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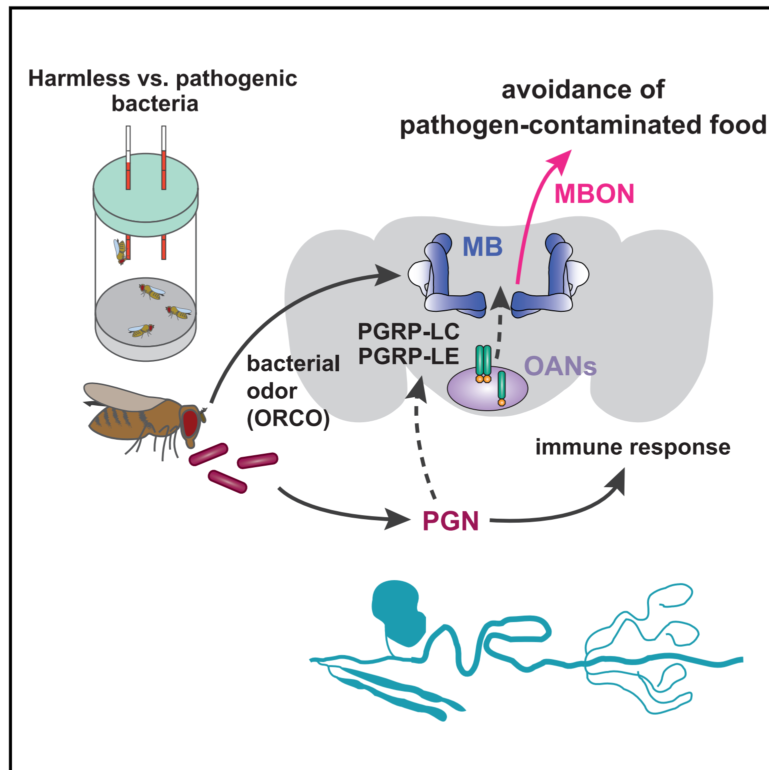
# Immune Receptor Signaling and the Mushroom Body Mediate Post-ingestion Pathogen Avoidance

## Graphical Abstract




## Highlights

- *Drosophila* flies are innately attracted to the odor of pathogenic bacteria
- Pathogen-ingestion induces a lasting avoidance of contaminated food
- This feeding suppression relies on ORCO, the mushroom body, and rutabaga
- Immune receptors PGRP-LC and -LE in octopaminergic neurons mediate this behavior

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## In Brief

Kobler et al. unravel a mechanism underpinning acquired avoidance of spoiled food. Flies innately prefer the odor of pathogenic bacteria and readily feed on them. This initial acceptance turns into a lasting feeding suppression after ingestion, a behavioral adaptation that relies on the mushroom body and immune receptors in octopaminergic neurons.

Kobler et al., 2020, Current Biology *30*, 4693–4709
December 7, 2020 © 2020 The Author(s). Published by Elsevier Inc.
https://doi.org/10.1016/j.cub.2020.09.022

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# Immune Receptor Signaling and the Mushroom Body Mediate Post-ingestion Pathogen Avoidance

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https://doi.org/10.1016/j.cub.2020.09.022

## SUMMARY

In spite of the positive effects of bacteria on health, certain species are harmful, and therefore, animals must weigh nutritional benefits against negative post-ingestion consequences and adapt their behavior accordingly. Here, we use *Drosophila* to unravel how the immune system communicates with the brain, enabling avoidance of harmful foods. Using two different known fly pathogens, mildly pathogenic *Erwinia carotovora* (*Ecc15*) and highly virulent *Pseudomonas entomophila* (*Pe*), we analyzed preference behavior in naive flies and after ingestion of either of these pathogens. Although survival assays confirmed the harmful effect of pathogen ingestion, naive flies preferred the odor of either pathogen to air and also to harmless mutant bacteria, suggesting that flies are not innately repelled by these microbes. By contrast, feeding assays showed that, when given a choice between pathogenic and harmless bacteria, flies—after an initial period of indifference—shifted to a preference for the harmless strain, a behavior that lasted for several hours. Flies lacking synaptic output of the mushroom body (MB), the fly's brain center for associative memory formation, lost the ability to distinguish between pathogenic and harmless bacteria, suggesting this to be an adaptive behavior. Interestingly, this behavior relied on the immune receptors PGRP-LC and -LE and their presence in octopaminergic neurons. We postulate a model wherein pathogen ingestion triggers PGRP signaling in octopaminergic neurons, which in turn relay the information about the harmful food source directly or indirectly to the MB, where an appropriate behavioral output is generated.

## INTRODUCTION

Food and feeding are essential for all animals. Food choices not only influence the energy budget but also the composition of the gut microbiome. However, feeding can be dangerous, through toxicity or bacterial infections that follow the ingestion of a spoiled food source. Some foods are innately avoided through the presence of a strongly aversive sensory cue. For instance, the fly *Drosophila melanogaster*, which feeds preferentially on rotting and fermenting food, recognizes the odor geosmin, which is produced by some highly toxic micro-organisms, as a danger signal and avoids foods containing it [1]. Frequently, however, toxins or pathogens do not present aversive cues recognizable by external sensory systems, and animals will consume them without expecting any harm or negative consequences. Importantly, such negative post-ingestion consequences can help to recognize and avoid food of bad quality and thereby significantly enhance survival chances of the individual and its species.

In many animals, including humans, pathogens that enter the digestive system via food can trigger malaise, infection, and even damage to internal organs, such as the gut [2]. The onset of these negative effects occurs, depending on the digestive system, at minimum several minutes after the food has been consumed, suggesting that animals can adapt their behavior by associating post-ingestion effects with the food's sensory characteristics. For instance, in rodents, the commonly used "conditioned taste aversion" assay relies on the strong and lasting association of the sweet taste of the non-nutritive artificial sweetener saccharin with the later occurring onset of chemically or radiation-induced nausea [3, 4]. The nematode *C. elegans* can associate an odor with the consumption of pathogenic bacteria and will avoid this odor in the future [5, 6]. Recent evidence posits that insects are also capable of adapting their behavior to avoid pathogens or toxins. Using the so-called proboscis extension assay (PER), honeybees can associate the post-ingestion effect of a toxin with an odor and avoid this odor in future trials [7]. Data from *D. melanogaster* larvae show that a food source contaminated with the pathogenic bacterium *Pseudomonas entomophila* (*Pe*) induces lasting avoidance [8]. A similar earlier study in adult flies saw odor-dependent avoidance of *Pe*-containing food upon prior exposure [9]. How ingested pathogens are recognized and how signals to the nervous system lead to a lasting avoidance of pathogen-containing food is not well understood in any organism.

In contrast to behavioral consequences, physiological consequences and defense mechanisms triggered when pathogens

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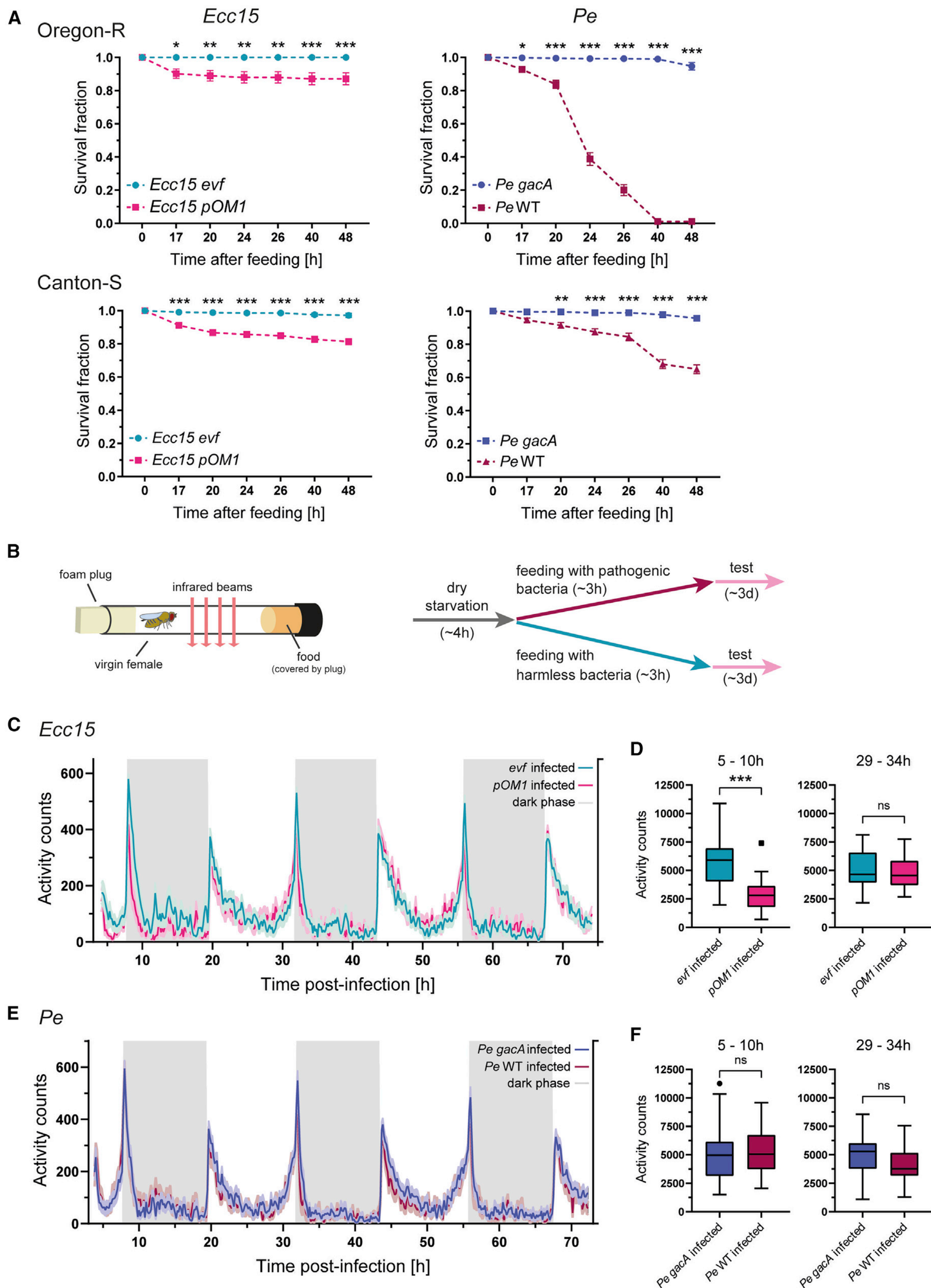


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enter the animal's digestive system are well studied in the fly [10, 11]. Two Gram-negative bacteria in particular and their interaction with the fly immune system have been characterized extensively [11]. The virulent generalist entomopathogen *Pe* and the mildly virulent opportunistic pathogen *Erwinia carotovora carotovora* (*Ecc15*) can infect *Drosophila* through feeding in laboratory conditions. Infections through ingestion of these Gram-negative bacteria trigger—within minutes a local and 1–4 h later a systemic—innate immune response through the activation of the so-called Imd (immune deficiency) pathway [12, 13]. More specifically, components of the bacterial cell wall, such as peptidoglycans (PGNs), are detected by peptidoglycan-recognition proteins (PGRPs) in the gut epithelium or the fatbody, i.e., the transmembrane receptor PGRP-LC and the cytosolic receptor PGRP-LE, which in turn activate the nuclear factor κB (NF-κB) transcription factor Relish, leading to the expression of antimicrobial peptides (AMPs), such as Diptericin and additional molecules [14]. Interestingly, PGRP-LC is also expressed in the nervous system, where it was recently shown to regulate synaptic plasticity, a key feature underpinning learning [15]. Furthermore, the Imd pathway acts in octopaminergic neurons in the brain, where it induces a reduction in egg laying upon septic injury with PGN or *E. coli* [16, 17]. Interestingly, PGRP-LC is not part of this mechanism; instead, the amidase PGRP-LB, which cleaves PGNs, and the intracellular receptor PGRP-LE mediate the process [16]. Whether PGRPs play a role in post-ingestion aversive learning is not known.

Here, we employed the powerful genetic model *Drosophila* with the aim of unraveling genetic and neuronal mechanisms underlying an acquired dislike of spoiled food. We show that flies innately prefer the odor of pathogenic bacteria over harmless mutant versions and readily feed on them. Interestingly, this initial acceptance turns into a lasting feeding suppression several minutes to hours after ingestion, a behavioral adaptation that relies on the mushroom body (MB) and PGRP-LC and -LE in octopaminergic neurons.

## RESULTS

### Locomotor Activity Is Mildly Affected upon Pathogen Ingestion

As mentioned above, two Gram-negative bacterial strains known to infect *Drosophila* are the phytopathogenic bacterium *Ecc15* [12] and the highly virulent soil bacterium *Pe* [13] (STAR Methods). To estimate the negative effect of pathogen ingestion, we first analyzed survival following pathogen feeding. In order to compare bacterial strains that were genetically and in terms of their nutritional value as similar as possible, we chose to always compare the impact of ingesting a pathogenic and a harmless mutant version of both *Ecc15* and *Pe*, respectively. For *Ecc15*, we compared a non-virulent mutant of *Ecc15*, referred to as *Ecc15 evf* from now on, where the *Erwinia virulence factor* (*evf*) was deleted [18]. As a pathogenic counterpart, we chose a strongly virulent form of *Ecc15* with an additional copy of *evf* introduced in form of a plasmid (*pOM1-evf*) [18]. We refer to this strain as *Ecc15 pOM1.* Similarly, we used a harmless avirulent form of *Pe*, *Pe gacA*, to be compared to the highly virulent wild-type (WT) version of *Pe*. The *Pe gacA* mutant strain is deficient for the GacS/GacA system, which controls the production of secondary metabolites and extracellular enzymes essential for its pathogenicity [13, 19].

Indeed, WT flies (OregonR [OrR] or CantonS [CS]) that had fed on *Ecc15 pOM1*, the pathogenic *Ecc15* strain [18], were affected, with about 5%–10% deceased flies at 17 h after ingestion (Figure 1A). As expected, WT flies that were fed the harmless mutant control strain *Ecc15 evf* were not impaired in their survival (Figure 1A). By contrast, the highly pathogenic *Pe* WT strain had killed all WT OrR flies at 40 h after feeding onset, although flies that had been fed the harmless *Pe gacA* mutant strain survived. Compared to the much weaker OrR fly strain, CS WT flies were less severely affected by infection with *Pe* WT, and at 24 h after ingestion, only ∼10% of flies had died (Figure 1A). Although *Ecc15* appeared to kill flies within the first few hours after ingestion, the largest *Pe*-induced death wave occurred later with 35% or 100% deceased flies at 40 h post-ingestion (Figure 1A).

We next sought to define the time window when pathogen ingestion first affects the flies to time all later experiments accordingly. Given recent work suggesting that changes to the microbiome or pathogen infection can influence sleep and motor activity of flies [20, 21], we monitored WT flies in the *Drosophila* Activity Monitor for 3 days following feeding on pathogenic or harmless *Ecc15* and *Pe* (Figure 1B). On average, flies orally infected with pathogenic *Ecc15* and *Pe* exhibited the same regular activity patterns as flies infected with the respective harmless control strains, with activity peaks at light onset and 12 h later at the beginning of the dark phase (Figures 1C, 1E, S1A, and S1B). Importantly, flies that had fed on pathogenic *Ecc15 pOM1* prior to activity monitoring were less active than *Ecc15 evf*-fed control flies during a 5-h time window comprising the first evening activity peak after infection (Figure 1D). This reduced activity lasted for up to 17 h upon infection (Figures 1C and S1C). By contrast, infection with *Pe* WT neither affected locomotor activity shortly after feeding nor 24 h later (Figures 1F and S1C). As infection with *Pe* is much more variable and extends over a longer period (the majority of *Pe*-infected flies died later than 24 h post-ingestion), brief periods of reduced activity could

**Figure 1. Feeding with Pathogenic Bacteria Causes Infection but Does Not Affect Circadian Rhythm**

(A) Survival of WT OregonR and CantonS flies after oral infection with pathogenic (*Ecc15 pOM1*, *Pe* WT) as compared to the corresponding harmless bacterial strains (*Ecc15 evf*, *Pe gacA*). Bacteria suspensions are optical density 600 ($OD_{600}$)»100 + 5% sucrose. *Ecc15*: n = 8 (OrR) and n = 9 (CS); *Pe*: n = 12 (OrR) and n = 12/14 (CS). 1 n corresponds to one bottle with 80 female flies. Error bars denote SEM; p values were calculated via repeated-measures two-way ANOVA followed by Bonferroni's post hoc test for multiple comparisons.

(B) Illustration of the *Drosophila* activity monitor and experimental protocol.

(C and E) Average locomotor activity of WT OrR flies after feeding with *Ecc15 evf* (n = 24) or *Ecc15 pOM1* (n = 17–24) and with *Pe gacA* (n = 23) or *Pe* WT (n = 17–23). Infected flies were excluded from the time of death onward; mean ± SEM.

(D and F) Total activity counts for two 5-h-long periods comprising the first two light-off phases after feeding. n = 24 (*Ecc15* 5–10 h), 24/17 (*Ecc15 evf*/*Ecc15 pOM1* 29–34 h), 23 (*Pe* 5–10 h), and 23/21 (*Pe gacA*/*Pe* WT 29–34 h). p values were calculated via unpaired t test with Welch's correction.

See also Figure S1.

potentially have been distributed over a larger time frame and thus evaded detection. In addition, we compared burstiness and periodicity for the different conditions [22] and found no difference between flies fed with pathogenic *Pe* or *Ecc15* (Figures S1D and S1E), indicating that infection with pathogenic bacteria does not affect circadian rhythm.

Taken together, these data suggested that flies orally infected with *Ecc15* or *Pe* were sufficiently fit to be analyzed in behavioral preference assays. In addition, the survival and activity data indicated that *Ecc15* infection affected flies up to 17 h after initial ingestion, while *Pe*-infected flies showed the largest increase in lethality later than 24 h after ingestion. These data are in line with prior work; Keesey et al. [23] showed that male pheromone production was increased up to ~8 h after septic infection with *Ecc15* but lasted longer than 1 day after septic infection with *Pe*.

## Flies Innately Prefer the Odor of Pathogenic Bacteria over Harmless Controls

Prior evidence suggests that flies detect the bacterial odor geosmin as a warning sign and innately avoid it [1]. To determine whether *Drosophila* was innately averse to the odor of the used pathogenic bacterial strains, we subjected WT OrR flies to different olfactory choices in a 4-field arena assay (Figure 2A). Starved flies preferred the odor of pathogenic *Pe* WT and pathogenic *Ecc15 pOM1*, respectively, over humidified air (Figure 2B) and also over the odor of LB medium (Figure S2A). These data confirmed that hungry flies are highly attracted to putative high-calorie and protein-rich food sources. Surprisingly, nevertheless, given a choice between the odor of pathogenic and that of harmless bacteria, flies preferred pathogenic bacteria over the respective harmless strains for both *Ecc15* and *Pe* (Figure 2C). This distinction required an OR-type receptor, as mutants for the obligate olfactory co-receptor ORCO were not able to distinguish between the odor of pathogenic and that of harmless bacteria (Figures 2C and S2B). Importantly, these results show that *Drosophila* is not innately repelled by the odors of pathogenic *Ecc15* or *Pe* and that it uses olfaction to distinguish between them.

We next asked whether flies can learn to avoid the harmful bacteria based solely on their odor. To test this, we fed OrR flies overnight with harmless or pathogenic bacteria and the next morning (i.e., 16–20 h after feeding onset) tested whether this prior experience would change their preferences toward bacterial odors (Figure 2D). Indeed, prior ingestion of pathogenic *Ecc15* or *Pe* significantly reduced the animals' preference for the odor of these bacteria as compared to the odor of harmless bacteria (Figure 2D). However, feeding with the respective harmless strain led to a similar reduction in preference for the odor of pathogenic over that of harmless bacteria for both *Ecc15* and *Pe* (Figure 2D). The reduced preference of bacteria-fed flies, irrespective of pathogenicity of the fed strain, as compared to naive flies is likely due to different degrees of starvation. Naive flies were wet starved and thus completely derived of nutrients, while bacteria-fed flies were offered the bacteria-sucrose mixture 16–20 h prior to the olfactory choice assay. Thus, although flies are innately attracted to the odor of pathogenic bacteria and even prefer it to that of harmless strains, prior oral infection with pathogenic bacteria, in contrast to a previous report [9], did not induce a specific avoidance of their odor in our hands and in our conditions. Rather, these and the results above highlight that a need of nutrients has a strong influence on choice behavior and that equivalent (i.e., in terms of protein and calorie content) food sources should be used as controls.

## Feeding Induces Lasting Aversion of Food Containing Pathogenic Bacteria

We next tested the hypothesis that flies could adapt their behavior to avoid detrimental food sources when provided with a more complete sensory and contextual experience. For instance, in the classical CTA carried out with rodents, two neighboring drinking bottles with sugar versus water are presented to the animal usually in the same context [3]. To mimic a similar situation for flies, we tested different feeding choices involving harmless and pathogenic bacterial strains in two-choice feeding assays: a slightly modified version of the CAFE (STAR Methods) [24] and the flyPAD [25]. In addition to the direct choice between harmless and harmful bacteria, we also analyzed the flies' preference for bacteria, which represent a protein-rich food source similar to yeast, versus sugar.

In the CAFE assay (Figure 3A), which allows monitoring choice behavior over many hours, we therefore tested three feeding choices: 5% sucrose versus 5% sucrose+harmless bacteria; 5% sucrose versus 5% sucrose+pathogenic bacteria; and 5% sucrose+harmless bacteria versus 5% sucrose+pathogenic bacteria. Importantly, all bacterial solutions were mixed with sucrose to boost feeding. In line with a lack of innate odor aversion and similar to the results obtained with larvae [8], WT flies initially fed from both *Ecc15* strains but then shifted their preference to harmless *Ecc15 evf* and consumed significantly more harmless than pathogenic *Ecc15* after 9 h (Figures 3B, 3C, and S3A). This time frame coincided with the time frame of reduced activity upon ingestion of *Ecc15 pOM1*, indicating that flies are indeed most affected during this time period (see Figure 1). Flies also clearly preferred harmless bacteria+sucrose over sucrose alone and also slightly preferred *Ecc15 pOM1*+sucrose to mere sucrose toward the end of the assay (Figure 3C). With respect to *Pe*, flies more clearly preferred feeding on harmless *Pe gacA*+sucrose to the highly virulent *Pe* WT+sucrose and even consumed significantly less *Pe* WT+sucrose compared to sucrose alone after 5 h (Figures 3B and 3D). This was not the case for *Pe gacA*+sucrose versus sucrose alone (Figure 3D).

Given the higher nutritive value (i.e., sugar plus protein) of the bacteria-sucrose mixture, it seems reasonable that severely starved flies (24 h on water plus 9 h CAFE assay) prefer sucrose containing harmful bacteria to mere sucrose toward the end of the assay, as starvation is even deadlier than feeding on a relatively mild pathogen, such as *Ecc15*. Yet, if given the choice between two food sources of equal caloric value, i.e., pathogenic and harmless bacteria mixed with sucrose, flies always preferred the non-hazardous food source by the end of the assay. These data show that flies can distinguish a pathogen-contaminated from a harmless protein source and prefer it if given the possibility to feed on them.

The results further suggested that flies use taste or post-ingestion signals to avoid pathogen-contaminated food. In order to distinguish between these two possibilities, we used the flyPAD to obtain higher resolution data of the time course of the feeding choice between harmless and pathogen-contaminated food

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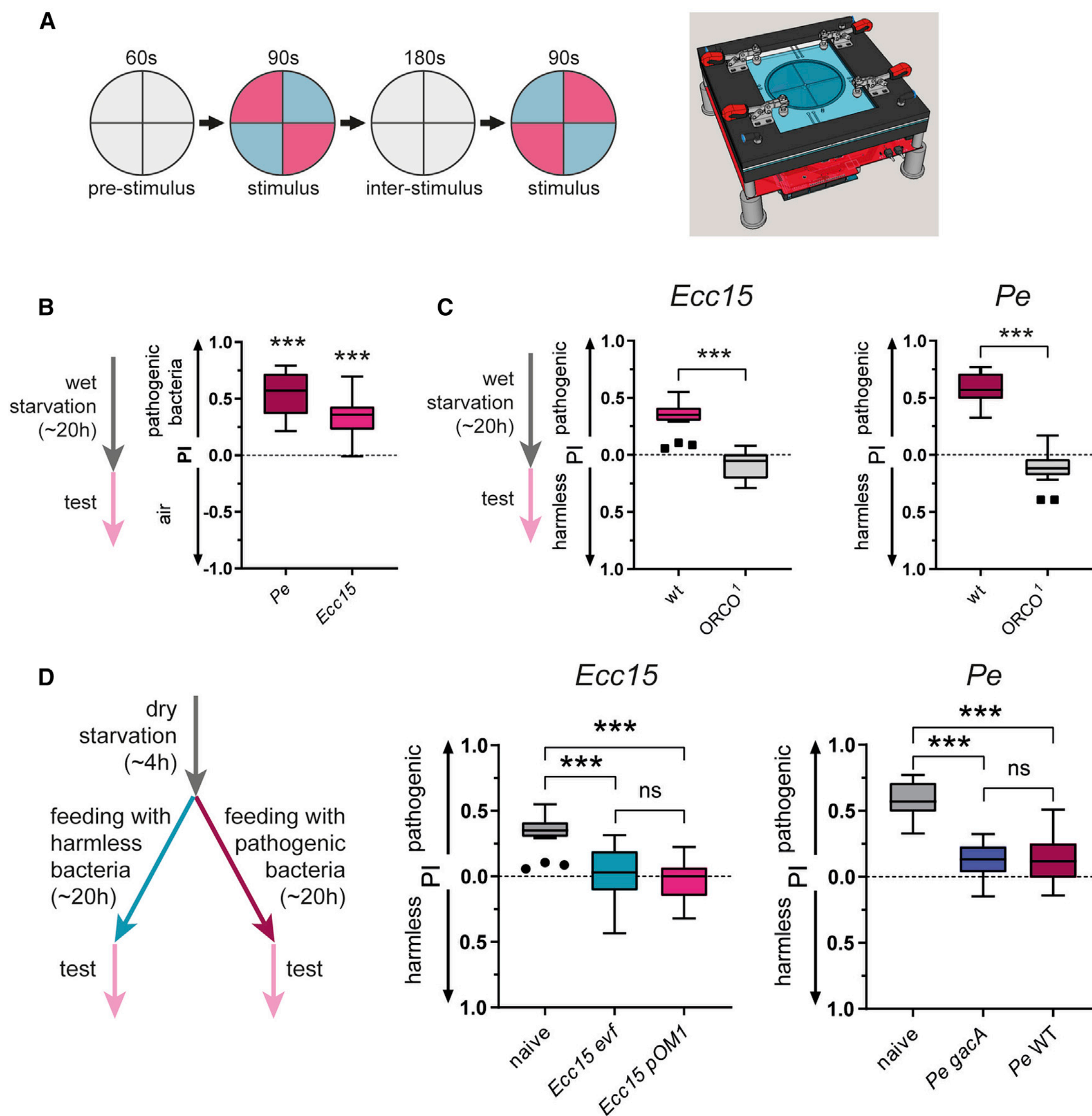


**Figure 2. Flies Innately Prefer the Odor of Pathogenic Bacteria**

(A) Illustration of the 4-field olfactory choice arena and the general experimental protocol for olfactory preference assays.

(B) Preferences of starved, naive OrR flies for the odor of pathogenic *Ecc15* and *Pe* over air only, respectively. n = 16; p values were calculated via one-sample t test comparing to 0.

(C) Preferences of starved, naive OrR, and anosmic ORCO$^{1}$-null mutant flies for the odor of the pathogenic *Ecc15* or *Pe* strain over the corresponding harmless strain (all n = 16). p values were calculated via unpaired t test with Welch's correction.

(D) Prior feeding with pathogenic and harmless *Ecc15* or *Pe* abolishes the olfactory preference for harmless over pathogenic bacteria seen in starved, naive flies. Naive control flies are from innate behavior experiments in (C); *Ecc15*: n = 16 (naive), n = 24 (*evf*), n = 25 (*pOM1*); *Pe*: all n = 16. p values were calculated via one-way ANOVA followed by Tukey's post hoc test for multiple comparisons.

See also Figure S2.

(Figure 3E). We hypothesized that an instantaneous preference for harmless over pathogenic bacteria would suggest a difference in innate (taste) preference for either of the two strains, whereas a delayed shift in preference would indicate the contribution of post-ingestion signals to a developed behavior. Due to technical limitations, the flyPAD assay cannot be run for longer than 60 min. Yet, similar to the CAFE assay, WT flies at the end of the assay had ingested significantly more harmless than pathogenic bacteria (always mixed with sucrose) in the case of both *Ecc15* and *Pe*, as seen from the number of sips taken during a 1-h feeding choice (Figures 3F, 3G, and S3B). Moreover, harmless *Ecc15 evf* and *Pe gacA* were associated with more feeding bursts (Figure 3H) and a higher number of activity bouts (Figure S3C), consistent with a higher preference for harmless strains. Flies also exhibited a higher motivation to feed on harmless as opposed to pathogenic bacterial strains, which is indicated by a higher linear coefficient [25] (Figure 3I). Similar to what was seen in the CAFE assay, flies thus clearly avoided

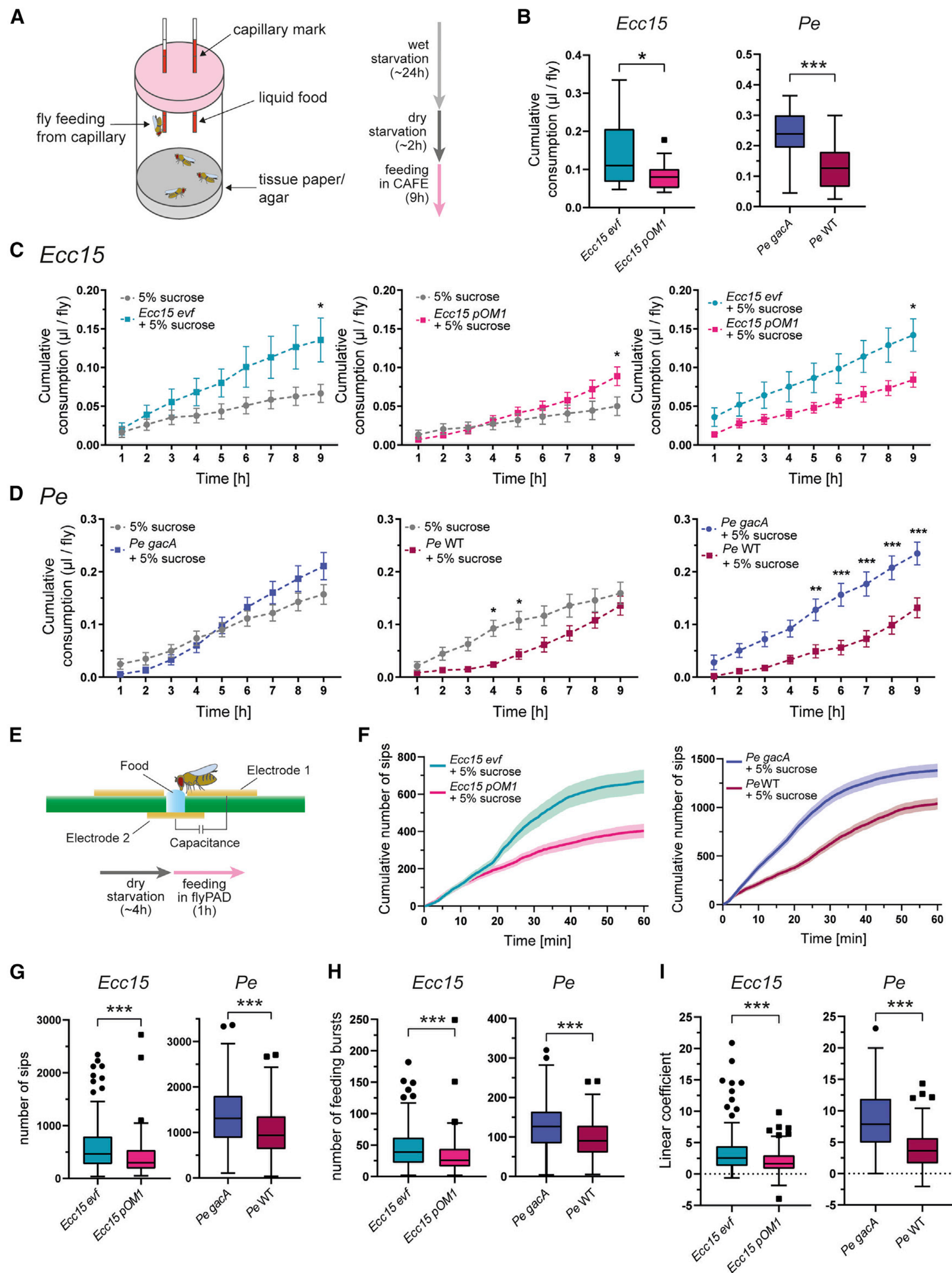


**Figure 3. Feeding Induces Lasting Preference for Harmless over Pathogenic Bacterial Strains**

(A) Illustration of a CAFE chamber and general experimental protocol.

(B) Total consumption in µL/fly of WT CS flies after 9 h in the CAFE for the feeding choice between *Ecc15 evf* and *Ecc15 pOM1* as well as *Pe gacA* and *Pe* WT; see also right panels in (C) and (D).

(C) *Ecc15* feeding in the CAFE. Cumulative consumption of WT CS flies in µL/fly for the feeding choices sucrose versus harmless *Ecc15 evf* (n = 16), sucrose versus pathogenic *Ecc15 pOM1* (n = 16), and *Ecc15 evf* versus *pOM1* (n = 17).

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feeding on pathogenic bacteria as compared to the corresponding harmless strains.

Remarkably, flies were not instantaneously repelled by pathogenic *Ecc15 pOM1* compared to the harmless control (Figure 3F). Instead, they fed equally from both *Ecc15 evf* and *Ecc15 pOM1* for the first 15 min of the feeding choice and shifted their preference to the harmless strain only thereafter (Figure S3D). Moreover, flies preferred pathogenic *Ecc15 pOM1* to the LB growth medium control, indicating that flies are not innately repelled by the taste of the pathogenic strain (Figure S3F). These data further suggested that the observed shift in feeding behavior was due to a difference in post-ingestion effects rather than mere taste. To corroborate the hypothesis that an innate preference would lead to an instantaneous difference in choice, we offered WT flies the choice between a higher and a lower sucrose concentration in the flyPAD. As previously observed [25], flies immediately favored the food substrate containing the higher concentration of sucrose (Figure S3E).

Surprisingly, avoidance in the flyPAD was more immediate in the case of highly pathogenic *Pe* than for *Ecc15*, as flies consumed more harmless *Pe gacA* almost from the start of the assay (Figures 3F and S3B). Thus, as opposed to the innate attraction to the odor of pathogenic *Pe*, flies avoided feeding on them and instead preferred the respective harmless strain, possibly due to an innate taste preference or the very fast onset of a post-ingestion effect.

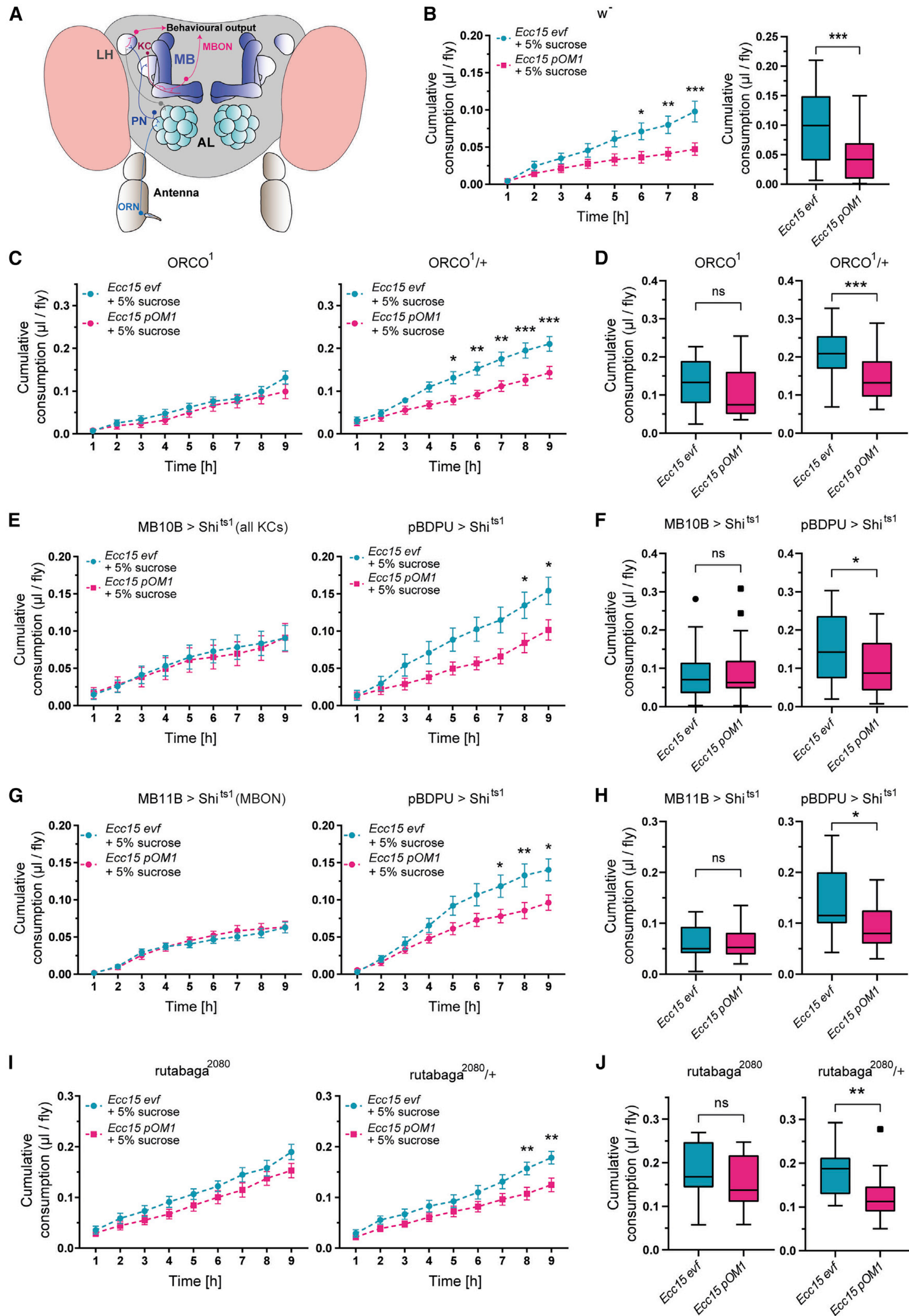
Taken together, although an innate taste aversion might contribute to the feeding behavior observed for *Pe*, the data on *Ecc15* are consistent with an acquired aversion of food contaminated with pathogenic bacteria—possibly due to post-ingestion signals.

### Flies Need ORCO and Their MB to Distinguish Good from Bad Bacteria

Instantaneous avoidance is likely due to a repulsive taste of bacteria in spite of the addition of sucrose. But how do flies adapt their behavior post-ingestion to bacteria that do not taste bad but instead smell good (Figure 2)? To answer this question, we focused on *Ecc15* for all of the following experiments, as aversion of these bacteria only started about 15 min after they were first ingested (Figures 3F and S3D).

Our data in the olfactory arena indicated that flies use olfactory information to distinguish between the pathogenic and harmless *Ecc15* strains (Figure 2). This was also the case in the CAFE assay (Figures 4C and 4D). Although flies heterozygous for an *ORCO* mutant allele (*ORCO*$^{1}$/+) showed the same developed avoidance as other control strains, *ORCO* mutants no longer distinguished between pathogenic and harmless *Ecc15* strains (Figures 4C and 4D). *ORCO* mutants also failed to distinguish bacteria from the sugar-only food source (Figure S4B), suggesting the odor is an essential cue for the flies to distinguish different food sources, including pathogenic from harmless *Ecc15* bacteria. These data, importantly, provide evidence that an olfactory preference is reduced for several hours by the negative post-ingestion effects of pathogenic bacteria. In insects, the MB is essential for associative olfactory learning as well as for the modulation of innate odor-driven behaviors [26, 27]. Following the binding of an odorant to the olfactory receptors on the antennae or maxillary palps, olfactory receptor neurons send their projections to the antennal lobe, where they synapse onto projection neurons that in turn propagate the olfactory information to the lateral horn (LH) and the MB (Figure 4A). We next tested whether the MB is required for the developed dislike of pathogenic bacteria-contaminated food. For that purpose, we blocked all synaptic output from the Kenyon cells (KCs), the principal cell type of the MB, by expressing the temperature-sensitive dynamin mutant allele *shibire*$^{ts1}$ [28]. Flies where MB output was inactivated by a temperature shift (30°C) during the *Ecc15* feeding choice in the CAFE assay did not prefer the harmless *Ecc15 evf* to the pathogenic *Ecc15 pOM1* and instead fed equally on both bacterial strains. Control flies carrying the so-called "empty" control transgene lacking the regulatory fragment to drive expression of the transcription factor Gal4, i.e., *pBDP-GAL4 > UAS-shi*$^{ts1}$, Gal4-only, and *w-* controls [29], exhibited the already observed feeding preference for the harmless strain (Figures 4B, 4E, 4F, S4A, and S4D). KCs provide output to MB output neurons (MBONs) [30]. In line with the requirement of KCs, inhibition of synaptic output of a subset of MBONs (i.e., MBON-β'2mp_bilateral, MBON-β'2mp, and MBON-γ5β'2a; all labeled by the Gal4 line MB11B) [30] also led to equal feeding on pathogenic and harmless *Ecc15* bacteria (Figures 4G, 4H, and S4D). To further corroborate that the distinction between good and bad bacteria indeed constitutes an adaptive, possibly learned, behavior, we also subjected *rutabaga* mutant flies to the *Ecc15* feeding choice in the CAFE assay. Rutabaga is a $Ca^{2+}$/calmodulin-dependent adenylyl cyclase that mediates synaptic plasticity and is necessary for short-term associative memory formation, in particular in the MB [31, 32]. The absence of *rutabaga* abolished the preference for harmless over pathogenic *Ecc15* (Figures 4I, 4J, and S4B). However, the lack of rutabaga, in contrast to lack of ORCO, did not affect the flies' preference for protein-rich food as compared to sucrose alone (Figure S4C), indicating that the animals were still able to distinguish food sources of different nutritional values. Interestingly, though flies

(D) *Pe* feeding in the CAFE. Cumulative consumption of WT OrR flies in μL/fly for the feeding choices sucrose versus harmless *Pe gacA* (n = 16), sucrose versus pathogenic *Pe* WT (n = 18), and *Pe gacA* versus *Pe* WT (n = 17).
(E) Illustration of the flyPAD and experimental protocol.
(F–I) Feeding preferences of WT CS flies in the flyPAD for the choice between harmless and pathogenic *Ecc15* and *Pe* strains, respectively. n = 132 (*Ecc15*); n = 137 (*Pe*).
(F) Cumulative number of sips; mean ± SEM.
(G) Total number of sips after 60 min; p values calculated by comparing feeding ratios to 1 via the Wilcoxon signed rank test.
(H) Number of feeding bursts.
(I) Linear coefficient, i.e., motivation to feed.
(B–D) p values calculated via repeated-measures two-way ANOVA followed by Bonferroni's post hoc test for multiple comparisons. (H and I) p values calculated via the Wilcoxon matched-pairs signed rank test. Extreme outliers in (G)–(I) were removed for plotting but are included in the statistical analysis. See also Figure S3.

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suppress feeding for many hours upon initial pathogen ingestion, they do not appear to form a 24-h long-lasting memory of this negative experience (Figures S3G and S3H).

Taken together, our results indicate that the suppression of feeding from a source containing detrimental bacteria indeed involves synaptic plasticity mechanisms and possibly short-term, but not long-term, associative olfactory memory formation.

## The Immune Receptors PGRP-LC and PGRP-LE Are Required for Suppression of Pathogen Feeding

We next asked how the brain of the fly senses that pathogens or toxins have been ingested. As part of the innate immune response, the peptidoglycan of Gram-negative bacteria is recognized by PGRPs and thereby triggers the activation of the Imd pathway, which culminates in the nuclear translocation of the NF-κB transcription factor Relish and the expression of AMPs and other immune effectors [33] (Figure 5A). To test whether the Imd pathway is involved in the observed feeding suppression of pathogenic Gram-negative bacteria, we tested mutants for different components of this pathway for their feeding behavior toward *Ecc15*. Indeed, flies lacking the transmembrane receptor PGRP-LC completely lost the ability to distinguish between harmless and pathogenic *Ecc15* in the CAFE assay and additionally preferred not only harmless but also pathogenic *Ecc15* to sucrose alone, respectively (Figures 5B and 5C). Similar to *PGRP-LC* mutants, the cytosolic immune receptor PGRP-LE was also required for the pathogen-harmless distinction in the feeding assay, as $PGRP\text{-}LE^{112}$ mutant flies fed equally on both harmless *Ecc15 evf* and pathogenic *Ecc15 pOM1* (Figures 5D and 5E). These data suggest that the Imd pathway is required to convey pathogen ingestion to the nervous system, including the MB. Importantly, *PGRP-LC* and *-LE* mutants were able to distinguish pathogenic from harmless *Ecc15* bacteria based solely on their odor and were still attracted to them (Figure S5D), suggesting that Imd signaling does not interfere with the animals' ability to tell the bacteria apart based on their smell.

We next tested downstream signaling partners of the Imd pathway (Figure 5A) in the same assay. Similar to *PGRP-LC* mutants, flies with a mutation in the NF-κB transcription factor Relish were not able to differentiate between the two strains (Figure S5B). In addition, we tested flies deficient for all immune-inducible AMPs (*ΔAMP*) except for the four cecropins, which are therefore highly susceptible to an infection with *Ecc15* [34]. Absence of most AMPs similarly rendered flies indifferent to the presented feeding choice, as food intake did not differ between harmless *Ecc15 evf* and pathogenic *Ecc15 pOM1* (Figure S5C). These results indicated that Imd signaling and—one or several yet to be identified—AMPs are required for flies to be able to reduce feeding on pathogen-contaminated food.

## PGRP-Mediated Feeding Choice Acts in Octopaminergic Neurons

Flies lacking the transmembrane immune receptors PGRP-LC and -LE did not suppress feeding on pathogenic bacteria. For the innate immune response, these PGRPs appear to act primarily in the fatbody and in cells in the gut [35]. New studies, however, showed that PGRP signaling is required in the nervous system to regulate synaptic plasticity and egg-laying behavior [15, 16]. To narrow down where PGRP-LC and -LE are required for the post-ingestion distinction between good and pathogenic bacteria, we downregulated PGRP-LC in enterocytes of the midgut (mex-Gal4), in the fatbody (Lpp-Gal4), and in the nervous system (nSyb-Gal4) using RNAi-mediated knockdown. Global downregulation of *PGRP-LC* using an actin-Gal4 driver confirmed the behavior seen in *PGRP-LC* mutant flies; they were unable to distinguish between harmless and pathogenic bacteria (Figures S6A and S6B). Nevertheless, these flies retained the ability to differentiate between sucrose containing harmless bacteria and sucrose alone (Figure S6A), again indicating that they were not affected in all of their feeding choices. Absence of PGRP-LC in the adult fatbody also reduced the feeding preference for harmless *Ecc15 evf* over pathogenic *Ecc15 pOM1* (Figure S6C) in line with the role of the fatbody in the innate immune defense [36]. We obtained the same results by knocking down PGRP-LE (Figure S6D). By contrast, mex-Gal4 > PGRP-LC RNAi flies lacking *PGRP-LC* in midgut enterocytes retained WT-like behavior and had consumed significantly more *Ecc15 evf* by the end of the assay (Figure S7C).

Interestingly, downregulation of *PGRP-LC* and *-LE* in the nervous system using the pan-neuronal driver nSyb-Gal4 rendered flies incapable of distinguishing between harmless and pathogenic *Ecc15* (Figure 6), suggesting that these PGRPs are required in the nervous system. Knowing that flies need their MB to distinguish between *Ecc15 evf* and *Ecc15 pOM1* (Figures 4E and 4F), we next asked whether PGRP signaling was required in the MB for adapting their feeding behavior of pathogenic bacteria. Downregulation of PGRP-LC in the MB indeed abolished the preference for harmless *Ecc15 evf* over pathogenic *pOM1* (Figure S7D). However, due to very low overall food intake and

**Figure 4. ORCO, the Mushroom Body, and Rutabaga Are Required for Feeding Aversion to Pathogens**

(A) Schematic representation of the *Drosophila* olfactory pathway. Odorants are sensed by olfactory receptor neurons (ORNs) at the antennae and maxillary palps. ORNs send their projections to the antennal lobe (AL), where they synapse onto projection neurons (PNs). PNs propagate olfactory information to the lateral horn (LH) and the Kenyon cells (KCs) of the mushroom body (MB), which connect to MB output neurons (MBONs) to generate the appropriate behavioral output.

(B) Feeding preferences of control $w^-$ flies for harmless *Ecc15 evf* over pathogenic *Ecc15 pOM1* (n = 22; experiment at 30°C).

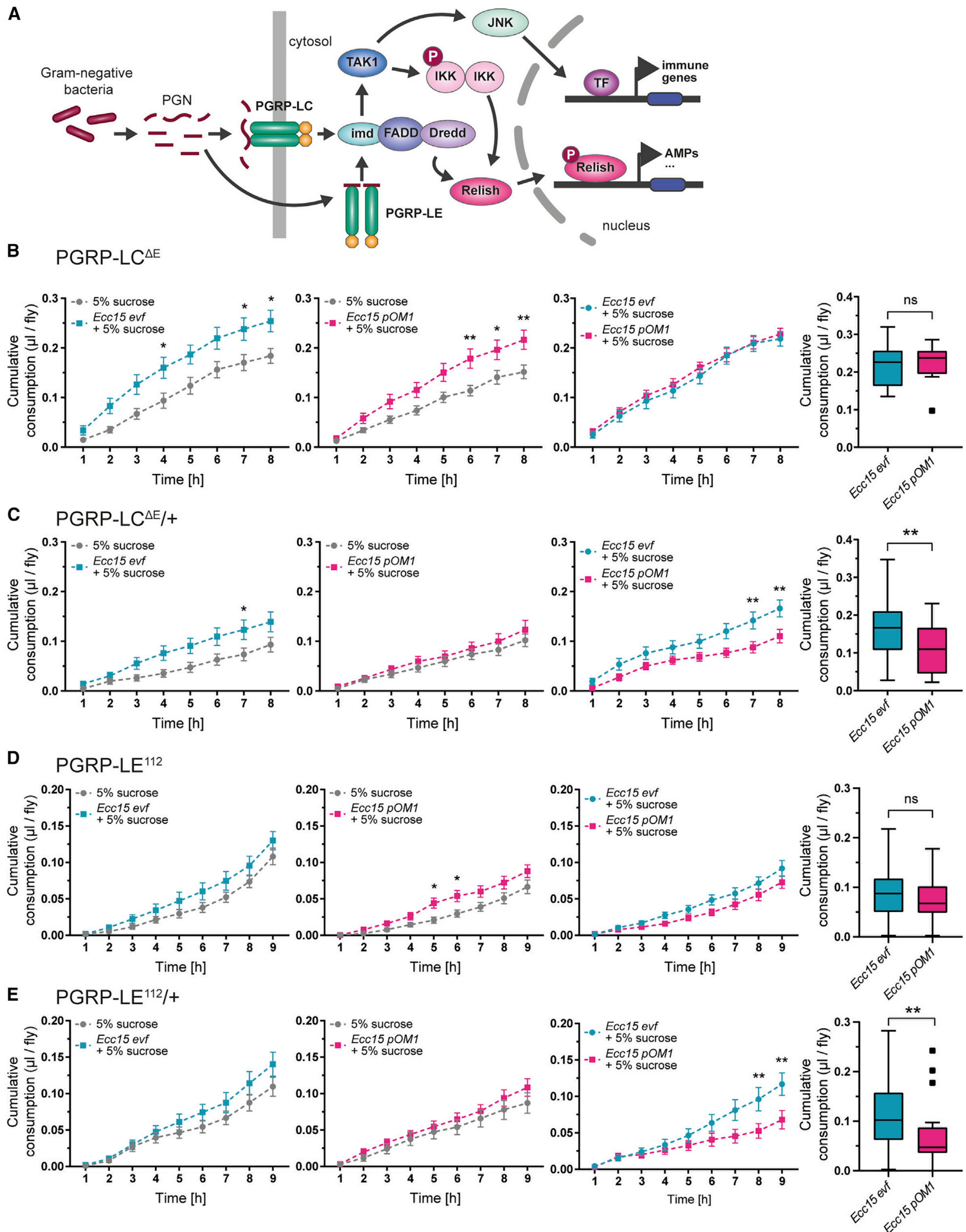
(C and D) Feeding preferences of anosmic $ORCO^1$ and heterozygous $ORCO^1$/+ control flies for the choice between *Ecc15 evf* and *Ecc15 pOM1* (n = 17).

(E and F) Feeding preferences for the choice between harmless *Ecc15 evf* and pathogenic *Ecc15 pOM1* upon inactivation of all MB KCs using MB10B > UAS-$shibire^{ts1}$ flies (n = 19). The control genotype is the empty driver pBDPU > UAS-$shibire^{ts1}$ (n = 24); experiment at 30°C.

(G and H) Feeding preferences for the choice between *Ecc15 evf* and *Ecc15 pOM1* upon inactivation of γ5β'2a, β'2mp, and β'2mp_bilateral MBON cell types using MB11B > UAS-$shibire^{ts1}$ (n = 18). The control genotype is the empty driver pBDPU > UAS-$shibire^{ts1}$ (n = 19); experiment at 30°C.

(I and J) Feeding preferences of flies deficient for the learning gene *rutabaga* and of heterozygous $rutabaga^{2080}$/+ control flies for the choice between harmless *Ecc15 evf* and pathogenic *Ecc15 pOM1* (n = 17).

(B–J) Cumulative consumption in μL/fly in the CAFE and boxplots of total consumption at the end of the experiment (8 or 9 h, respectively). p values calculated via repeated-measures, two-way ANOVA followed by Bonferroni's post hoc test for multiple comparisons. See also Figure S4.

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poor health of these flies, we cannot say with certainty whether PGRP-LC was indeed necessary in the MB.

Given the requirement of KCs and MBONs, we also analyzed flies with inhibited dopaminergic signaling, as dopamine is a major player in associative memory formation [37]. To this end, we simultaneously inhibited the synaptic output of the majority of dopaminergic neurons (DANs) in the fly nervous system (TH-Gal4,58E02-Gal4 > $shi^{ts1}$) and tested their behavior in the CAFE assay (Figure S7A). Surprisingly, these flies behaved just like the controls (Figure S7B), ruling out a strong contribution of dopamine to the acquired behavior.

Another important player in MB-mediated behavioral adaptation is octopamine [38–41]. Moreover, octopaminergic neurons are known to convey post-ingestion and feeding-related information to higher brain circuits. And third, recent important publications showed that octopaminergic neurons (OANs) were involved in regulating microbe-dependent behavior [16, 17, 20]. We thus tested the hypothesis that PGRP signaling could be required in octopaminergic neurons to allow flies to differentiate post-ingestion between pathogenic and harmless bacteria by downregulating PGRP-LC or -LE in octopaminergic neurons using the Tdc2-Gal4 driver. Indeed, flies that lacked PGRP-LC or -LE exclusively in OANs still preferred *Ecc15 evf* and *Ecc15 pOM1* over sucrose, respectively, but fed equally from both harmless and pathogenic bacteria when given a direct choice between the two (Figures 7A–7C). These data are consistent with the interpretation that PGRP-LC and -LE are required in OANs for flies to acquire a post-ingestion dislike of a detrimental food source.

## DISCUSSION

The ability to adapt behavior in order to avoid contaminated or dangerous food is highly conserved across species, regardless of their preferred food source, lifespan, or anatomy [3, 5]. In spite of the prevalence of this type of behavioral adaptation, how animals make the connection between the presence of toxic or pathogenic substances in their digestive system and the neural circuits that guide feeding and food choice remains incompletely understood. Here, we have provided evidence in *Drosophila melanogaster* that pathogen ingestion is recognized by the nervous system through the immune receptors PGRP-LC and -LE in neuromodulatory (i.e., octopaminergic) neurons. Based, in addition, on our data implicating the sense of smell and the insect's learning and memory center, the MB, we propose a model wherein pathogen ingestion is detected by immune receptors in octopaminergic neurons that in turn convey this information to the MB, where it lastingly modulates feeding behavior potentially through mechanisms analogous to short-term olfactory associative learning (Figure 7D). Of note, this mechanism is less important for other feeding decisions. In line with a previous study [42], PGRP-deficient flies were still able to distinguish protein-enriched sucrose from mere sucrose, suggesting a specific requirement in the detection of harmful foods. In fact, flies appear to use the bitter taste receptor Gr66a to distinguish *Ecc15* bacteria diluted in sucrose from a sucrose-only solution [42]. It is worth noting, however, that our feeding substrates contain a ∼3 times higher concentration of sugar, and hence, this higher sugar concentration might strongly suppress the bitter taste of the bacteria in our assay.

At this point, many questions remain unanswered that will be the focus of future studies. Pathogenic bacteria enter the body through ingestion and subsequently interact with the digestive system. Albeit a prominent expression and role of PGRP-LC and -LE in cells inherent to the gut [35], our genetic data primarily implicate PGRP-LC and -LE signaling in neurons and the fatbody (Figures 6 and S6). PGRPs were previously shown to be expressed in the nervous system [15], and recent work indicates that exogenous PGN suppresses calcium levels through PGRP-LE and a specific isoform of the amidase PGRP-LB in octopaminergic neurons to reduce egg-laying behavior upon infection through injury [16, 17]. Moreover, pathogens and bacterial components, such as lipopolysaccharides (LPSs), induce grooming via PGRP-LC, but interestingly, neither PGRP-LB nor intracellular Imd signaling components appear to be involved [43]. Whether, upon bacterial ingestion, PGN or another metabolite or ligand of PGRP-LC and/or -LE travels from the digestive system to the brain to activate PGRP-LC signaling in the nervous system to suppress feeding of harmful food needs further investigation. In mammals, for example, bacterial products, such as PGN, seem to travel to the brain, where they regulate social behavior through direct interaction with specific pattern-recognition receptors, the mammalian homolog of PGRPs, of the innate immune system [44]. In insects, PGN is released into the hemolymph and reaches other organs [12]. Similarly, recent work suggests that tracheal cytotoxin, a monomeric PGN released by Gram-negative bacteria, acts as a long-distance signal for PGRP-LC [33]. Furthermore, PGRP-LC signaling is not only

**Figure 5. PGRPs of the Imd Pathway Are Necessary to Distinguish Pathogenic from Harmless Bacteria**

(A) Schematic representation of the *Drosophila* Imd pathway. Peptidoglycan (PGN) from Gram-negative bacteria is recognized by transmembrane PGRP-LC or cytosolic PGRP-LC and activates the downstream Imd signaling cascade via the Imd/FADD/Dredd complex. Cleavage of the NF-κB transcription factor Relish by Dredd and phosphorylation via the TAK1-activated IKK complex induces nuclear translocation of Relish and transcription of immune effector genes, such as AMPs. Note that TAK1 can also activate JNK signaling.

(B) Feeding preferences of flies deficient for the transmembrane receptor PGRP-LC for the choices sucrose versus harmless *Ecc15 evf*, sucrose versus pathogenic *Ecc15 pOM1*, and *Ecc15 evf* versus *Ecc15 pOM1* (all n = 16).

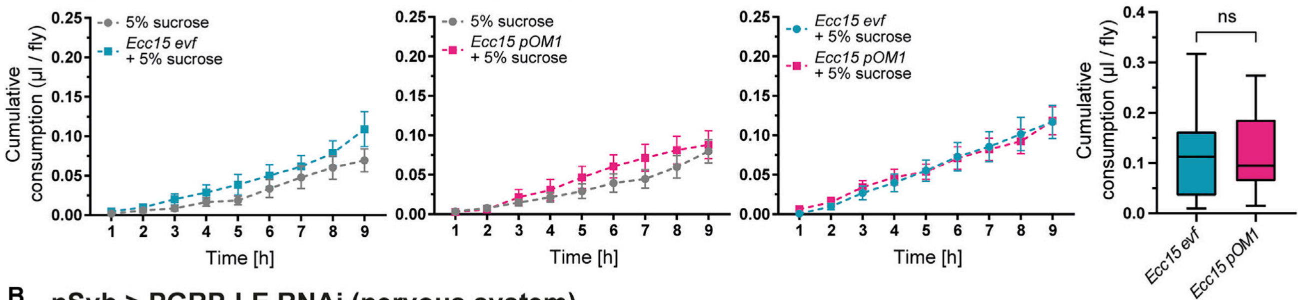
(C) Feeding preferences of heterozygous PGRP-$LC^{\Delta E}$/+ controls for the choices between sucrose and *Ecc15 evf*, sucrose and *Ecc15 pOM1*, as well as *Ecc15 evf* and *Ecc15 pOM1* (all n = 24).

(D) Feeding preferences of flies deficient for the cytosolic receptor PGRP-LE for the choices sucrose versus *Ecc15 evf*, sucrose versus *Ecc15 pOM1*, and *Ecc15 evf* versus *Ecc15 pOM1* (all n = 24).

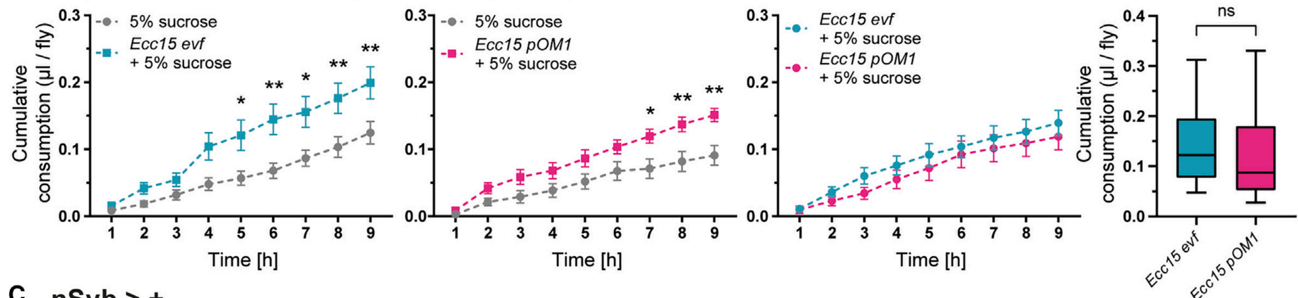
(E) Feeding preferences of heterozygous PGRP-$LE^{112}$/+ controls for the choices between sucrose and *Ecc15 evf* (n = 24), sucrose and *Ecc15 pOM1* (n = 22), and *Ecc15 evf* and *Ecc15 pOM1* (n = 23).

(B–E) Cumulative consumption in μL/fly in the CAFE and boxplots of total consumption at the end of the experiment (8 or 9 h, respectively). p values calculated via repeated-measures, two-way ANOVA followed by Bonferroni's post hoc test for multiple comparisons. See also Figure S5.

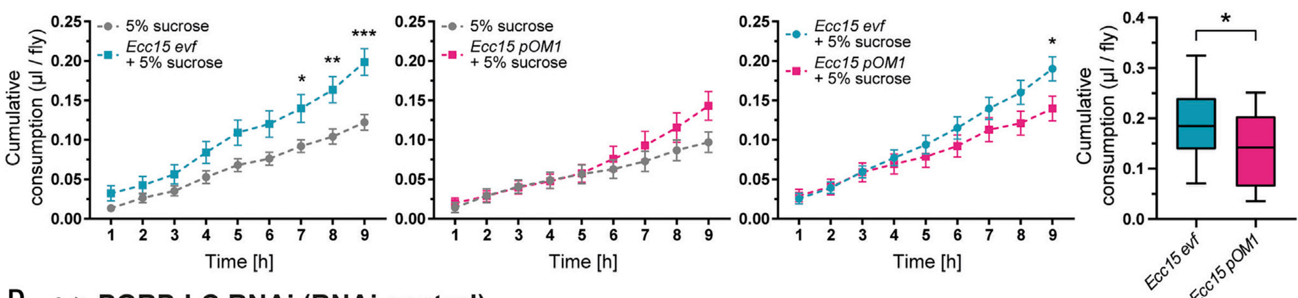
**A** nSyb > PGRP-LC RNAi (nervous system)



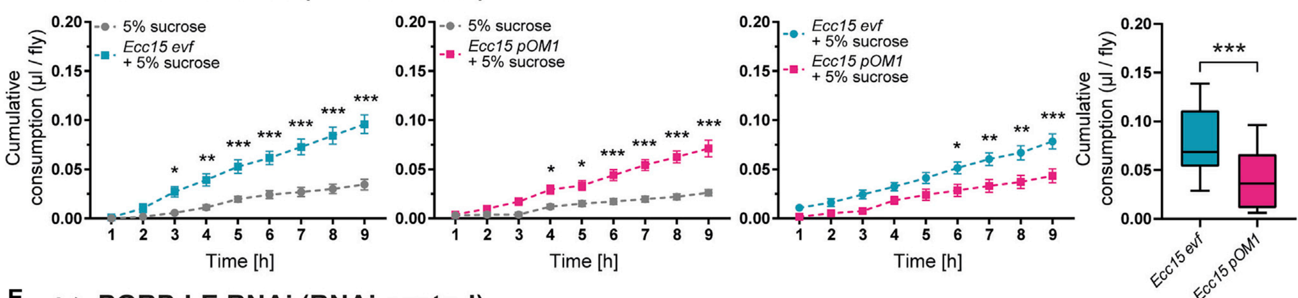

**B** nSyb > PGRP-LE RNAi (nervous system)



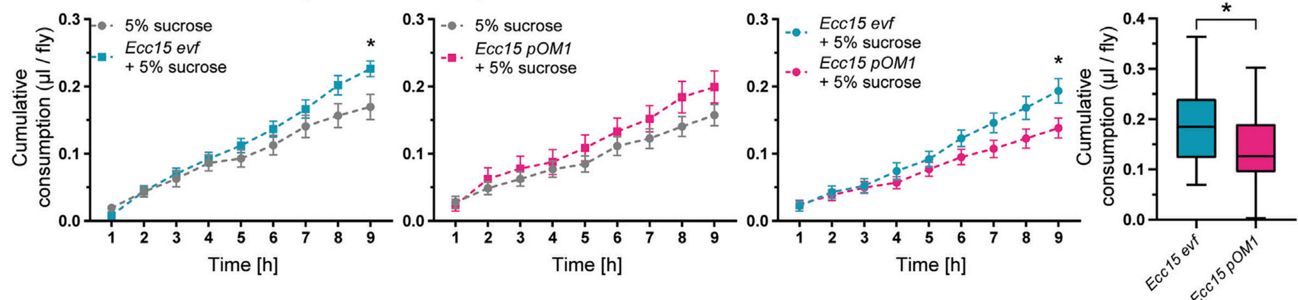

**C** nSyb > +




**D** + > PGRP-LC RNAi (RNAi control)




**E** + > PGRP-LE RNAi (RNAi control)

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induced by PGN, but acetate produced by bacteria in the gut can stimulate PGRP-LC or its expression and thereby induce the expression of the neuropeptide tachykinin (TK) in enteroendocrine cells locally [45]. Tachykinins, or substance P as it is known in vertebrates, are known modulators of gut and brain function impacting strongly on sensory perception and feeding-related behavior [46]. Of note, TK release at the level of the antennal lobe modulates attraction to food odors in *Drosophila* [47, 48]. Moreover, TK signaling regulates insulin-producing cells and boosts male fly aggression and arousal [49, 50]. Hence, it is conceivable that PGRP-LC-induced TK—or other neuropeptides—modulate not only gut function, as suggested by Kamareddine et al. [45], but in addition, PGRP-LC expression in neurons could activate neuropeptide signaling in the brain.

Our data also implicate a role for PGRP signaling in the fatbody in feeding-induced pathogen aversion (Figures S6C and S6D). Although it is known that the fatbody, for instance through the production of AMPs, forms an essential part of the fly's innate immune response [51], we currently do not understand how it contributes to processes governed by the brain. It is conceivable that AMPs produced in the fatbody in response to oral pathogen infection and IMD signaling reach the brain [52, 53]. This could happen in parallel to a possibly AMP-independent signaling of PGRP-LC and -LE in OANs. Given the new tools now available to test the function of individual AMPs [34], future work will hopefully provide an answer to these questions.

An alternative mechanism to diffusion or transport of a secreted factor, such as a bacterial metabolite or neuropeptide, could be a neuronal pathway, such as the vagus nerve, bringing information from organs to the brain [54, 55]. A possible counterpart to the vagus has been described in larval *Drosophila* [56, 57], which was hypothesized to mediate their ability to avoid pathogens (i.e., *Pe*) in food [8]. Furthermore, octopaminergic neurons are found throughout the nervous system of the fly innervating muscles and reproductive and sensory organs [58–60]. A prominent group of octopaminergic neurons is located in the fly's putative brain stem, the so-called subesophageal zone (SEZ), from where they innervate multiple regions of the brain, including the MB [58]. One of these neurons, VPM4, forms direct synaptic connections with a specific output neuron of the MB (i.e., MBON-$\gamma$1pedc > $\alpha\beta$) to presumably convey the presence of food or feeding to the higher brain centers [40, 61]. Although our current data do not support a role of this particular neuron in pathogen avoidance behavior, it is possible that other neurons in this SEZ cluster are involved in projecting or receiving the information that pathogens are being ingested. A neuronal connection as opposed to mere diffusion would be consistent with our flyPAD feeding data showing that pathogen feeding, although not instantaneous, is reduced after about 15 min and lasts for hours (Figure 3). Given that the passage of food into the fly digestive system takes only a few minutes, a 15-min delay between start of feeding and avoidance is congruent with behavioral adaptation post-ingestion. Finally, whether or why transmembrane PGRP-LC and intracellular PGRP-LE are required in the same octopaminergic neurons or different ones is not known. Given the large number of such neurons, it is conceivable that these two receptors work in distinct neuronal subsets or at distinct time points.

In line with the spontaneous feeding on pathogenic bacteria, our olfactory arena data indicate that flies are not innately averse to the pathogenic bacteria used here. To the contrary, naive flies, including flies without PGRP-LC or -LE, appear to be attracted to the odor of the pathogenic forms of *Ecc15* and *Pe* over their harmless counterparts—a behavior that depends on ORCO-mediated olfaction (Figure 2). Although we currently ignore the reasons for this innate attraction, it is tempting to speculate that, in particular, host-dependent bacteria, including pathogens, might have an evolutionary advantage over bacteria innately avoided based on their unpleasant smell.

Importantly, our data also show that flies use their sense of smell to distinguish and suppress feeding on the pathogenic *Ecc15* strain upon ingestion (Figure 4). Though the MB and its role in learning and behavioral adaptation are primarily studied in the context of olfaction [26, 27], the MB receives multisensory information, including post-ingestion signals regarding the value of food [39, 62–70]. These previous data and our data are consistent with the interpretation that post-ingestion signals and chemosensory information (e.g., odor) are combined at the level of the MB to choose and adapt behavior accordingly. Although we currently do not know the actual post-ingestion signals that are received by MB neurons, MB neuron inhibition and *rutabaga* mutant data (Figure 4) implicate a short-term associative learning mechanism [31]. Nevertheless, our results also show that flies do not retain a long-term negative memory of pathogenic *Ecc15* beyond the 17-h period when they are still acutely affected by the infection (Figure S3). Given the relatively mild impact on fly survival, it is perhaps not surprising that the animals do not avoid feeding on this putative food source once the negative consequences disappear or are overcome.

Nevertheless, long-term associative learning induces the expression of specific AMPs—their knockdown in the head fatbody indeed impairs learning and memory [52] consistent with

**Figure 6. Neuronal PGRP-LC and PGRP-LE Mediate Feeding Aversion to Pathogens**

(A) Feeding preferences of nSyb > PGRP-LC$^{RNAi}$ flies that lack PGRP-LC exclusively in neurons when offered a choice between sucrose and harmless *Ecc15 evf* (n = 19), sucrose and pathogenic *Ecc15 pOM1* (n = 19), or *Ecc15 evf* and *Ecc15 pOM1* (n = 20).

(B) Feeding preferences of nSyb > PGRP-LE$^{RNAi}$ flies that lack PGRP-LE exclusively in neurons when offered a choice between sucrose and *Ecc15 evf* (n = 18), sucrose and *Ecc15 pOM1* (n = 17), or *Ecc15 evf* and *Ecc15 pOM1* (n = 17).

(C) Preferences of nSyb > + control flies for the feeding choices sucrose versus *Ecc15 evf* (n = 19), sucrose versus *Ecc15 pOM1* (n = 19), and *Ecc15 evf* versus *Ecc15 pOM1* (n = 21).

(D) Feeding preferences of + > PGRP-LC RNAi control flies for the choices sucrose versus *Ecc15 evf* (n = 17), sucrose versus *Ecc15 pOM1* (n = 19), and *Ecc15 evf* versus *Ecc15 pOM1* (n = 17).

(E) Feeding preferences of + > PGRP-LE RNAi control flies for the choice between sucrose and *Ecc15 evf* (n = 22), sucrose and *Ecc15 pOM1* (n = 21), and *Ecc15 evf* and *Ecc15 pOM1* (n = 22).

Graphs show the cumulative consumption in μL/fly in the CAFE and boxplots of total consumption at the end of the experiment (9 h). p values were calculated via repeated-measures, two-way ANOVA followed by Bonferroni's post hoc test for multiple comparisons. See also Figure S6.

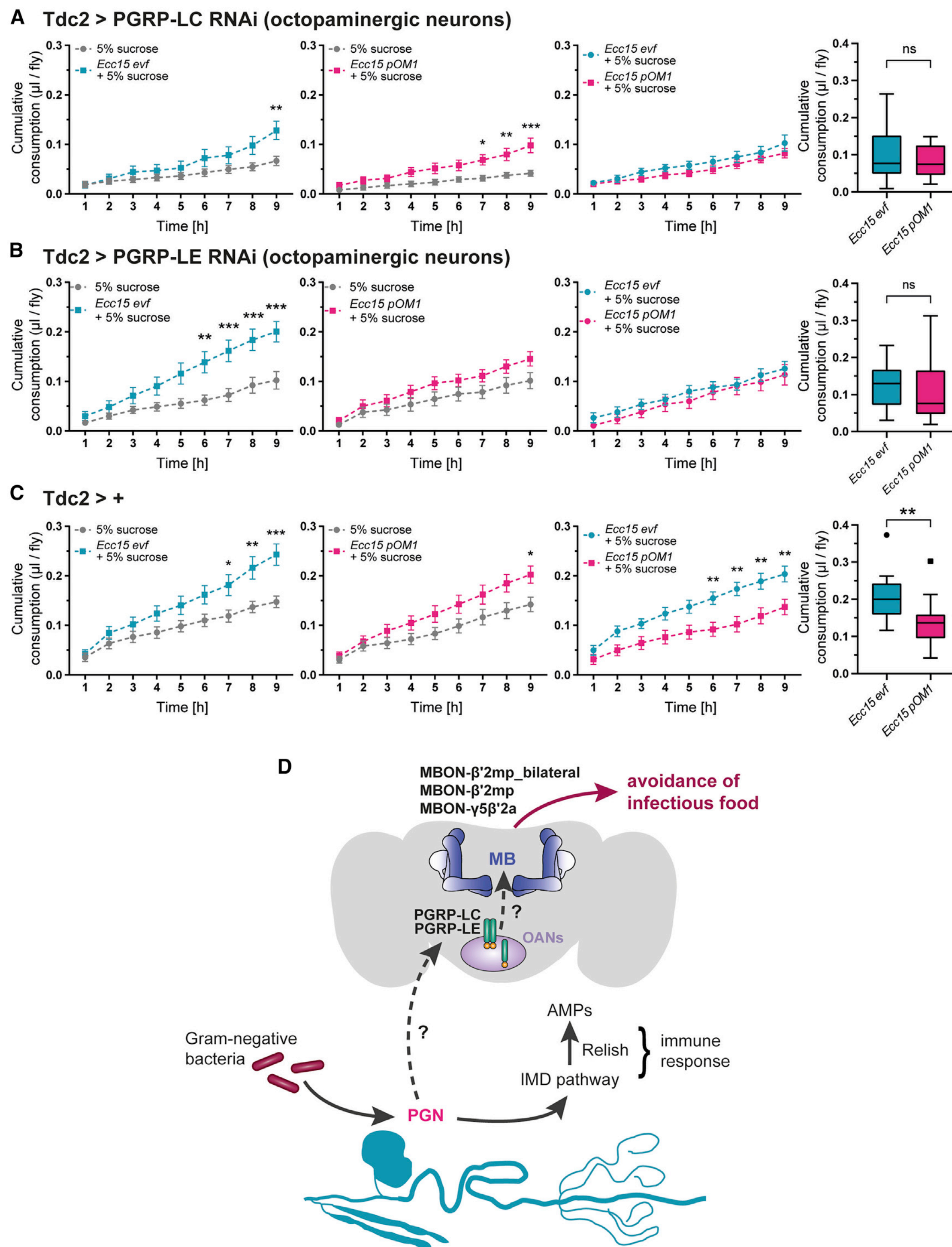


**Figure 7. PGRP-LC and PGRP-LE Are Required in Octopaminergic Neurons to Distinguish Harmless from Pathogenic Bacteria**

(A) Feeding preferences of Tdc2 > PGRP-LC$^{RNAi}$ flies that lack PGRP-LC specifically in octopaminergic neurons for the choices between sucrose and harmless *Ecc15 evf* (n = 19), sucrose and pathogenic *Ecc15 pOM1* (n = 17), and *Ecc15 evf* and *Ecc15 pOM1* (n = 20).

(B) Feeding preferences of Tdc2 > PGRP-LE$^{RNAi}$ flies that lack PGRP-LE specifically in octopaminergic neurons for the choices between sucrose and harmless *Ecc15 evf*, sucrose and pathogenic *Ecc15 pOM1*, and *Ecc15 evf* and *Ecc15 pOM1* (all n = 18).

(C) Feeding preferences of Tdc2 > + control flies for the choices sucrose versus *Ecc15 evf* (n = 17), sucrose versus *Ecc15 pOM1* (n = 17), and *Ecc15 evf* versus *Ecc15 pOM1* (n = 16).

(A–C) Cumulative consumption in µL/fly in the CAFE and boxplots of total consumption at the end of the experiment (9 h). p values were calculated via repeated-measures, two-way ANOVA followed by Bonferroni's post hoc test for multiple comparisons.

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our data suggesting that AMPs are involved in pathogen avoidance behavior (Figure S5). Of note, the MB also regulates circadian behavior and activity in flies [71]. And hence, it is possible that the effect of *Ecc15 pOM1* ingestion on activity (Figure 1) also involves the MB. Equally noteworthy, the time window during which *Ecc15* ingestion reduces activity and pathogen feeding correlates with the increase of the Imd-dependent pheromone production upon *Ecc15* infection [23]. Changes in behavior during this time window might thus be critical to help flies fight infection and increase chances of survival. Finally, in line with our data suggesting a function in behavioral plasticity, PGRP-LC signaling was found recently to regulate synaptic plasticity at the neuromuscular junction of *Drosophila* [15, 72].

Taken together, we suggest that the use of immune receptors by neurons to detect signals coming from pathogenic infection or the microbiome might present a conserved solution of post-ingestion gut-brain signaling. The tools for genetic and neural circuit dissection available in the fly should help to better understand the role of immune receptors in the nervous system and to gain fundamental mechanistic insights in the interaction between gut, immune system, and brain with wider implications also for higher animals, including humans.

## STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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  - Flies
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- METHOD DETAILS
  - Natural bacterial infection
  - Survival analysis
  - *Drosophila* Activity Monitor
  - Olfactory choice assay
  - Capillary feeding assays
  - flyPAD
- QUANTIFICATION AND STATISTICAL ANALYSIS

## SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.cub.2020.09.022.

## ACKNOWLEDGMENTS

We thank Heidi Miller-Mommerskamp for excellent technical support throughout this project and Sophie Aimon for helping characterize the activity monitor data with burstiness and rhythmicity. We are grateful to Marc Eppler for initial experiments for this project. We are indebted to Francois Leulier, Bruno Lemaitre, Julien Royet, Luis Teixeira, Mark Hanson, Irene Miguel-Aliaga, and Michael Pankratz for providing fly stocks, bacteria, and advice. We thank Kausik Si, François Leulier, and Nicolas Gompel for important feedback on the manuscript and helpful discussions. We also thank members of the Grunwald Kadow lab, in particular Sophie Aimon, Jean-François De Backer, Paul Bandow, Francisco Rodriguez Jimenez, and Sydney Hunt, for providing comments on the manuscript. We further thank Harald Luksch, Bertram Gerber, and Michael Schemann for support and important suggestions during the course of the project. The work was supported by the German Research Foundation (DFG) (FOR2705) and an ERC starting grant (FlyContext; H2020) to I.C.G.K.

## AUTHOR CONTRIBUTIONS

I.C.G.K. and J.M.K. conceived the project and planned the experiments. J.M.K. carried out all experiments with the exception of some flyPAD experiments, which were performed by I.P., and some olfactory choice experiments, which were conducted by F.J.R.J.

## DECLARATION OF INTERESTS

The authors declare no competing interests.

Received: March 27, 2020
Revised: July 31, 2020
Accepted: September 7, 2020
Published: October 1, 2020

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(D) Model for PGRP-LC- and PGRP-LE-mediated acquired feeding aversion. Gram-negative bacteria that are taken up with food induce the activation of the immune response and trigger PGRP-LC and PGRP-LE signaling in octopaminergic neurons (OANs), for example, via the detection of bacterial PGN. OANs could convey this information to the MB, where the appropriate behavioral output, i.e., the lasting avoidance of infectious food, is generated.
See also Figure S7.

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# STAR★METHODS

## KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---|---|---|
| Deposited Data | | |
| All data | Mendeley Data | http://dx.doi.org/10.17632/bdw29yxwxp.1 |
| Experimental Models: Organisms/Strains | | |
| Canton-S | Bloomington DSC | FBst0064349 |
| Oregon-R | Gift from Nicolas Gompel | FBsn0000276 |
| $w^{1118}$ | Bloomington DSC | FBst0003605 |
| UAS-shibire$^{ts1}$ | Bloomington DSC | FBst0044222 |
| ORCO$^{1}$ | Bloomington DSC | FBst0023129 |
| Dredd$^{B118}$ | Bloomington DSC | FBst0055712 |
| PGRP-LC$^{\Delta E}$ | Bloomington DSC | FBst0055713 |
| PGRP-LE$^{112}$ | Bloomington DSC | FBst0033055 |
| Rel$^{E20}$ (isogenized) DrosDel | Gift from Mark Hanson | N/A |
| $w^{1118}$ (isogenized) DrosDel | Gift from Mark Hanson | N/A |
| ΔAMPs | Gift from Mark Hanson | N/A |
| PGRP-LC RNAi | Bloomington DSC | FBst0033383 |
| PGRP-LE RNAi | Bloomington DSC | FBst0060038 |
| actin-GAL4 | Bloomington DSC | FBst0004414 |
| Lpp-GAL4 | Gift from Irene Miguel-Aliaga | N/A |
| mex-GAL4 | Gift from François Leulier | N/A |
| nSyb-GAL4 | Bloomington DSC | FBst0051635 |
| Tdc2-GAL4 | Bloomington DSC | FBst0009313 |
| pBDP-GAL4U | Bloomington DSC | FBst0068384 |
| MB10B-GAL4 | Janelia Farm Research Campus | FBst0068293 |
| MB11B-GAL4 | Janelia Farm Research Campus | FBst0068294 |
| rutabaga$^{2080}$ | Bloomington DSC | FBst0009405 |
| TH58E02-GAL4 | Gift from Siju Kunhi Purayil | N/A |

## RESOURCE AVAILABILITY

### Lead Contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Ilona C. Grunwald Kadow (ilona.grunwald@tum.de).

### Materials Availability

This study did not generate new unique reagents.

### Data and Code Availability

All datasets generated for the current study are available at Mendeley Data: http://dx.doi.org/10.17632/bdw29yxwxp.1.

## EXPERIMENTAL MODEL AND SUBJECT DETAILS

### Flies

*Drosophila melanogaster* stocks were raised on standard cornmeal medium (per 100 L: 1170 g agar, 10kg corn flour, 1kg soya flour, 1850 g brewers' yeast, 4kg diamalt, 4kg sugar beet syrup, 250 g methyl paraben and 1L 10% phosphoric acid) at 25°C in a 60% humidified incubator and a 12h/12h light-dark cycle. Experimental flies were collected directly after hatching, sorted on ice and aged 3-7 days before experiments. All experiments were conducted with mated female flies.

### Bacteria

The bacteria used are the *Erwinia carotovora carotovora 15* (*Ecc15*) strains *Ecc15 pOM1-evf* (abbreviated as *Ecc15 pOM1*, pathogenic, spectinomycin-resistant) and *Ecc15 evf* (avirulent, rifampicin-resistant) (kindly provided by François Leulier) and the *Pseudomonas entomophila* strains *Pe* WT (pathogenic) and *Pe gacA* (avirulent) (both rifampicin-resistant, kindly provided by Bruno Lemaitre). *Ecc15* are phytopathogenic Gram-negative enterobacteria which can cause a local and systemic immune response in *Drosophila* [12, 73]. The *Erwinia* virulence factor (*evf*) is responsible for the infectious properties of *Ecc15*, as it increases persistence of the bacteria in the *Drosophila* gut, and the avirulent *evf*-deficient strain *Ecc15 evf* is rapidly cleared from the gut [18]. While feeding on wild-type *Ecc15* is typically not life-threatening for adult *Drosophila* (e.g., [74, 75]), overexpression of *evf* in the *Ecc15 pOM1-evf* strain increases persistence in the gut and thereby lethality after feeding [18, 76, 77]. By contrast, *Pe* are highly virulent entomopathogenic bacteria due to their capacity to counteract the immune response, destroy the gut epithelium through pore-forming toxins and the consequential overshooting stress response in the host [13, 19, 74, 78–80]. The *Pe gacA* mutant strain is deficient for the GacS/GacA system, which regulates a wide range of processes necessary for *Pe* virulence, and is thus completely avirulent [13, 19].

*Ecc15* were grown on standard lysogeny broth (LB) agar plates with 100μg/ml antibiotics at 29°C for 24h. For the preparation of a concentrated bacterial pellet, the *Ecc15* strains were cultured overnight at 29°C and shaking at 220rpm in LB medium containing 100μg/ml of the respective antibiotic. The optical density at 600nm was measured and bacterial cultures were spun down at 3500rpm and 4°C for 20min, washed in PBS and again pelleted at 4000rpm and 4°C for 20min. Bacterial pellets were resuspended in PBS, adjusted to an $OD_{600}$ of ~ 200. *Pe* were grown on LB agar plates with 100μg/ml antibiotic and 1% skim milk at 30°C for 24-30h. *Pe* cultures were prepared by inoculating a pre-culture containing 100μg/ml antibiotic with a protease-positive *Pe* WT (i.e., clear colony) or a *Pe gacA* clone, followed by incubation at 30°C and 250rpm for 8-12h. The pre-culture was diluted 1:16 in LB to prepare an overnight culture (30°C, 250rpm, ~16h). The optical density was measured at 600nm and bacterial cultures were pelleted (2500 g, 4°C, 15min), resuspended in the remaining medium and adjusted to the desired $OD_{600}$ »200 with PBS. All concentrated bacteria suspensions were stored at 4°C for a maximum of 24h until usage.

## METHOD DETAILS

### Natural bacterial infection

For the bacterial infection of flies via pathogen feeding, flies were dry starved, i.e., incubated in empty vials for 3-5h at 25°C to increase the motivation to feed. Flies were placed into standard fly bottles with 1.5% agarose to provide humidity and a filter paper soaked with bacteria-sucrose solution. This solution was prepared by mixing the concentrated bacteria ($OD_{600}$~200) in equal parts with a 10% sucrose solution to obtain a final concentration of 5% sucrose (146 mM) and an $OD_{600}$ »100. Flies were left to feed on the bacteria-sucrose solution for a minimum of 3h at 25°C and 60% humidity.

### Survival analysis

Groups of 80 female flies (wild-type, OrR and CS) were fed either the pathogenic bacterial strains (*Ecc15 pOM1*, *Pe* WT) or the respective harmless strains (*Ecc15 evf*, *Pe gacA*), incubated on the bacteria-sucrose solutions for 24h and then transferred back onto standard food. Dead flies were counted at specified time points post infection.

### *Drosophila* Activity Monitor

The locomotor activity and circadian rhythm of infected wild-type OrR flies was monitored via the MB5 Multibeam Activity Monitor (TriKinetics, Waltham, MA USA), which consists of 16 separate tubes bisected each by 17 infrared beams. Interruptions of the infrared beams by flies walking along the tubes are recorded as activity counts. Activity monitor experiments are conducted at 25°C in a 60% humidified incubator and a 12h/12h light-dark cycle. To avoid distortions of the recorded activity counts by larvae, virgin female flies had to be used. Flies were dry starved for ~4h and allowed to feed on either pathogenic (*Ecc15 pOM1*, *Pe* WT) or harmless bacteria (*Ecc15 evf*, *Pe gacA*) for 3h before being transferred into activity monitoring tubes, where they were monitored for approximately 3 days. The raw data was processed using *DAM File Scan* (TriKinetics). Burstiness was defined as:

$$B = \frac{\sigma_\tau - m_\tau}{\sigma_\tau + m_\tau},$$

where the interevent time $\tau$ is the time between two successive events [22], and $\sigma_\tau$ and $m_\tau$ are the standard deviation and mean of its distribution. We characterized the periodicity of the circadian rhythm as the maximum of the activity autocorrelation. Of note, we considered only flies living more than 24h to avoid artifacts due to dead animals. We found that flies could be divided into three groups: flies with an autocorrelation peak at 24h, flies with a peak at 12h, and flies with an autocorrelation peak inferior to 12h that suggested no periodicity. We used a chi-square test of independence to determine if the repartition of flies in the 24h or the 12h-and less group was affected by infection with the specific bacterial strain and found p = 0.8676.

### Olfactory choice assay

The 4-field olfactory choice arena is a custom-made behavioral assay to monitor the preference behavior of freely moving adult *Drosophila* upon olfactory and/or optogenetic stimulation and has been described previously [81]. For this project, only the olfactory stimulation was used. In short, an air/odor delivery system relying on passive suction by a rotary vane pump (G12/01-4 EB, Gardner

Denver Thomas GmbH) is connected to the center of the arena. At a flow rate of ~200ml/min, the pump creates negative pressure and sucks in air from the four odor input connectors located at each quadrant, which are in turn connected to solenoid valves (MFH-3-MF, Festo) via PTFE tubing. The valves allow rapid switching between odor (bacterial supernatant after pelleting of overnight cultures) and air channels (filtered tap water). Background illumination was provided by infrared LEDs and behavior was recorded with an infrared camera (Flea3 USB3 1.3MP Mono, FLIR Systems). Data acquisition and analysis as well as hardware control were realized via Arduino Mega and a custom-made MATLAB script. Behavioral attraction or aversion was determined by calculating a preference index ((number of flies in stimulus quadrants – number of flies in non-stimulus quadrants) / total number of flies). The general protocol for olfactory choice experiments consisted of 60 s acclimatization, 90 s stimulus phase in two opposing quadrants, 180 s inter-stimulus phase and another 90 s for the second stimulus phase with odor being presented in the remaining two quadrants. To determine olfactory preference behavior toward bacterial odors, flies were starved with only water for ~20 h before the olfactory choice assay to increase the motivation to respond to odors.

### Capillary feeding assays

Food intake during bacterial feeding choices was monitored using a slightly modified version of the CAFE assay [24]. Standard fly vials where humidity was provided by tissue paper soaked in filtered water served as CAFE chambers. Two disposable graduated glass micro-capillaries (#022.7142, CAMAG, Muttenz, Switzerland) per chamber were poked through soft foam plugs and then filled with 5μl of liquid food. To facilitate visualizing the descent of the meniscus during experiments, bacteria-sucrose and pure sucrose solutions were supplemented with standard red food dye (0.5% Allura Red AC, #458848, Sigma-Aldrich, St. Louis, MO USA). Prior to feeding in the CAFE, flies were wet starved (~24h) and shortly dry starved (~1-3h). Feeding choices tested in the CAFE were 5% sucrose versus harmless bacteria (*Ecc15 evf* or *Pe gacA*), 5% sucrose versus pathogenic bacteria (*Ecc15 pOM1* or *Pe* WT) or harmless versus pathogenic bacteria (*Ecc15 evf* versus *pOM1* or *Pe gacA* versus WT). All bacteria preparations were $OD_{600} \sim 100$ + 5% sucrose. For each feeding choice, a CAFE chamber without flies served as an evaporation control. Flies were tested in groups of 10 per vial in a standard incubator (25°C, 60% humidity), and liquid decrease in the capillaries was measured on an hourly basis for 8-10 hours. Experiments using shibire$^{ts1}$ were conducted at 30°C. The cumulative consumption per fly was calculated by subtracting the liquid decrease in the evaporation controls from the decrease in experimental CAFE chambers and dividing this value by the total number of flies. Cases where the cumulative consumption per fly for both capillaries was lower than 0.03μl after 9h as well as cases where flies did not eat at all were excluded from further analysis. CAFE assays were conducted on a minimum of two experimental days using different batches of flies and bacteria.

### flyPAD

The flyPAD is an automated behavioral assay, which uses capacitive measurements to determine the physical interaction of single flies with food [25]. In short, the flyPAD assay records changes in capacitance between an electrode containing a gelled food substrate and an electrode on which the fly stands during feeding. Periods of activity as well as sips generate characteristic patterns in the capacitance signal and can hence be extracted via an algorithm to detect activity and feeding. The food choices tested in the flyPAD were 1% sucrose versus 10% sucrose, *Ecc15 pOM1* + 5% sucrose versus LB medium + 5% sucrose, *Ecc15 evf* + 5% sucrose versus *Ecc15 pOM1* +5% sucrose and *Pe gacA* +5% sucrose versus *Pe* WT + 5% sucrose. All food substrates additionally contained 1% agarose (low gelling temperature, A9414, Sigma-Aldrich). For one experiment, each electrode was filled with 5μl of the respective food substrate, individual flies were then aspirated into the arenas and feeding was recorded for 60min. All flyPAD experiments were conducted at 25°C in a 60% humidified climate chamber. For feeding choice experiments following overnight bacterial infection, flies were dry starved for ~5h, followed by natural, oral bacterial infection with either pathogenic *Ecc15 pOM1* or harmless *Ecc15 evf*. The next morning, both groups were tested for their feeding preference in an *Ecc15* versus *Ecc15 pOM1* choice. All flyPAD data were acquired using the open-source software Bonsai [82] via a script provided by Pavel Itskov. The post-analysis was conducted using a custom-made MATLAB script again provided by Pavel Itskov.

## QUANTIFICATION AND STATISTICAL ANALYSIS

All statistical analyses were conducted via the GraphPad Prism8 software. Datasets were tested for normality using the Anderson-Darling and the D'Agostino & Pearson test.

The burstiness parameters from DAM experiments as well as the PIs from olfactory choice assays were compared via the unpaired t test with Welch's correction for unequal variances (two groups) or the one-way ANOVA followed by Tukey's multiple comparisons test (three or more groups). CAFE assay data were analyzed with a repeated-measures two-way ANOVA followed by the Bonferroni's post hoc test for multiple comparisons. The one sample t test or the Wilcoxon signed-rank test were employed to compare datasets to a hypothetical value (0 for PIs or 1 for ratios). For the feeding choices in the flyPAD, the ratio between the number of sips of the two feeding substrates was calculated for specific time points. The other flyPAD parameters were analyzed via the Wilcoxon matched-pairs signed rank test.

The significance threshold (α) was set to 0.05 according to standard statistical conventions; the statistical notations were as follows: 'ns' $p > 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Error bars in the graphs represent the standard error of the mean (SEM). For boxplots, the whiskers are drawn according to Tukey, i.e., correspond to 1.5 x IQR (interquartile range). All values outside of this range are displayed as outliers.

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## Supplemental Information

# Immune Receptor Signaling and the Mushroom Body Mediate Post-ingestion Pathogen Avoidance

**Johanna M. Kobler, Francisco J. Rodriguez Jimenez, Irina Petcu, and Ilona C. Grunwald Kadow**

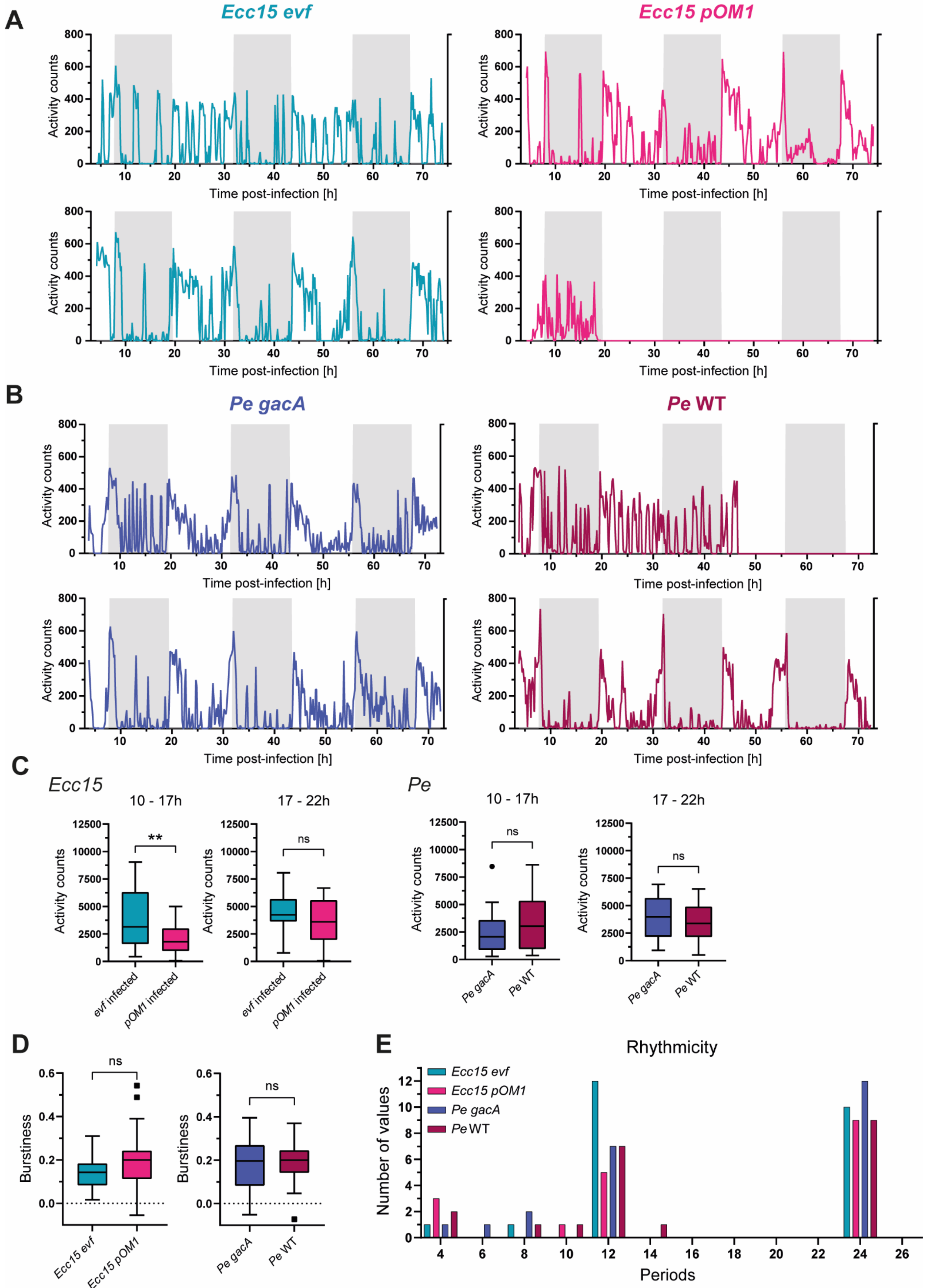

A
Ecc15 evf
Ecc15 pOM1
Activity counts
Time post-infection [h]
B
Pe gacA
Pe WT
C
Ecc15
Pe
10 - 17h
17 - 22h
**
ns
evf infected
pOM1 infected
Pe gacA
Pe WT
D
Burstiness
Ecc15 evf
Ecc15 pOM1
E
Rhythmicity
Number of values
Periods

**Figure S1: Analysis of effect of pathogen ingestion on fly activity. Related to Figure 1.**

**(A)** and **(B)** Activity counts of exemplary single flies after feeding with *Ecc15 evf* or *Ecc15 pOM1* as well as *Pe gacA* or *Pe* WT. **(C)** Total activity counts in the *Drosophila* activity monitor for two 5h-long periods after *Ecc15* or *Pe* feeding. *Ecc15 evf/pOM1*: n=24/21 (10-17h) and n=24/20 (17-22h); *Pe*: all n = 23. p-values calculated via unpaired t-test with Welch's correction. **(D)** Burstiness of locomotor activity patterns after bacteria feeding, n=24 (*Ecc15*), n=23 (*Pe*), p-values calculated via unpaired t-test with Welch's correction. **(E)** Circadian rhythms of infected flies. Chi-square test of independence showed no significant differences for periodicity of flies fed with different bacterial strains (p=0.8676).

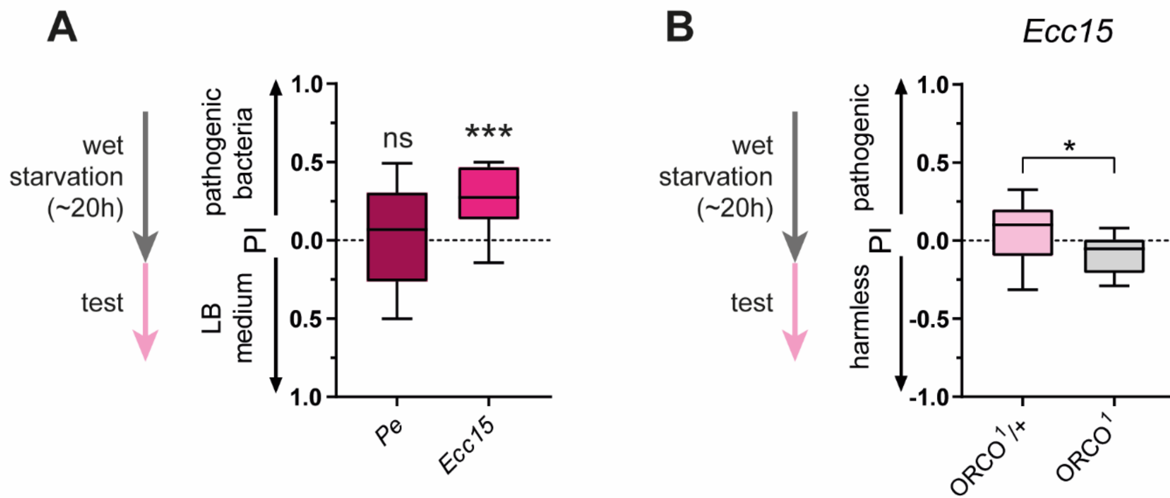
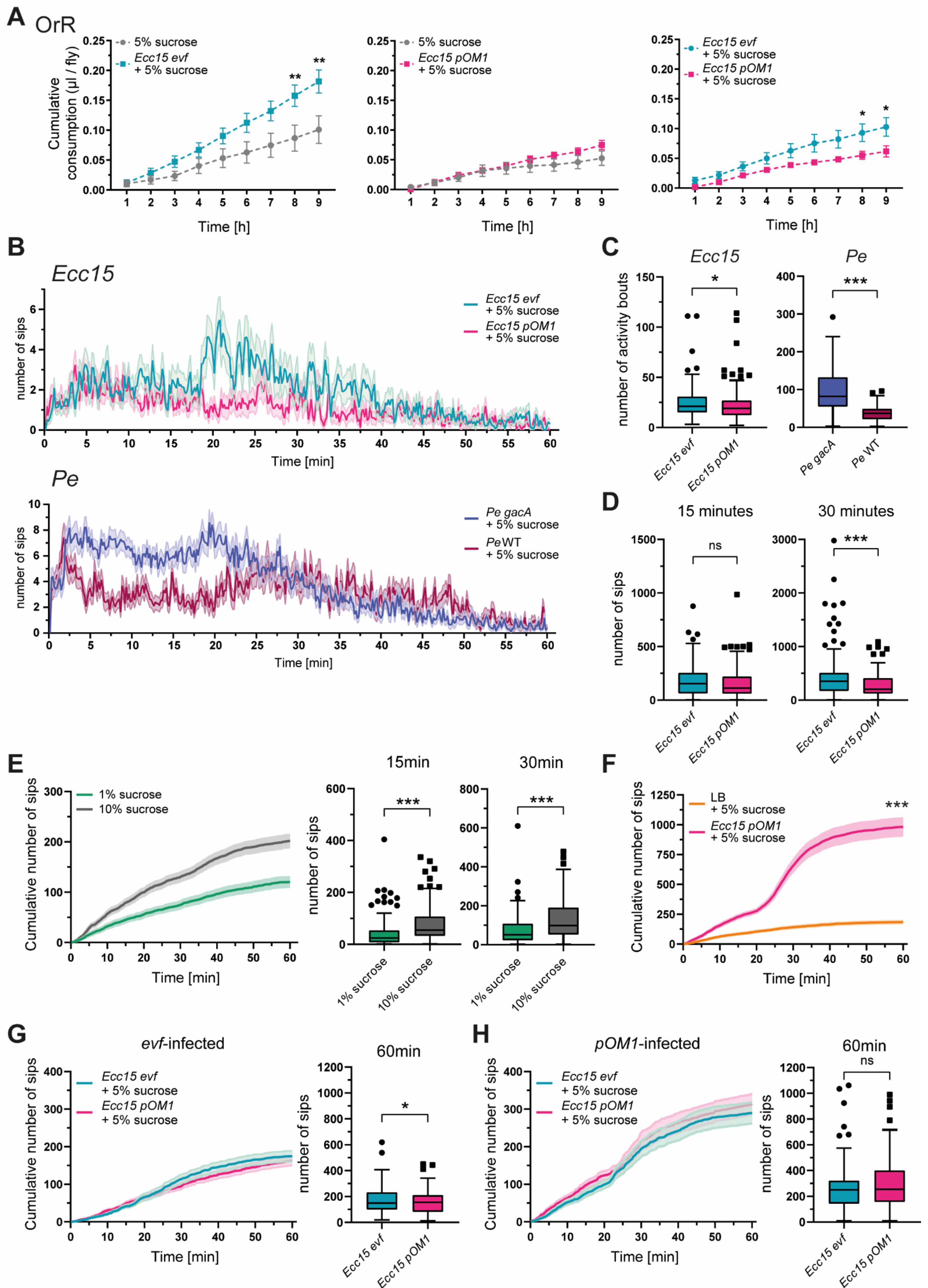



**Figure S2: Olfactory choices of control and mutant flies in olfactory arena assay. Related to Figure 2.**

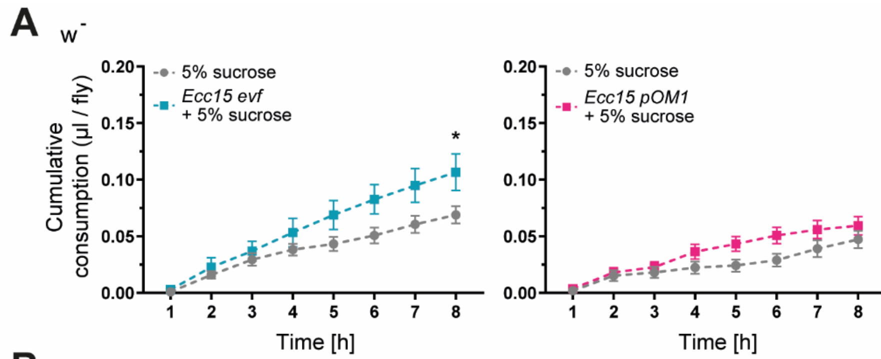
**(A)** Preferences of starved, naïve OrR flies for the olfactory choice between pathogenic *Pe* WT or *Ecc15 pOM1* and LB medium, respectively. n=21 (*Pe*), n=16 (*Ecc15*). p-values calculated via one-sample t-test comparing to 0. **(B)** Preferences of heterozygous $ORCO^1$/+ and anosmic $ORCO^1$ null mutant flies for the olfactory choice between harmless *Ecc15 evf* and pathogenic *Ecc15 pOM1*. n=16, p-value calculated via unpaired t-test with Welch's correction.



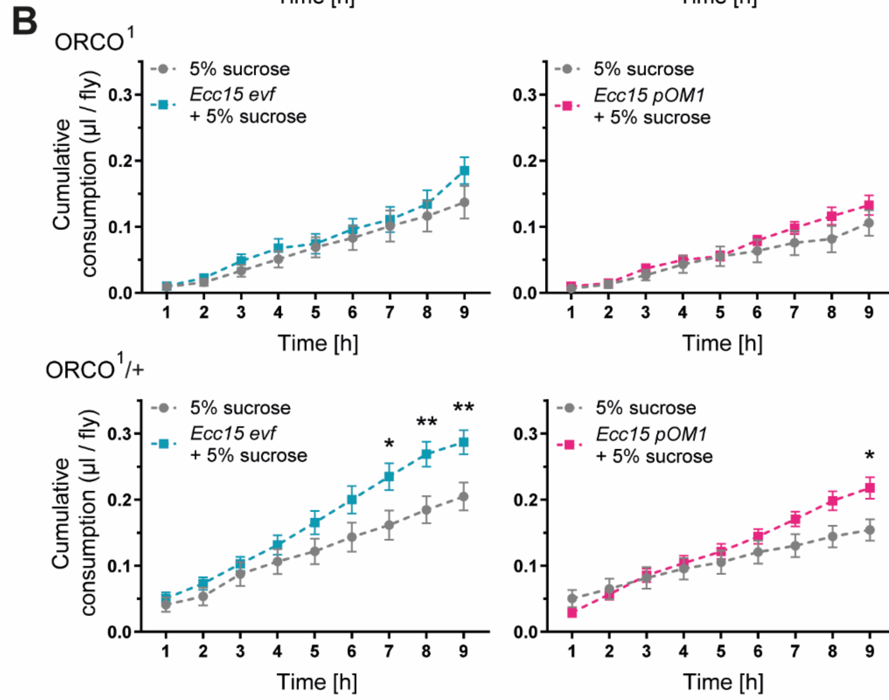
A OrR
5% sucrose
Ecc15 evf + 5% sucrose
Ecc15 pOM1 + 5% sucrose
Cumulative consumption (µl / fly)
Time [h]
B
Ecc15
Pe
number of sips
Time [min]
Pe gacA + 5% sucrose
Pe WT + 5% sucrose
C
number of activity bouts
Pe gacA
Pe WT
D
15 minutes
30 minutes
number of sips
E
1% sucrose
10% sucrose
Cumulative number of sips
15min
30min
F
LB + 5% sucrose
G
evf-infected
60min
H
pOM1-infected
ns

**Figure S3: Feeding induced changes in behavior to pathogenetic bacteria. Related to Figure 3.**

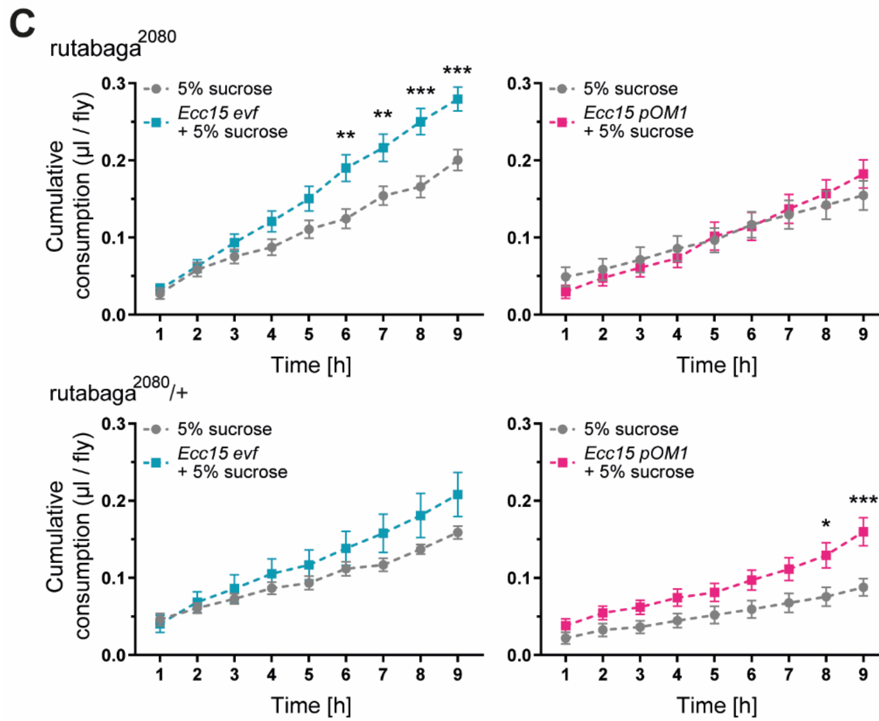
**(A)** *Ecc15* feeding of wild-type OrR flies in the CAFE. Cumulative consumption in µl/fly for the feeding choices sucrose vs. harmless *Ecc15 evf* (n=19), sucrose vs. pathogenic *Ecc15 pOM1* (n=20) and *Ecc15 evf* vs. *Ecc15 pOM1* (n=20). p-values calculated via repeated-measures two-way ANOVA followed by Bonferroni's post hoc test for multiple comparisons. **(B-D)** Additional parameters for the bacterial feeding preferences of wild-type CS flies in the flyPAD shown in Figure 3F-I, n=132 (*Ecc15*), n=137 (*Pe*). **(B)** Non-cumulative number of sips for the feeding choice between harmless and pathogenic *Ecc15* or *Pe*, respectively. **(C)** Number of activity bouts, p-value calculated via the Wilcoxon matched-pairs signed rank test. **(D)** Number of sips 15 and 30 minutes after feeding onset for the feeding choice between harmless *Ecc15 evf* and pathogenic *Ecc15 pOM1*. **(E)** Cumulative feeding of wild-type CS flies and number of sips 15 and 30 minutes after feeding onset for the choice between 1% and 10% sucrose in the flyPAD, n=126. **(F)** Cumulative feeding of wild-type CS flies for the choice between pathogenic *Ecc15 pOM1* and LB medium in the flyPAD, n=133. **(G), (H)** Feeding preferences of wild-type CS flies for the choice between harmless *Ecc15 evf* and pathogenic *Ecc15 pOM1* in the flyPAD following overnight feeding on *Ecc15 evf* (G) or *Ecc15 pOM1* (H). Cumulative feeding and total number of sips after 60 minutes, n=65. **(D-H)** p-values calculated by comparing feeding ratios to 1 via the Wilcoxon signed rank test.



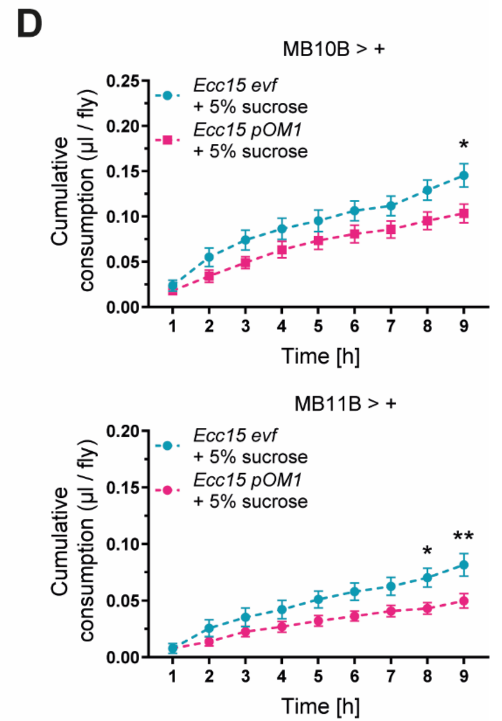
A
w-
Cumulative consumption (µl / fly)
5% sucrose
Ecc15 evf + 5% sucrose
Ecc15 pOM1 + 5% sucrose
Time [h]

B
ORCO1
ORCO1/+
Cumulative consumption (µl / fly)
5% sucrose
Ecc15 evf + 5% sucrose
Ecc15 pOM1 + 5% sucrose
Time [h]

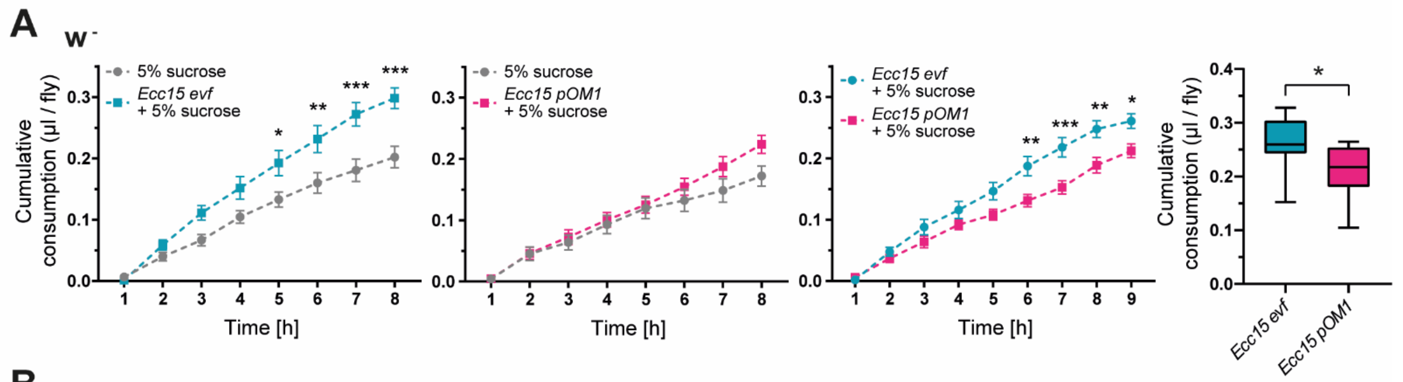
C
rutabaga2080
rutabaga2080/+
Cumulative consumption (µl / fly)
5% sucrose
Ecc15 evf + 5% sucrose
Ecc15 pOM1 + 5% sucrose
Time [h]

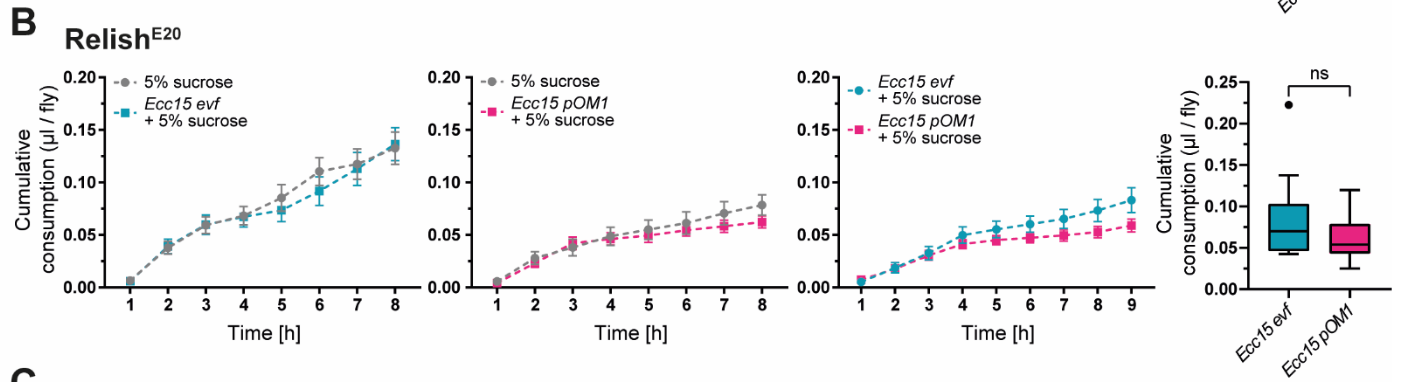
D
MB10B > +
MB11B > +
Cumulative consumption (µl / fly)
Ecc15 evf + 5% sucrose
Ecc15 pOM1 + 5% sucrose
Time [h]

**Figure S4: The mushroom body is involved in adaptive post-ingestion behavior to pathogenic bacteria. Related to Figure 4.**

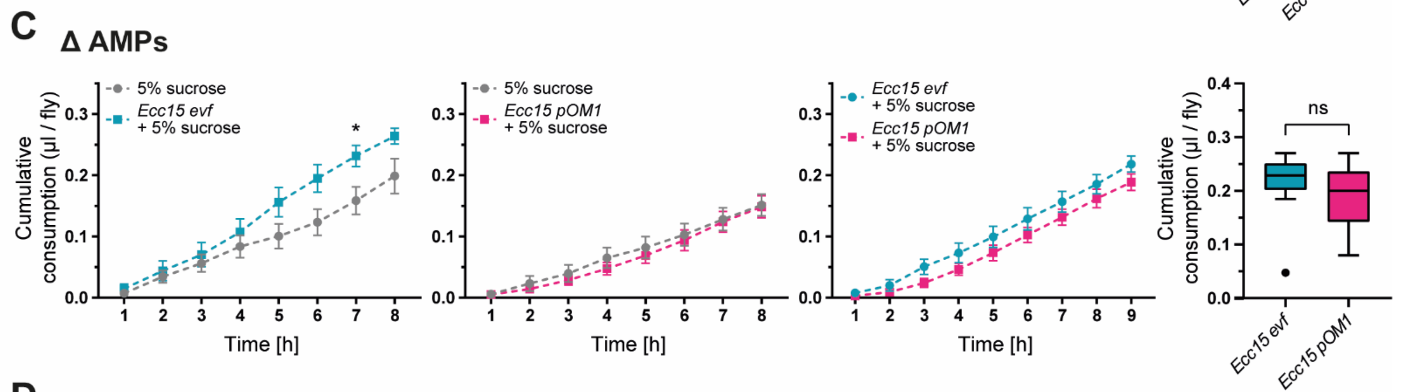
**(A)** Feeding preferences of control $w^-$ flies for the choice between sucrose and harmless *Ecc15 evf* (n=22) as well as between sucrose and pathogenic *Ecc15 pOM1* (n=23), (experiment at 30°C). **(B)** Feeding preferences of anosmic $ORCO^1$ and heterozygous $ORCO^1$/+ control flies for the choices sucrose vs. *Ecc15 evf* and sucrose vs. *Ecc15 pOM1* (all n=17). **(C)** Preferences of $rutabaga^{2080}$ learning mutant flies (n=17) and of heterozygous $rutabaga^{2080}$/+ control flies (n=16/17) for the feeding choice between sucrose and harmless *Ecc15 evf* and between sucrose and pathogenic *Ecc15 pOM1*. **(D)** GAL4-driver controls for MB10B and MB11B Split-GAL4 lines. Feeding preferences of MB10B > + (n=21) and MB11B > + (n=19) flies for the choice between harmless *Ecc15 evf* and pathogenic *Ecc15 pOM1*. **(A-D)** cumulative consumption in µl/fly in the CAFE, p-values calculated via repeated-measures two-way ANOVA followed by Bonferroni's post hoc test for multiple comparisons.



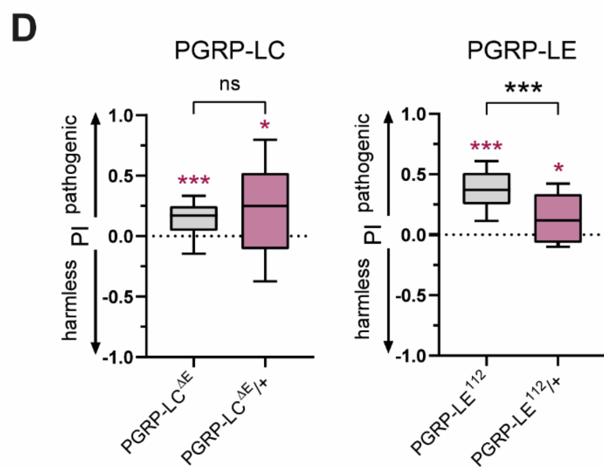
A
w-
5% sucrose
Ecc15 evf + 5% sucrose
Ecc15 pOM1 + 5% sucrose
Cumulative consumption (µl / fly)
Time [h]
0.0
0.1
0.2
0.3
0.4
1 2 3 4 5 6 7 8 9
* ** *** ***
** *** ** *
*
Ecc15 evf
Ecc15 pOM1

B
Relish E20
5% sucrose
Ecc15 evf + 5% sucrose
Ecc15 pOM1 + 5% sucrose
Cumulative consumption (µl / fly)
Time [h]
0.00
0.05
0.10
0.15
0.20
0.25
1 2 3 4 5 6 7 8 9
ns
Ecc15 evf
Ecc15 pOM1

C
Δ AMPs
5% sucrose
Ecc15 evf + 5% sucrose
Ecc15 pOM1 + 5% sucrose
Cumulative consumption (µl / fly)
Time [h]
0.0
0.1
0.2
0.3
0.4
1 2 3 4 5 6 7 8 9
*
ns
Ecc15 evf
Ecc15 pOM1

D
PGRP-LC
PGRP-LE
ns
***
*
PI
pathogenic
harmless
1.0
0.5
0.0
-0.5
-1.0
PGRP-LC ΔE
PGRP-LC ΔE/+
PGRP-LE 112
PGRP-LE 112/+

**Figure S5. Analysis of immune signalling components in feeding assay. Related to Figure 5.**

**(A)** Feeding preferences of isogenized control $w^{-}$ flies for the choices sucrose vs. harmless *Ecc15 evf* (n=15), sucrose vs. pathogenic *Ecc15 pOM1* (n=14) and *Ecc15 evf* vs. *Ecc15 pOM1* (n=16). **(B)** Feeding preferences of flies lacking the NF-κB transcription factor Relish for the choices sucrose vs. *Ecc15 evf*, sucrose vs. *Ecc15 pOM1* and *Ecc15 evf* vs. *Ecc15 pOM1* (all n=16). **(C)** Feeding preferences of flies deficient for all AMPs except for cecropins for the choice between sucrose and *Ecc15 evf*, sucrose and *Ecc15 pOM1*, and between *Ecc15 evf* and *Ecc15 pOM1* (all n=16). **(D)** Preferences of starved, naïve PGRP-$LC^{\Delta E}$ and PGRP-$LE^{112}$ mutant flies as well as of corresponding heterozygous controls for the odor of pathogenic *Ecc15 pOM1* over the odor of harmless *Ecc15 evf* in the 4-field arena (all n=16). p-values calculated via one-sample t-test comparing to 0 are indicated in red; p-values for group comparisons calculated via unpaired t-test with Welch's correction are indicated in black. **(A-C)** cumulative consumption in µl/fly in the CAFE and box plots of total consumption at the end of the experiment (9h). p-values calculated via repeated-measures two-way ANOVA followed by Bonferroni's post hoc test for multiple comparisons.

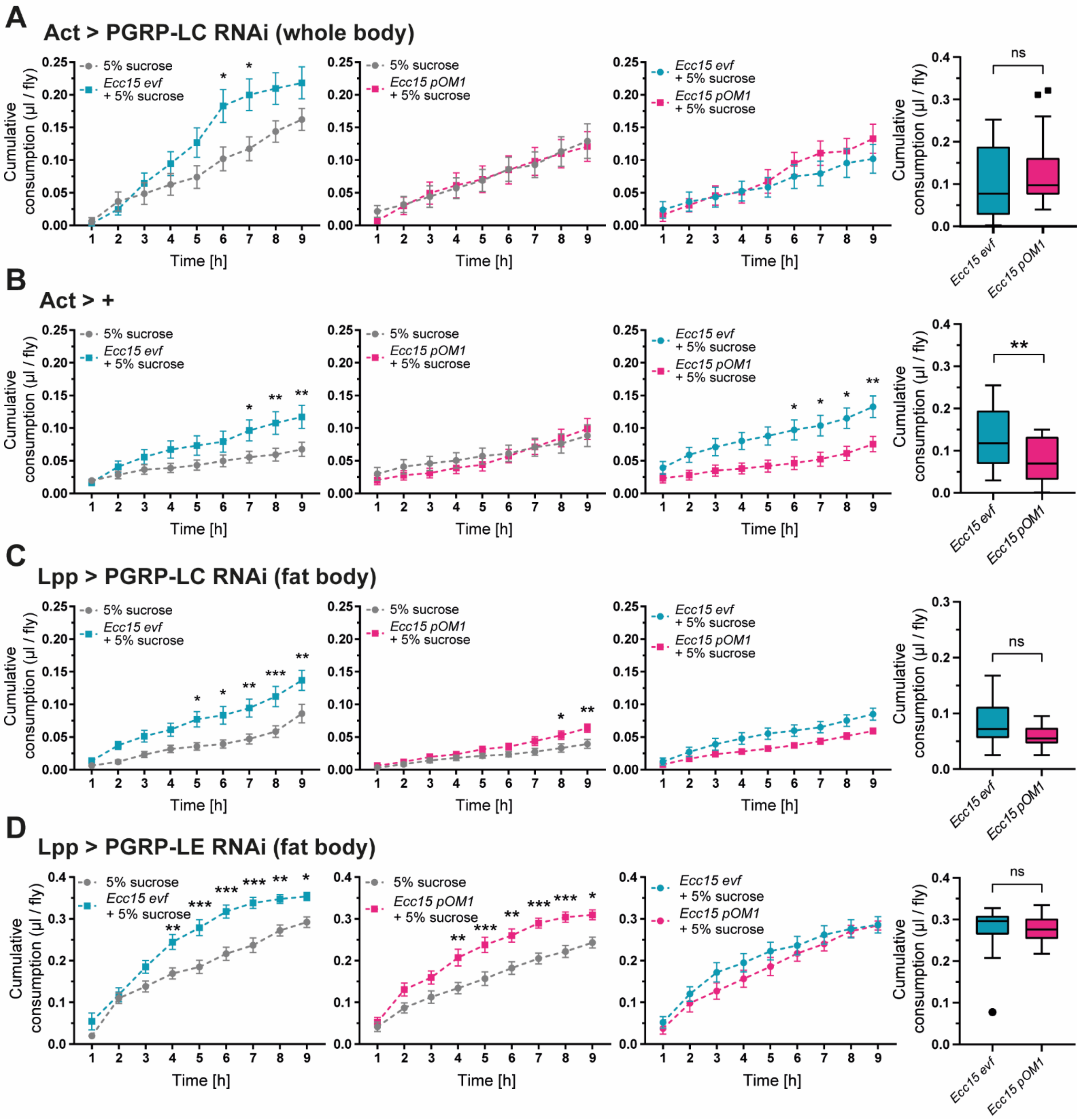
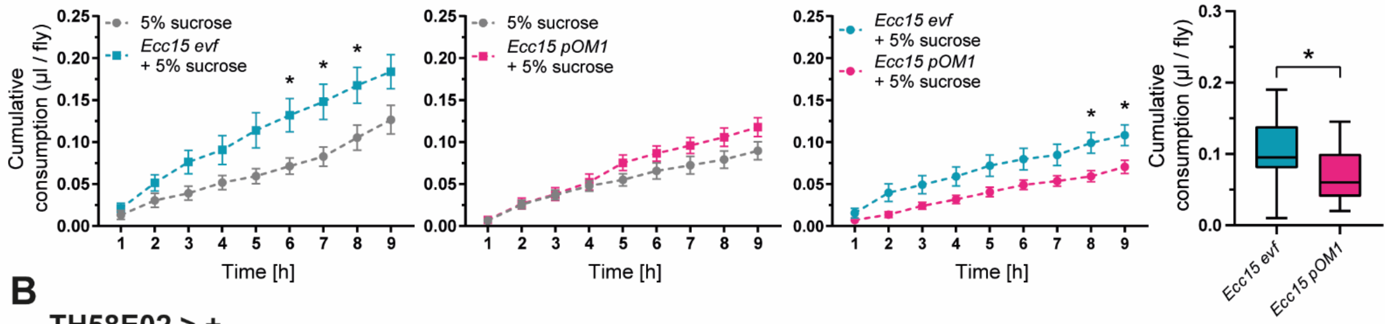
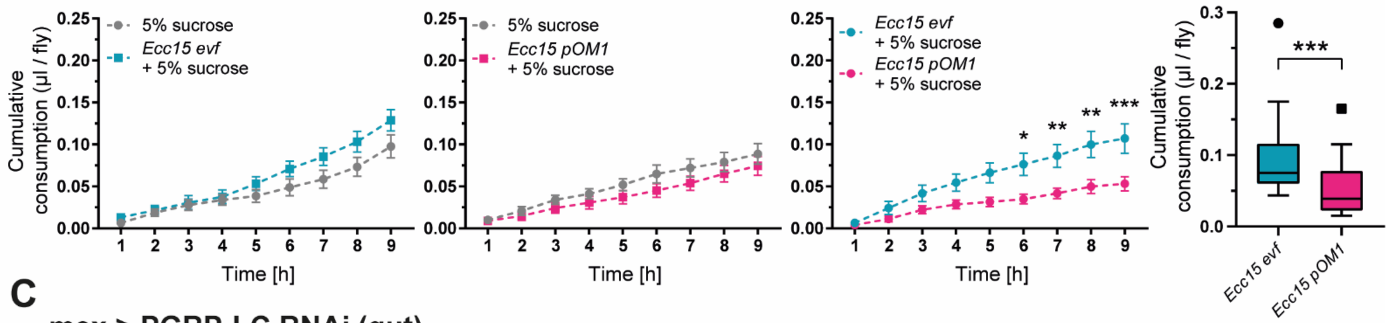
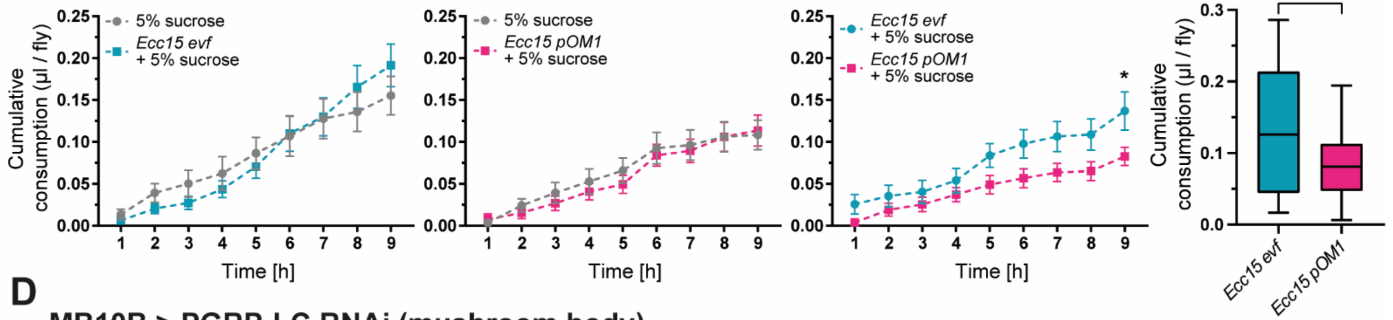

A
Act > PGRP-LC RNAi (whole body)
B
Act > +
C
Lpp > PGRP-LC RNAi (fat body)
D
Lpp > PGRP-LE RNAi (fat body)
5% sucrose
Ecc15 evf + 5% sucrose
Ecc15 pOM1 + 5% sucrose
Cumulative consumption (µl / fly)
Time [h]
ns
**
Ecc15 evf
Ecc15 pOM1

**Figure S6: Imd signaling components are involved in pathogen feeding choice. Related to Figure 6.**
**(A)** Feeding preferences of Act > PGRP-LC$^{RNAi}$ flies that similarly to PGRP-LC$^{\Delta E}$ mutant flies lack PGRP-LC in the whole body for the choice between sucrose and harmless *Ecc15 evf*, sucrose and pathogenic *Ecc15 pOM1* or between harmless and pathogenic *Ecc15* (all n=16). **(B)** Feeding preferences of Act > + control flies for the choices sucrose vs. *Ecc15 evf* (n=19), sucrose vs. *Ecc15 pOM1* (n=20) and *Ecc15 evf* vs. *Ecc15 pOM1* (n=20). **(C)** Feeding preferences upon downregulation of PGRP-LC specifically in the fat body using Lpp > PGRP-LC$^{RNAi}$ flies for the choices sucrose vs. *Ecc15 evf* (n=20), sucrose vs. *Ecc15 pOM1* (n=19) and *Ecc15 evf* vs. *Ecc15 pOM1* (n=20). **(D)** Preferences of Lpp > PGRP-LE$^{RNAi}$ flies that lack PGRP-LE specifically in the fat body for the feeding choices between sucrose and *Ecc15 evf* (n=14), sucrose and *Ecc15 pOM1* (n=17) and between *Ecc15 evf* and *Ecc15 pOM1* (n=16). **(A-D)** cumulative consumption in µl/fly in the CAFE and box plots of total consumption at the end of the experiment (9h). p-values calculated via repeated-measures two-way ANOVA followed by Bonferroni's post hoc test for multiple comparisons.


A
TH58E02 > shibire (dopaminergic neurons)
Cumulative consumption (µl / fly)
5% sucrose
Ecc15 evf + 5% sucrose
Ecc15 pOM1 + 5% sucrose
Time [h]
Ecc15 evf
Ecc15 pOM1
*


B
TH58E02 > +
Cumulative consumption (µl / fly)
5% sucrose
Ecc15 evf + 5% sucrose
Ecc15 pOM1 + 5% sucrose
Time [h]
Ecc15 evf
Ecc15 pOM1
***


C
mex > PGRP-LC RNAi (gut)
Cumulative consumption (µl / fly)
5% sucrose
Ecc15 evf + 5% sucrose
Ecc15 pOM1 + 5% sucrose
Time [h]
Ecc15 evf
Ecc15 pOM1
*

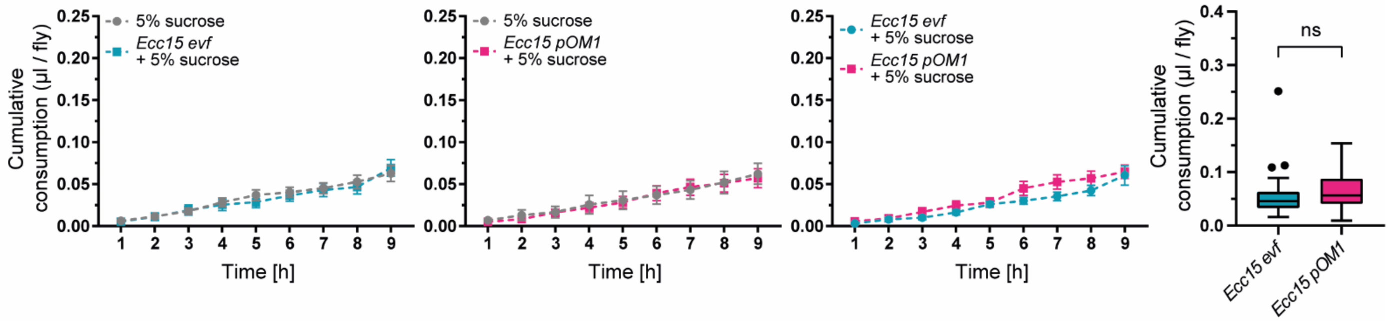

D
MB10B > PGRP-LC RNAi (mushroom body)
Cumulative consumption (µl / fly)
5% sucrose
Ecc15 evf + 5% sucrose
Ecc15 pOM1 + 5% sucrose
Time [h]
Ecc15 evf
Ecc15 pOM1
ns

**Figure S7: Dopamine is not essential for pathogenic bacteria feeding suppression. Related to Figure 7.**

**(A)** Preferences for the feeding choice between sucrose and harmless *Ecc15 evf* (n=21), sucrose and pathogenic *Ecc15 pOM1* (n=21) and between *Ecc15 evf* and *Ecc15 pOM1* (n=23) upon inactivation of broad clusters of dopaminergic neurons using TH58E02 > shibire$^{ts1}$ flies. **(B)** Feeding preferences of TH58E02 > + control flies for the choices sucrose vs. *Ecc15 evf* (n=22), sucrose vs. *Ecc15 pOM1* (n=21) and *Ecc15 evf* vs. *Ecc15 pOM1* (n=21). **(C)** Feeding preferences of mex > PGRP-LC$^{RNAi}$ flies that lack PGRP-LC specifically in midgut enterocytes for the choices between sucrose and harmless *Ecc15 evf*, sucrose and pathogenic *Ecc15 pOM1* and between *Ecc15 evf* and *Ecc15 pOM1* (all n=16). **(D)** Feeding preferences of MB10B > PGRP-LC$^{RNAi}$ flies that are deficient for PGRP-LC in all KCs of the MB for the choices sucrose vs. *Ecc15 evf* (n=18), sucrose vs. *Ecc15 pOM1* (n=16) and *Ecc15 evf* vs. *Ecc15 pOM1* (n=20). However, note the overall low consumption. **(A-D**) cumulative consumption in µl/fly in the CAFE and box plots of total consumption at the end of the experiment (9h). p-values calculated via repeated-measures two-way ANOVA followed by Bonferroni's post hoc test for multiple comparisons.