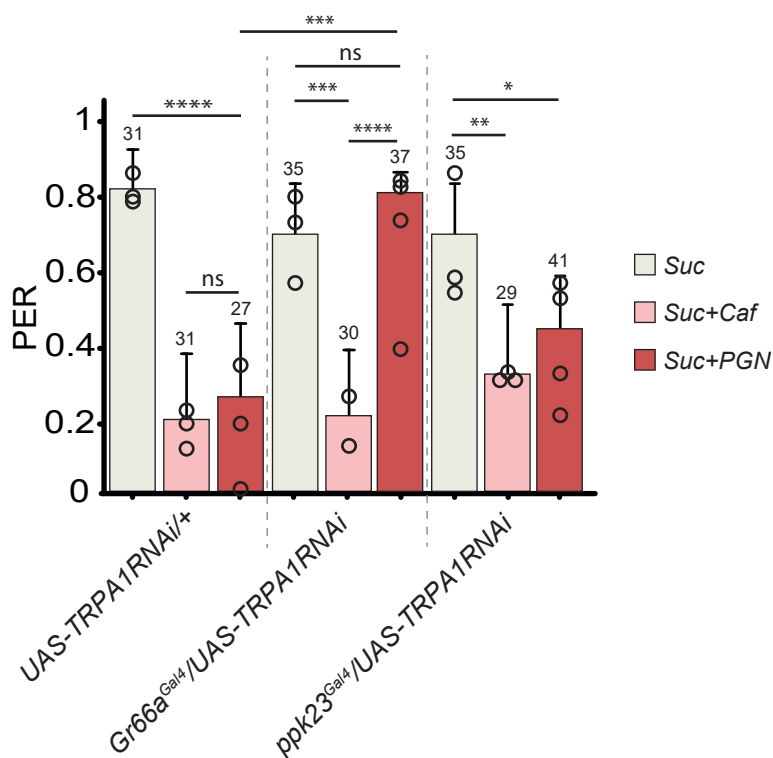
A



B



C



D

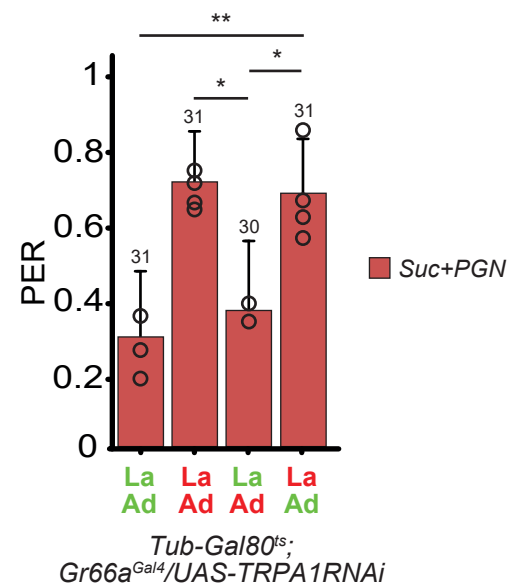


FIGURE 5

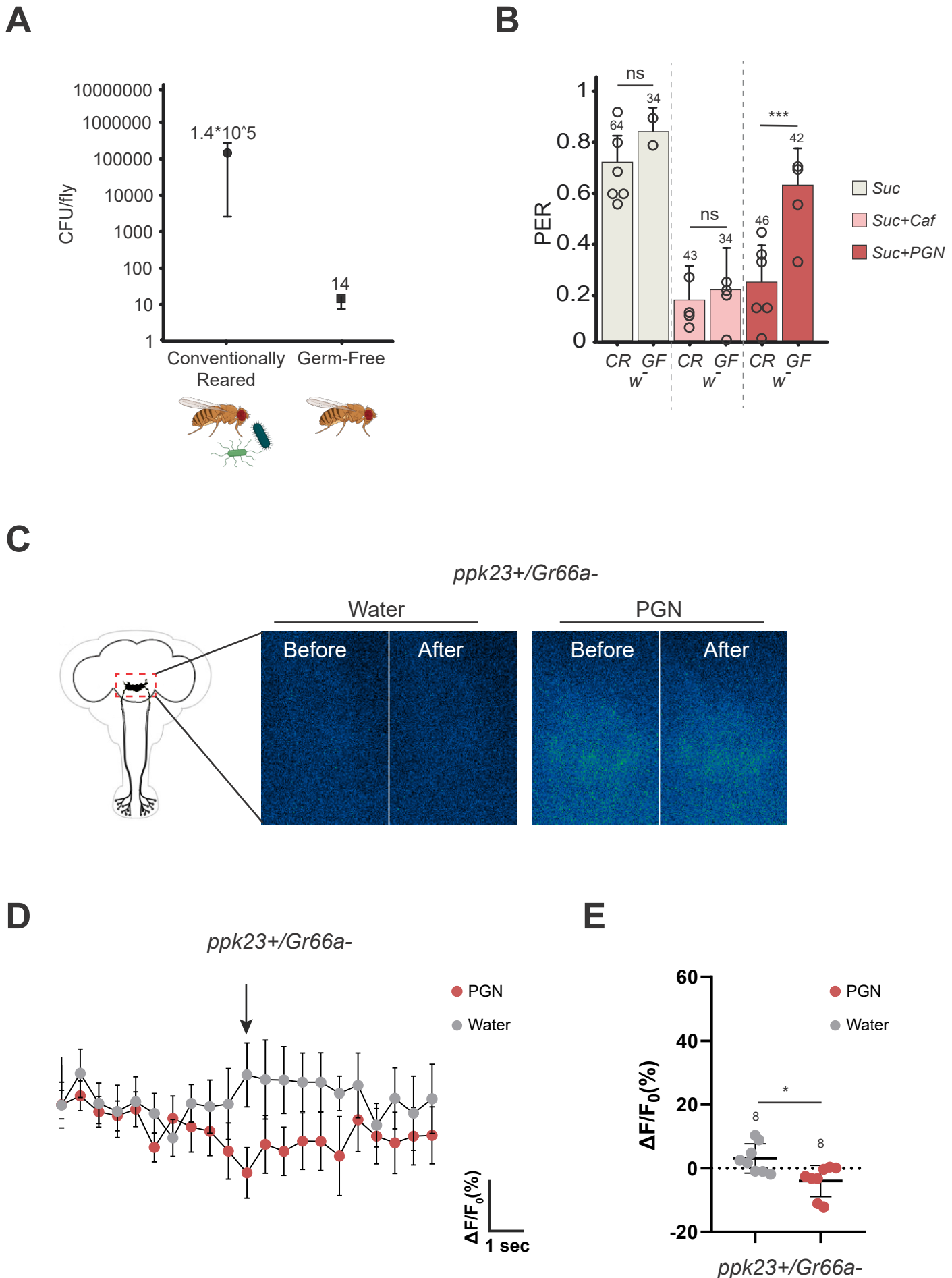
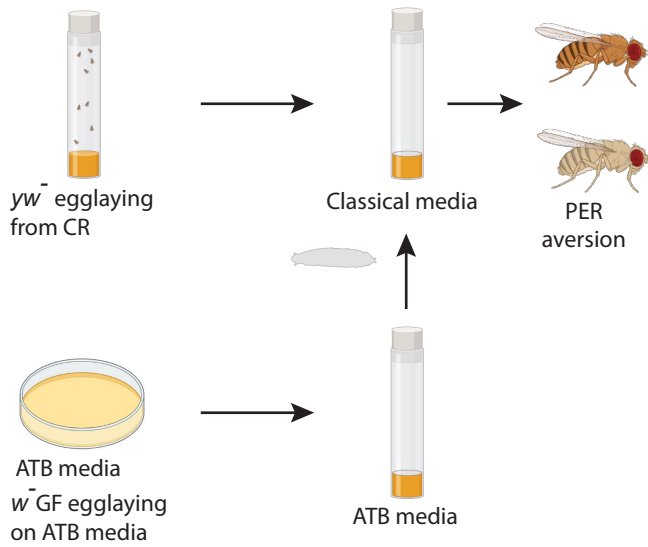
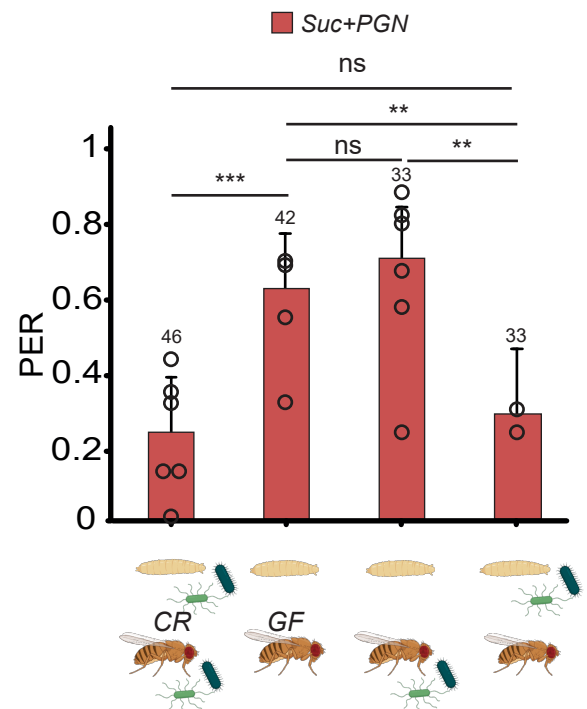


FIGURE 6

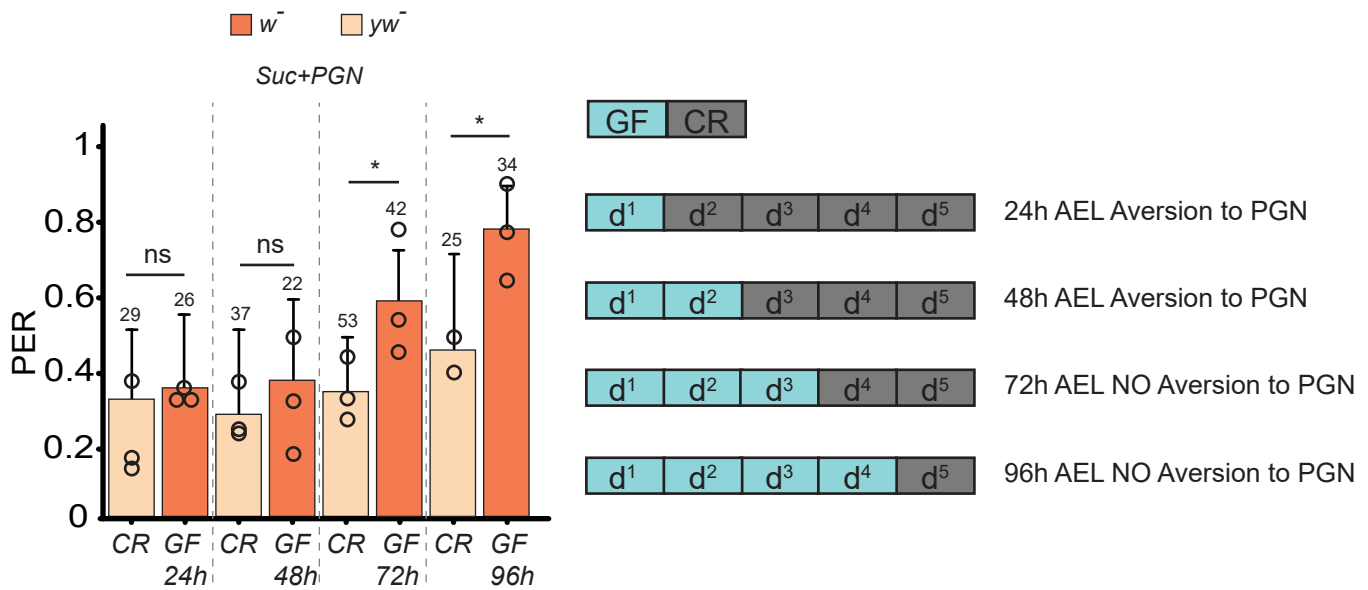
A



B



C



D

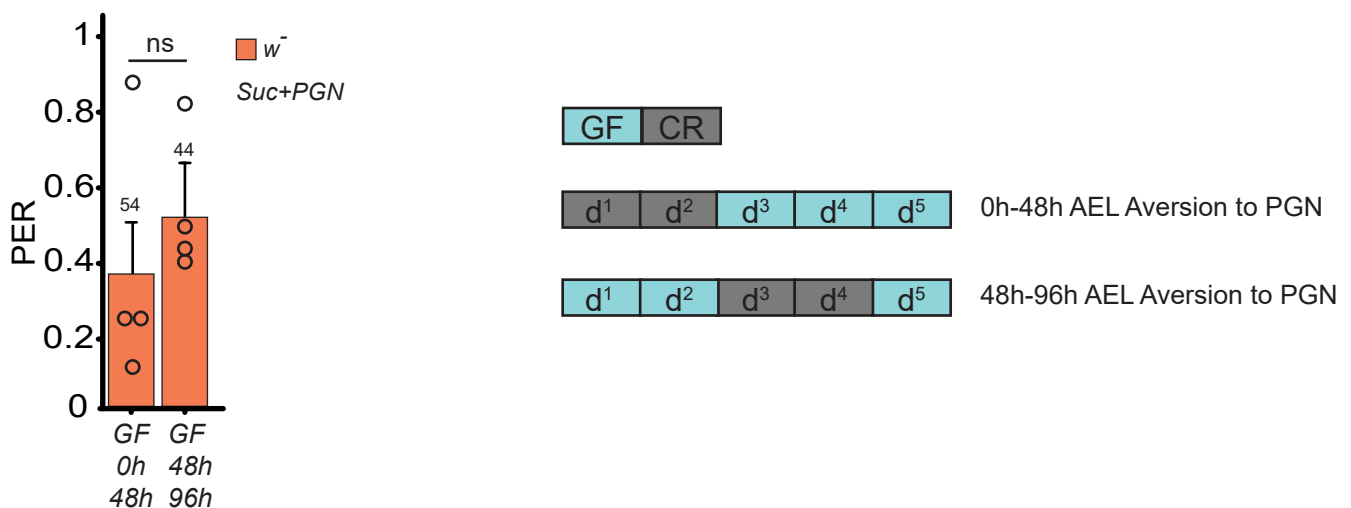
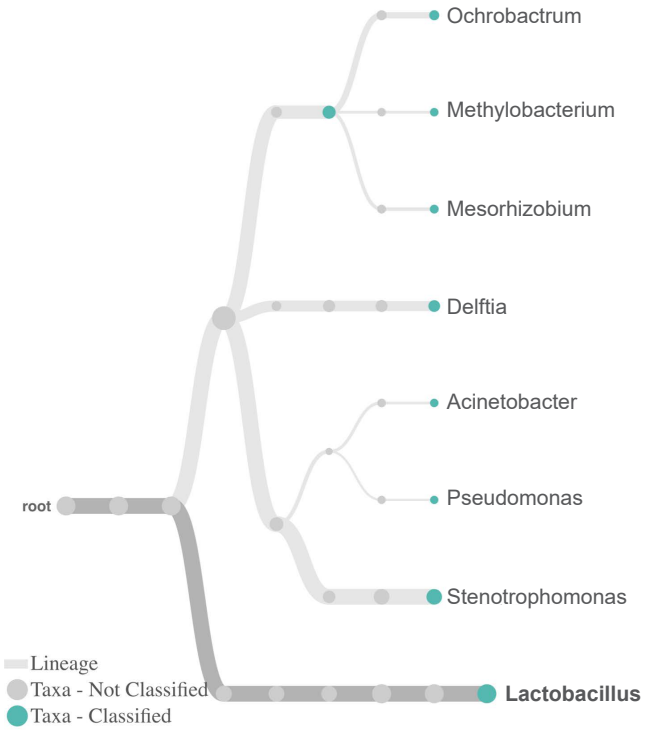
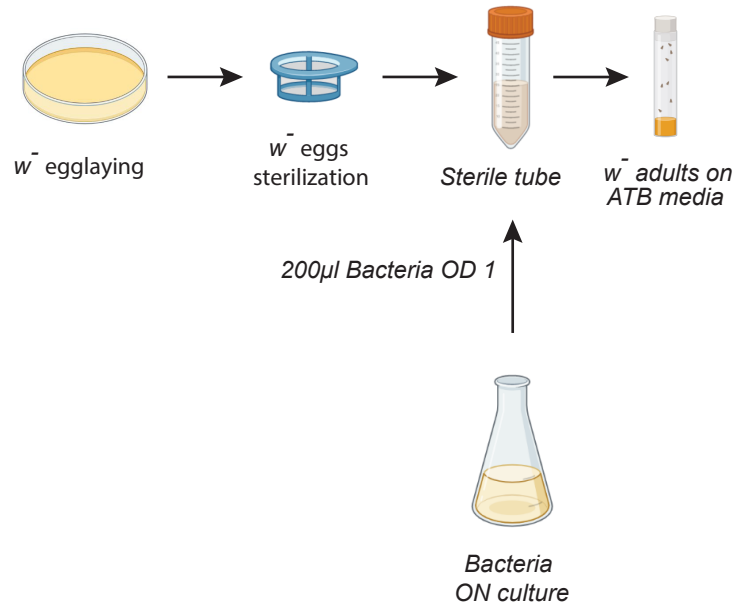


FIGURE 7

A



B



C

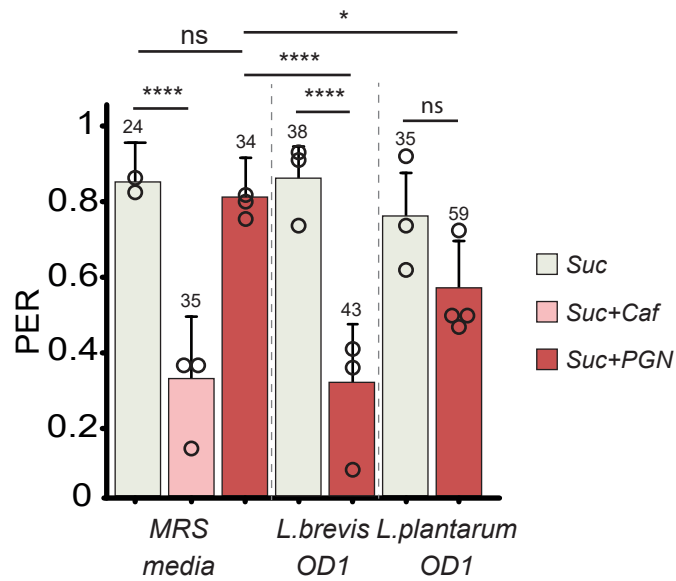
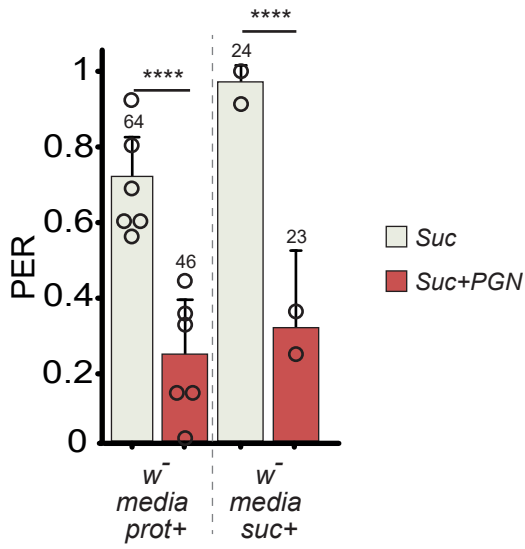
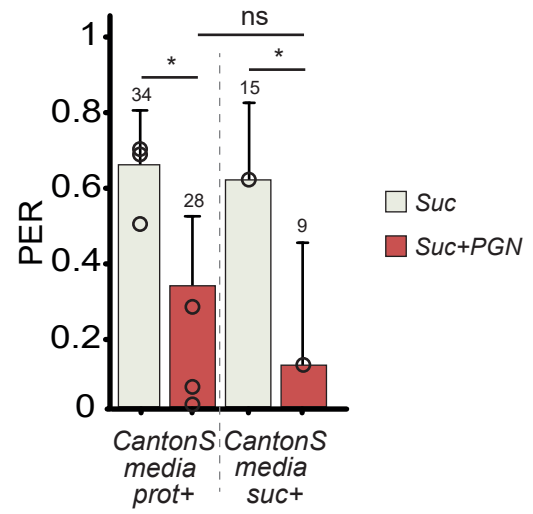


FIGURE S1

A



B



C

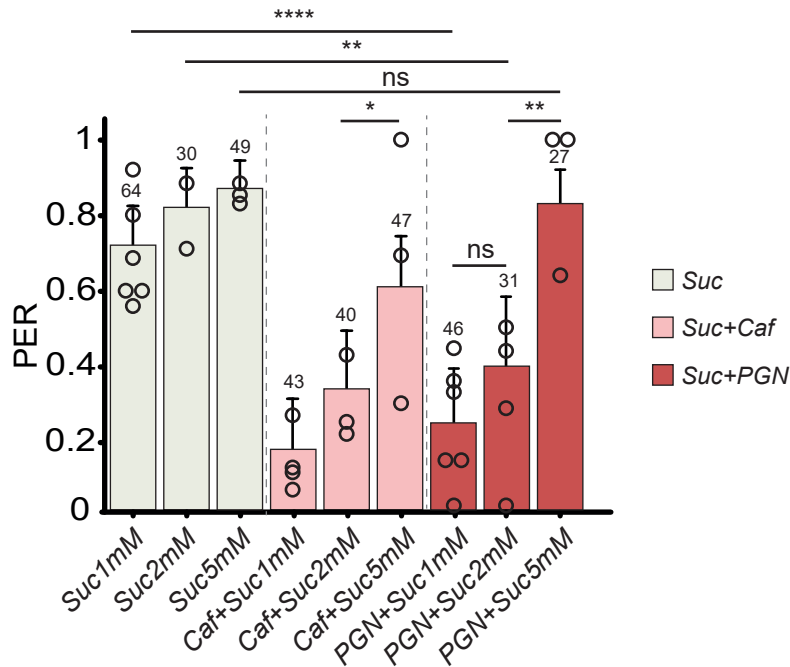
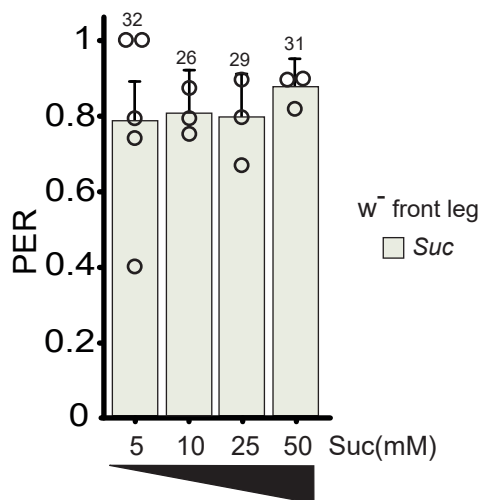
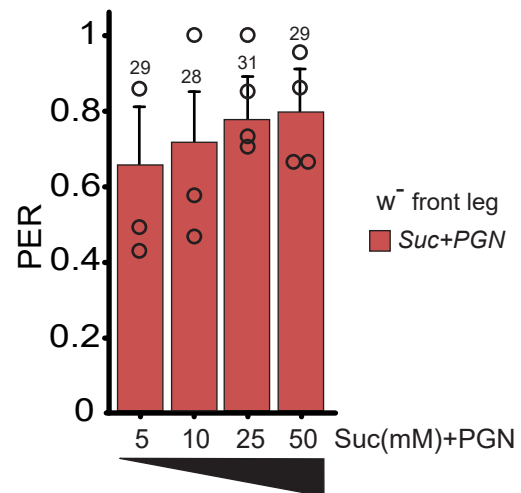


FIGURE S2

A



B



C



FIGURE S3

A

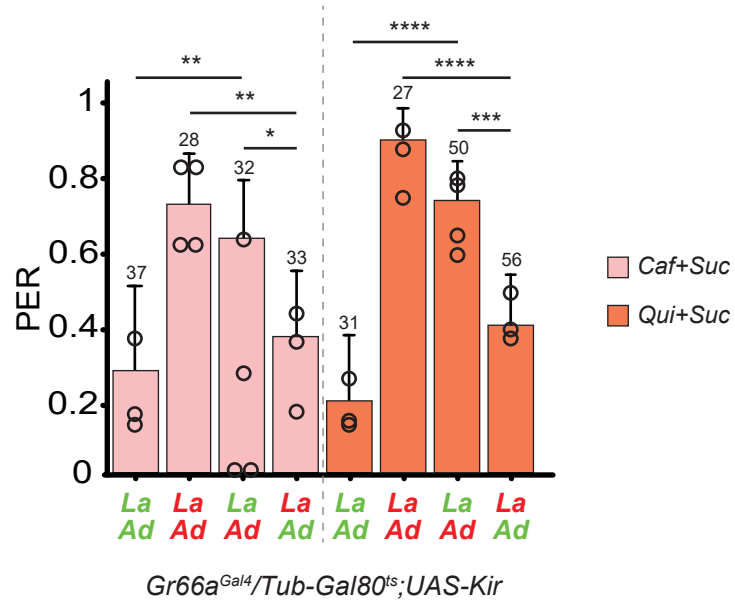
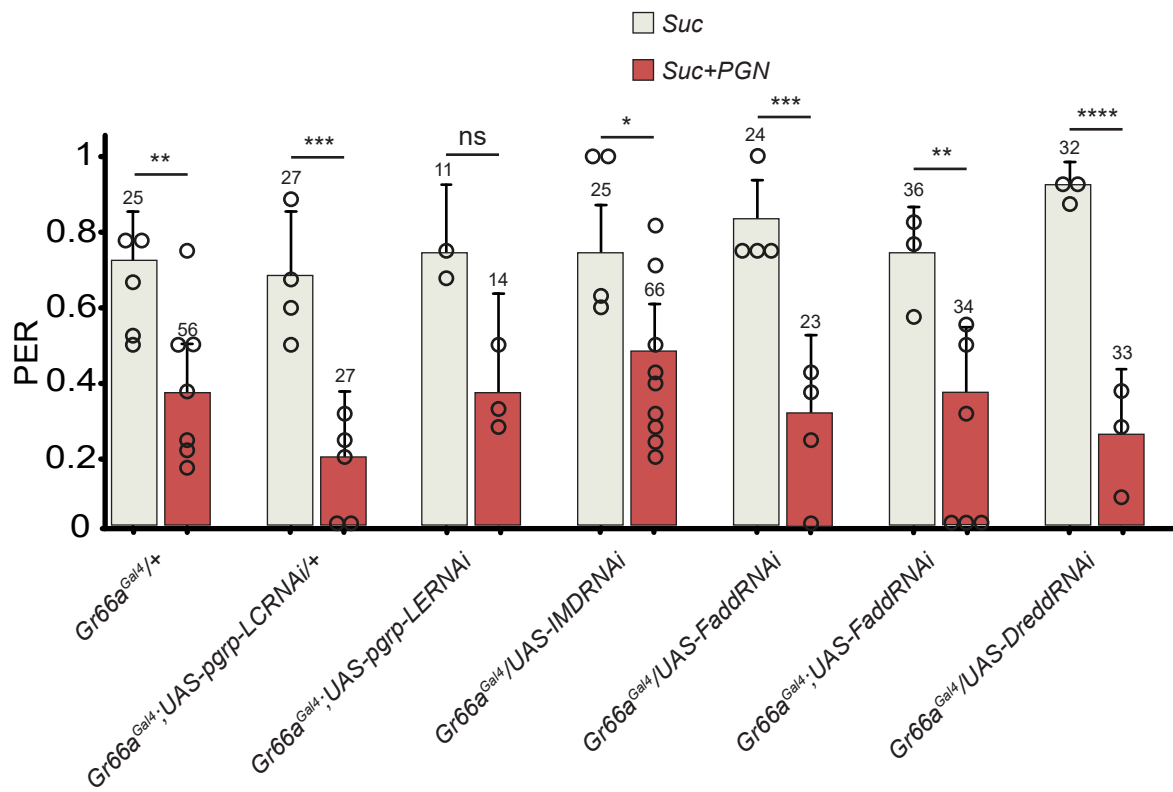
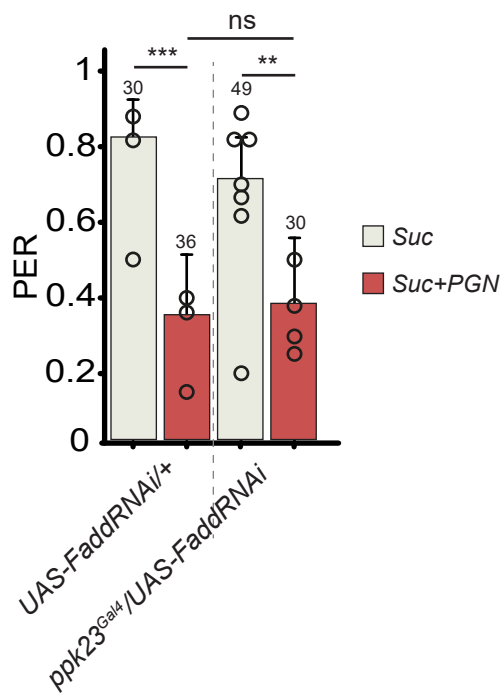


FIGURE S4

A



B



C

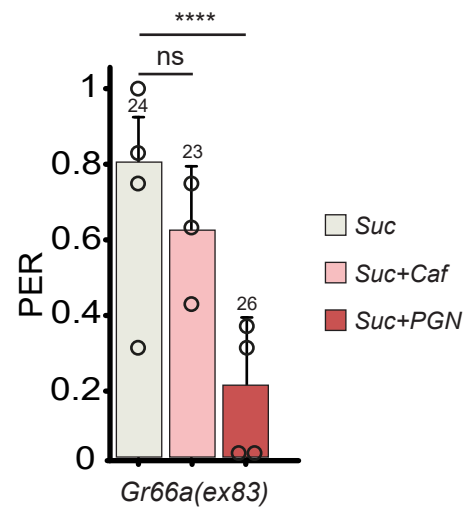


FIGURE S5

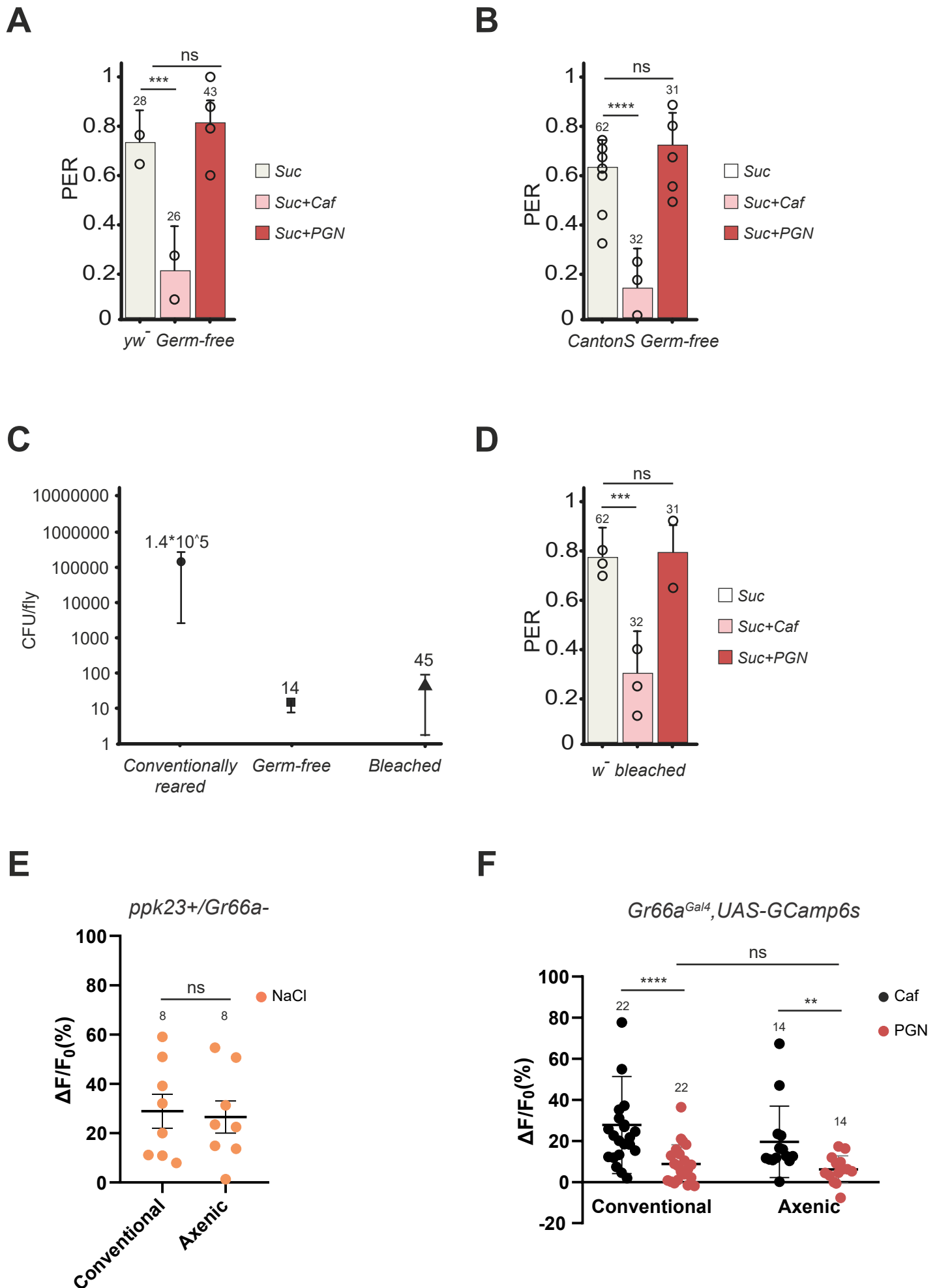


FIGURE S6

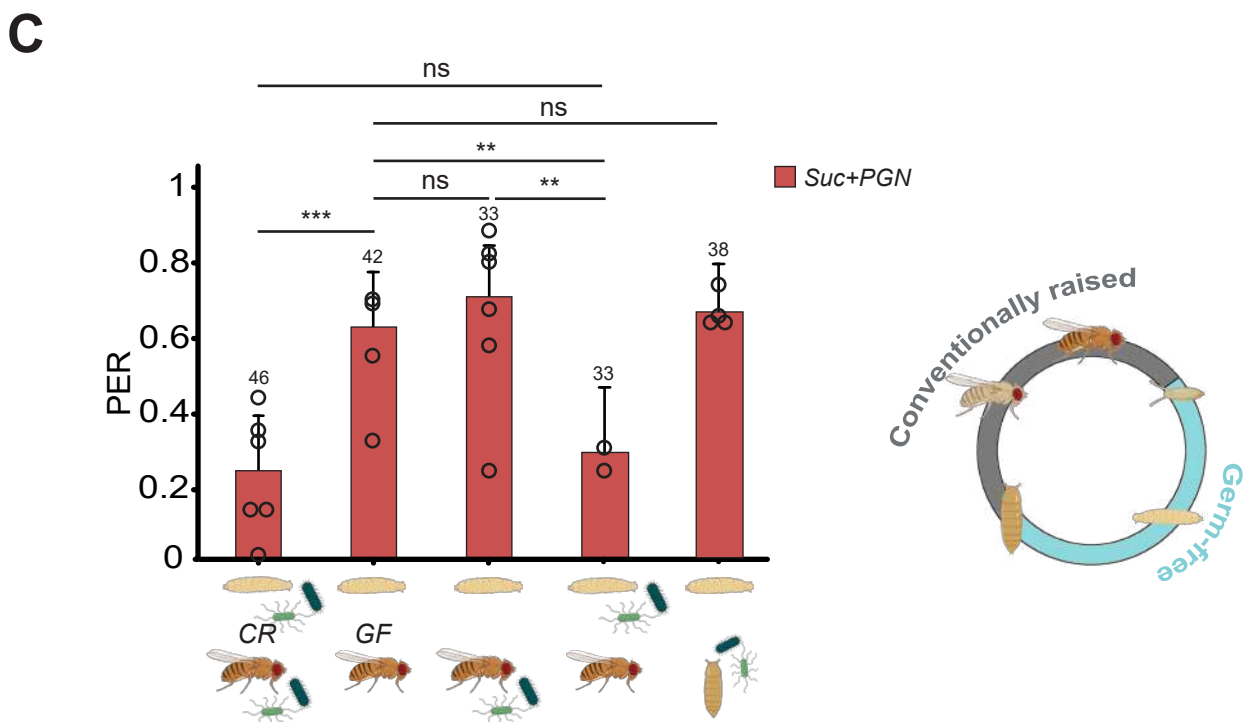
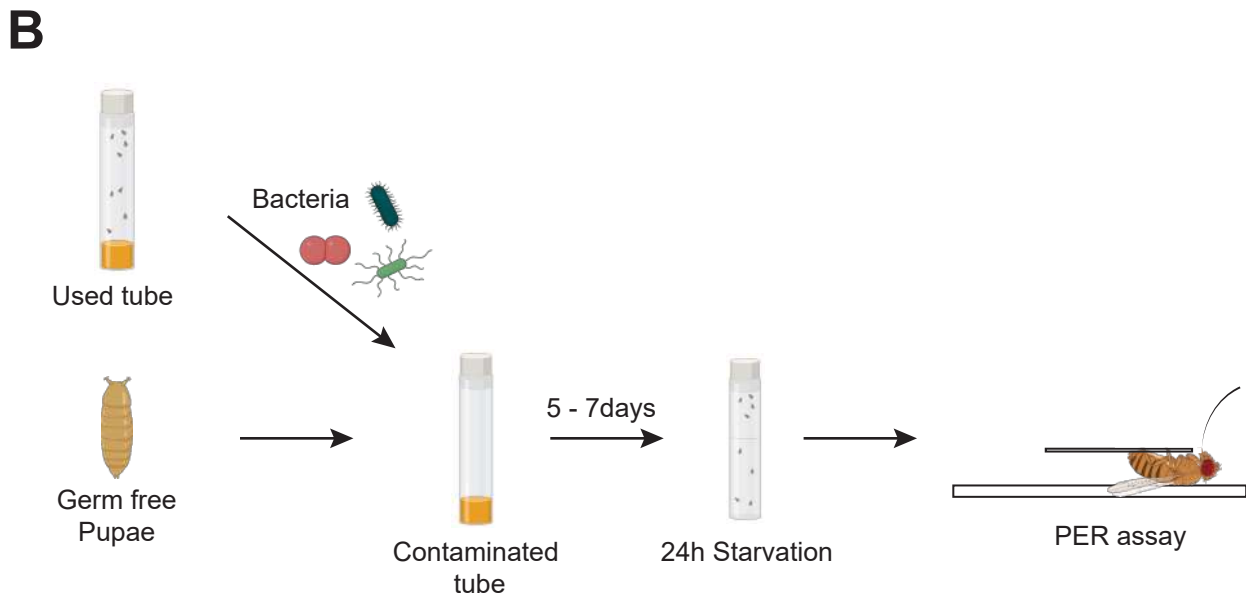
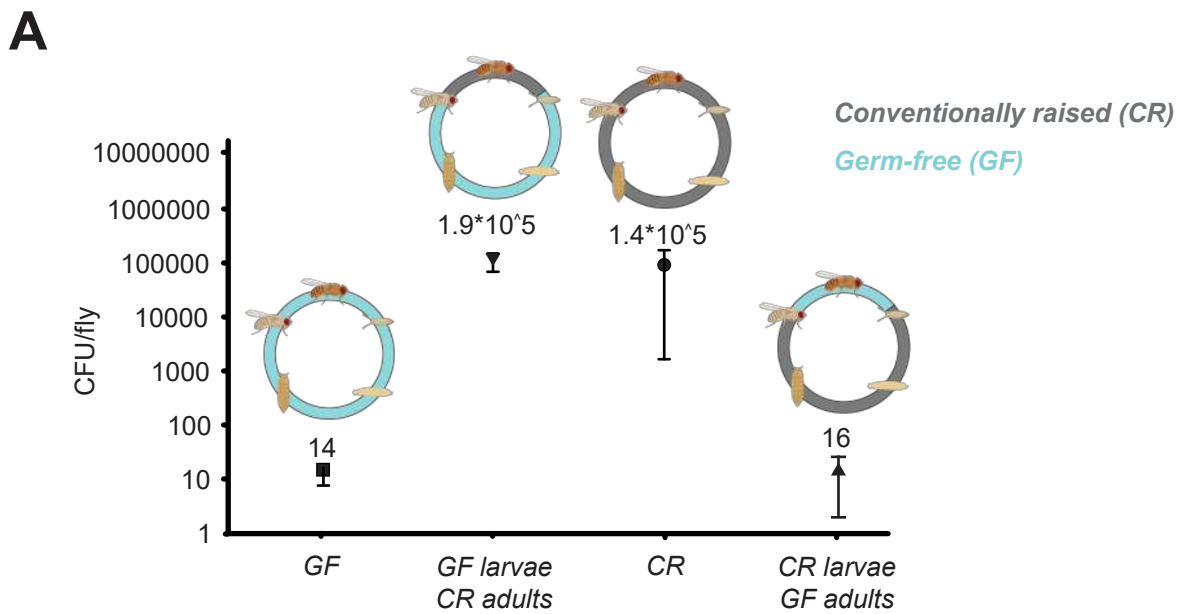
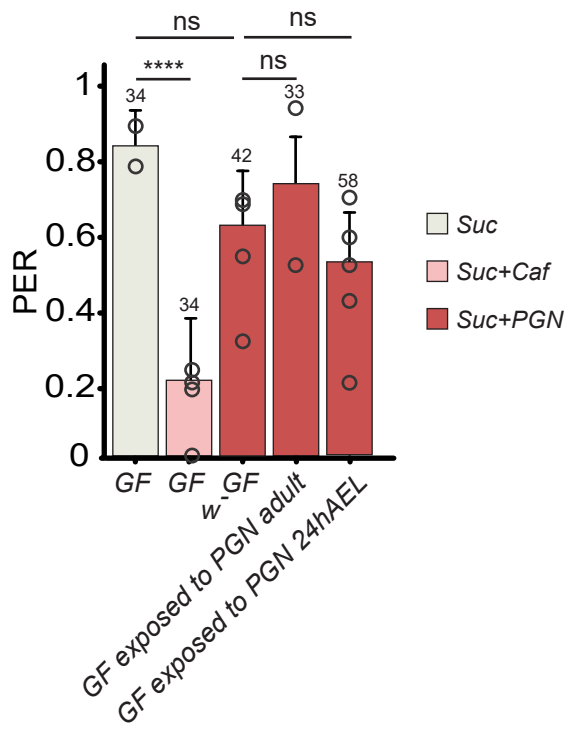


FIGURE S7

A



B

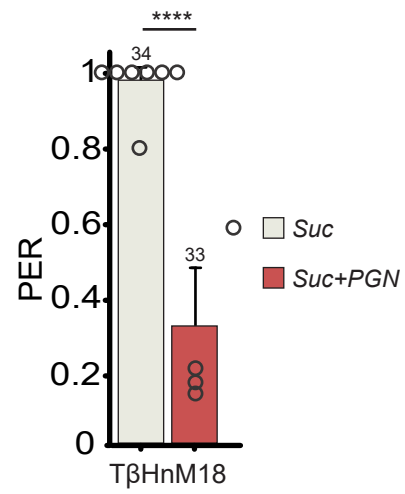
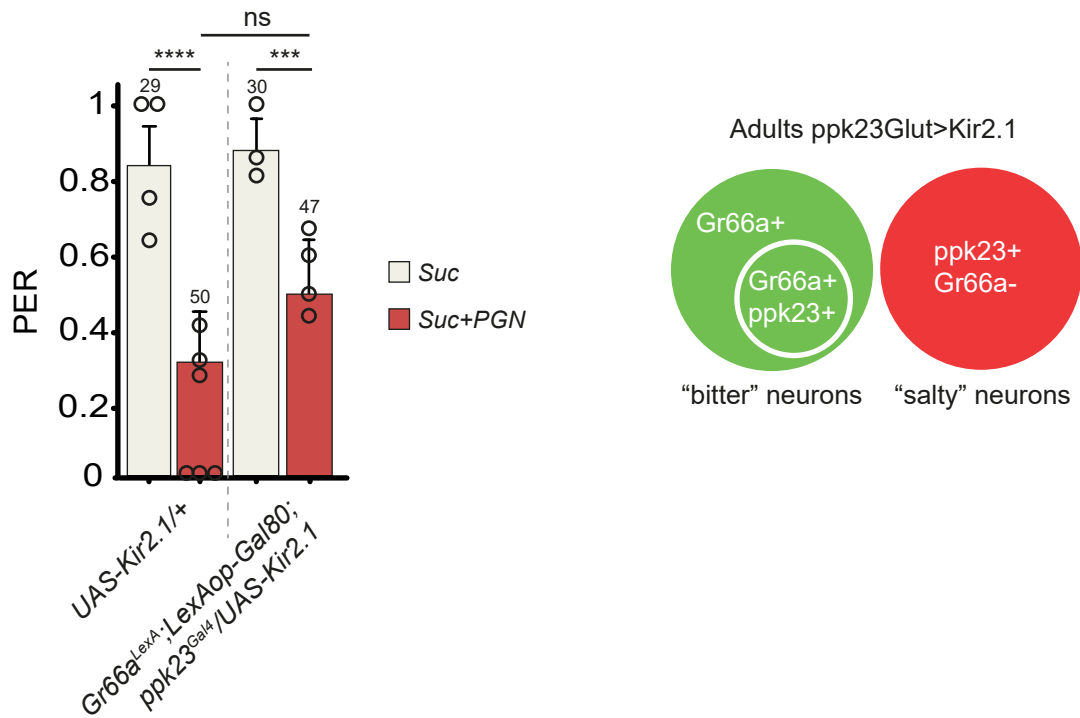
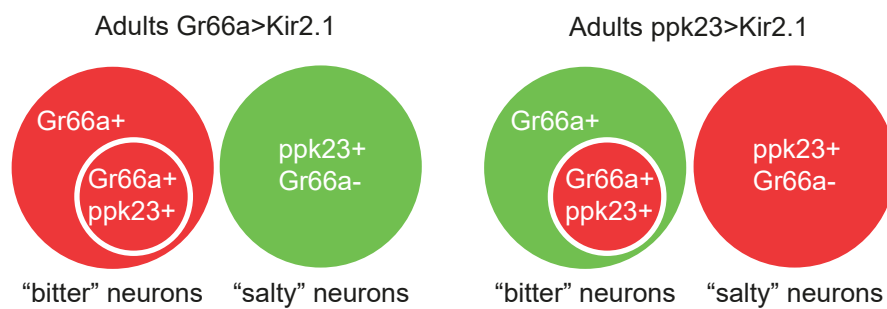


FIGURE S8

A



B



C

