NEURAL MECHANISMS OF PATHOGEN-MODULATED FEEDING BEHAVIOUR IN *DROSOPHILA*

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Glossary and abbreviations

AD	activation domain [of the GAL4 transcription factor]
AL	antennal lobe
AMP	antimicrobial peptide
ANOVA	analysis of variance
CAFE	capillary feeder
CI	confidence interval
CS	conditioned stimulus / Canton-Special
CTA	conditioned taste aversion
cAMP	cyclic adenosine monophosphate
DAM	*Drosophila* activity monitor
DAP	diaminopimelic acid
DBD	DNA-binding domain [of the GAL4 transcription factor]
Duox	dual oxidase
Ecc15	*Erwinia carotovora carotovora 15*
Evf	*Erwinia* virulence factor
FADD	Fas-associated death domain-containing protein
flyPAD	fly proboscis and activity detector
GABA	γ-aminobutyric acid
GR	gustatory receptor
GRN	gustatory receptor neuron
Hor	homologue of Rap
IKK	IκB kinase
Imd	immune deficiency [pathway]
IQR	inter-quartile range
IL	interleukin
IR	ionotropic receptor
JAK-STAT	Janus kinase - signal transducer and activator of transcription
JNK	c-Jun N-terminal kinase
MB	mushroom body
MBON	mushroom body output neuron
LB	lysogeny broth

LH	lateral horn
Lpp	lipophorin
LPS	lipopolysaccharides
NADPH	nicotinamide adenine dinucleotide phosphate [reduced form]
NF-κB	nuclear factor κ-light-chain-enhancer of activated B cells
ns	not significant
OA	octopamine
OD	optical density
ON	overnight
OR	olfactory receptor
ORCO	odorant receptor co-receptor
ORN	olfactory receptor neuron
OrR	Oregon-R
PAM	protocerebral anterior medial [dopaminergic neuron cluster]
Pe	*Pseudomonas entomophila*
PI	preference index
PGN	peptidoglycan
PGRP	peptidoglycan recognition protein
PN	projection neuron
PPL	protocerebral posterior lateral [dopaminergic neuron cluster]
PTFE	polytetrafluoroethylene
pvf	*Pe* virulence factor
RNAi	RNA interference
ROS	reactive oxygen species
SEM	standard error of the mean
SEZ	subesophageal zone
TAK1	transforming growth factor β-activated kinase 1
Tdc2	tyrosine decarboxylase 2
TNFR	tumour necrosis factor receptor
to	takeout
TRP	transient receptor potential
UAS	upstream activating sequence
Upd	unpaired
US	unconditioned stimulus

Abstract

Food provides animals with essential nutrients as well as beneficial microbes, but also carries the risk of ingesting detrimental pathogens or toxins. Animals hence constantly need to balance their nutritional needs against possible negative post-ingestive effects. Importantly, if pathogens in a food source evade the detection by the external sensory systems such as smell or taste, they are inadvertently taken up with the food. Pathogens that enter the body via the digestive system trigger an immune response, cause an infection, and potentially damage internal organs. Thus, in order to ensure survival, most animals adapt their behaviours upon pathogen exposure to alleviate the impact of an infection or remember the chemosensory perception of the food source that made them sick in order to be able to avoid it in the future. The acquired avoidance of food that has caused intestinal malaise is wide-spread across the animal kingdom and hence not only present in vertebrates, but also in invertebrates such as *Caenorhabditis elegans* or the honeybee (Y. Zhang et al., 2005; Wright et al., 2010). However, it is not known how pathogens detected in the periphery signal to the brain and which neural circuits are responsible for pathogen-modulated behaviours. While *Drosophila melanogaster* larvae for instance show an evasion behaviour to pathogen-contaminated food (Surendran et al., 2017), it is uncertain whether the adult fruit fly, too, can adapt its behaviour to avoid spoiled food. Due to the large genetic toolset available in *Drosophila*, we used the fruit fly to unravel how immune system, gut and brain interact to guide this essential adaptive behaviour.

To examine the behavioural adaptations following oral infection, I fed flies with two common fly pathogens, namely the mildly virulent bacterial strain *Erwinia carotovora carotovora 15* (*Ecc15*) and the highly pathogenic *Pseudomonas entomophila* (*Pe*) as well as harmless mutant control strains. Monitoring survival validated the efficiency of the oral infection paradigm. Nevertheless, pathogen ingestion did not affect the circadian rhythm of infected flies and only transiently reduced locomotor activity in the case of *Ecc15* infection. In an olfactory choice setting, naïve flies preferred the odour of pathogenic bacteria not only to air but surprisingly also to the odour of the respective harmless mutant strains, indicating that flies are not innately repelled by the odour of these detrimental microbes. By contrast, capillary feeder (CAFE) and flyPAD feeding assays showed that, when given a choice

between pathogenic and harmless *Ecc15* or *Pe*, flies preferred feeding on the harmless instead of the pathogenic strains. While this preference was immediate in the case of *Pe*, the observation that flies exhibited a delayed feeding preference for harmless over pathogenic *Ecc15* strains suggests this behaviour to be the result of an acquired aversion instead of a choice based on taste alone.

Further support for the hypothesis that the aversion to pathogen-infested food sources constitutes an acquired behaviour came from experiments showing that flies deficient for the learning and memory gene *rutabaga* as well as flies lacking synaptic output from the fly's brain centre for associative memory formation, the mushroom body, were unable to distinguish between food containing pathogenic or harmless bacteria. Distinguishing between good and bad food sources might thus require an associative learning mechanism.

Interestingly, this feeding choice also relied on several components of the immune deficiency (Imd) immune signalling pathway, which is activated upon detection of bacterial components by peptidoglycan-recognition proteins (PGRPs). Specifically, *Drosophila* relied on the two immune receptors PGRP-LC and PGRP-LE as well as the NF-κB transcription factor Relish and antimicrobial peptides (AMPs), which are the central effectors of the immune response, to differentiate between food sources containing harmless or pathogenic bacteria. Knockdown experiments showed that PGRP-LC signalling was necessary in the fat body and in neurons, in particular in octopaminergic neurons, but not in midgut enterocytes, to distinguish non-hazardous and detrimental food sources.

Based on the data collected in this thesis, I postulate a model wherein pathogen ingestion, via the presence of bacterial peptidoglycan and possibly other, yet unknown factors, triggers PGRP-LC signalling in octopaminergic neurons. These neurons could in turn relay the information about the detrimental food source and the infection to the mushroom body, where feeding behaviour is lastingly modulated, potentially via an associative learning mechanism, and where the appropriate behavioural output is generated.

1. Introduction

1.1 Friend or foe? Evaluating the quality of a food source

Food is undeniably one of the most basic needs of any animal, and the uptake of relevant nutrients is essential for survival, growth and reproduction. Accordingly, animals spend a considerable amount of time and energy on finding food to avoid starvation. Yet feeding itself can be dangerous, too: unknown food can contain toxins, parasites or pathogenic bacteria that induce sickness or could even kill the animal. While satiated animals might disregard a less ideal food source, hungry animals will take greater risks in order to appease their hunger and nonetheless feed on it. Animals thus constantly need to balance their nutritional needs with the threat posed by the ingestion of potentially detrimental food.

How can animals evaluate whether the food in front of them is good or bad, what happens when they nevertheless consume spoiled food and how can they avert those dangers? When navigating their environment, animals rely on their sensory systems, i.e. vision, olfaction, gustation, audition and somatosensation. The external sensory systems provide them with a picture of the current state of their surroundings, which they integrate into their decisions – where to move, where to find food or a mating partner, where to rest. In an ever-changing environment, sensory input enables an animal to constantly update and adjust these decisions. The initial evaluation of a food source happens via the senses, in particular smell and taste. During foraging, odours serve as salient cues to find food, but are likewise important first indicators of a potential contamination. Some odours are for example innately avoided because they indicate the presence of harmful microbes (Stensmyr et al., 2012). Apart from smell, taste can be a further sign that a food source is spoiled or toxic. For many animals, bitterness is an alarm signal for the presence of noxious or otherwise damaging compounds; and food that tastes bitter is commonly avoided across the animal kingdom (Yarmolinsky et al., 2009). However, while several bitter substances are not toxic, many compounds that are toxic in turn do not have a characteristic bitter taste (Nissim et al., 2017). Similarly, a contaminated food source does not necessarily emit specific odours. Thus, it may well be that the fact that a food source is detrimental evades the recognition via the olfactory or gustatory system. If an animal fails to detect contaminants via its external

sensory system, it will ingest them together with the food. Once taken up through the digestive system, pathogens or toxins can induce negative post-ingestive consequences such as infection and sickness, and damage internal organs. When pathogens enter the body, the innate immune system steps in as an immediate, yet non-specific first line of defence. While vertebrates can additionally resort to their adaptive immune system to combat infectious microorganisms, invertebrates only possess an innate immune system.

Apart from these direct measures to fight an infection, animals also employ behavioural strategies to alleviate the post-ingestive consequences of feeding on contaminated food. So-called sickness behaviours accompanying an infection, such as reduced activity and appetite as well as increased sleep, have been acknowledged not as an unintended, maladaptive response but instead as a way to reduce the impact of the infection and accelerate recovery (Hart, 1988). In mammals, the behavioural immune system has been postulated to encompass all mechanisms that allow the detection of pathogens in the environment and that trigger corresponding emotional and cognitive responses, thus helping to avoid an infection or mitigate its aftermath (Hart, 2011; Schaller et al., 2011). Insects, too, engage in specific behaviours to prevent infections, for example via spatial avoidance, or to alleviate the consequences once an infection has taken place, e.g. by increasing grooming or by fecundity compensation (de Roode et al., 2012).

In addition to the immediate behavioural adaptations aimed at reducing the exposure to a pathogen, animals have to be able to remember the food that made them sick in order to avoid it in the future and thus ensure survival. To this end, they have to be able to associate the post-ingestive effects of pathogen uptake, which typically occur minutes up to hours after feeding, with sensory cues present at feeding, such as the smell or taste of the food. The ability to avoid spoiled food due to its negative post-ingestive effects is vital and hence not only present in humans (Garb et al., 1974; Klosterhalfen et al., 2000), but for example also in rodents (Garcia et al., 1955), crows (Nicolaus et al., 1983), honeybees (Wright et al., 2010), and even the roundworm *Caenorhabditis elegans* (Y. Zhang et al., 2005).

How do animals manage to associate a feeding event with a later occurring malaise? Forming memories of a harmful food source and adapting behaviours to avoid it requires the coordination of all involved bodily systems and processes by the brain. The smell and taste of the food is registered via the olfactory and gustatory system, the pathogens enter via

the digestive system and trigger an immune response, and all of these processes culminate in the avoidance of said food source. The brain hence has to integrate the information it receives from the senses about the external environment and from the immune system and the gut about the infection to generate the appropriate behavioural output. Yet even though behavioural immunity as well as memories of spoiled food are essential for survival and wide-spread across the animal kingdom, the underlying molecular and neural circuit mechanisms are poorly understood. For example, what role does the immune system play and how does the information about pathogens detected in the body reach the brain? Which neuronal mechanisms and which neural circuits induce lasting behavioural changes and orchestrate acquired food aversions?

One organism that could offer answers to these fundamental questions is the fruit fly *Drosophila melanogaster*. *Drosophila* feeds on rotten fruit and is hence constantly in danger of ingesting pathogens. Moreover, the fly, too, has been shown to develop an olfactory avoidance of bacteria-contaminated food and avoids feeding on pathogenic bacteria (Babin et al., 2014; Surendran et al., 2017). Just like other animals, a fly that has successfully found a food source will have to evaluate whether to feed on it or refrain from feeding because of an indication of contamination. Past or current post-ingestive effects of pathogen ingestion can be such indicators and save the fly from prolonged exposure to the pathogen as well as from repeated infections or even death. Investigating these acquired behavioural adaptations induced by pathogen ingestion and infection can tell us how the immune system, gut and brain interact to guide adaptive sensory behaviour.

1.2 *Drosophila* as a model organism

The discovery of the *white* mutation and with it the role of chromosomes in heredity by Thomas Hunt Morgan (Morgan, 1910) heralded the rise of the fruit fly *Drosophila melanogaster* as one of the most powerful model organisms, initially in classical genetics, but later in many areas of biological research such as immunology, developmental biology and neuroscience. There are many reasons for this success story; part of it certainly being the fly's short generation time and the simple and inexpensive rearing conditions, but in particular the sophisticated and unmatched genetic toolset as well as the relative simplicity of its genome and nervous system. The *Drosophila* genome was one of the first animal genomes ever to be

sequenced (Adams et al., 2000), and while only ~5% the size of the human genome, approximately 75% of human disease-associated genes have been suggested to have a functional equivalent in the *Drosophila* (Reiter et al., 2001).

Drosophila has four pairs of chromosomes, with the fourth being so small it is rarely used for genetic interventions (Roote et al., 2013). Yet the numerical simplicity of the fruit fly genome is what has facilitated genetic manipulations and allowed researchers to study the function of genes and the effect of mutations. Much of what we now know about the innate immune system we owe to the fly: the mechanisms governing the activation of the innate immune response were for example initially discovered in *Drosophila* (Lemaitre et al., 1995; Lemaitre et al., 1996). The easy genetic accessibility is what also paved the way for the emergence of behavioural genetics in the 1960s and 1970s, which set out to unravel the genetic basis of behaviour by generating behavioural mutants and tracing the phenotypes back to genes. This led to the discovery of the first learning mutants, *dunce* (Dudai et al., 1976) and *rutabaga* (Livingstone et al., 1984), which encode a cyclic adenosine monophosphate (cAMP) phosphodiesterase (Byers et al., 1981; Davis et al., 1981; C.-N. Chen et al., 1986) and an adenylyl cyclase (Levin et al., 1992), respectively, and are both part of the cAMP cascade involved in learning and memory. These findings considerably contributed to the establishment of the fruit fly as a popular and valuable model not only for the study of associative olfactory learning, but for neuroscientific research in general. The fly brain with its approximately 100.000 neurons (Simpson, 2009) is comparably small, yet this is precisely what facilitates investigating the fundamental principles governing how the brain integrates and processes information from the periphery and the environment and generates appropriate behavioural output.

Crucially, it is not only *Drosophila*'s genetic tractability but in particular the numerous technical advances in the fly community over the previous decades that have provided researchers with a large number of experimental tools. A major breakthrough in fly genetics was the development of P-element-mediated transgenesis, which enabled the stable introduction of any desired exogenous DNA sequence into the germline, meaning that it would then be transferred across generations (Rubin et al., 1982; Spradling et al., 1982). Most importantly, it was the discovery of binary expression systems like the GAL4-UAS system that has enabled the targeting and manipulation of specific cell populations or even single

cells with high spatial and temporal resolution. GAL4 is a transcriptional activator originally derived from yeast, which recognizes specific GAL4-binding sites, namely the Upstream Activation Sequence (UAS), and upon binding drives the transcription of downstream effectors (Brand et al., 1993). In a first fly line, GAL4 is expressed under the control of an endogenous, typically cell- or region-specific enhancer, while a second fly line carries the UAS sequence and a downstream effector transgene, which is silent in the absence of GAL4. Crossing these two fly lines results in offspring where the target gene is transcribed exclusively in cells expressing GAL4 (Figure 1A).

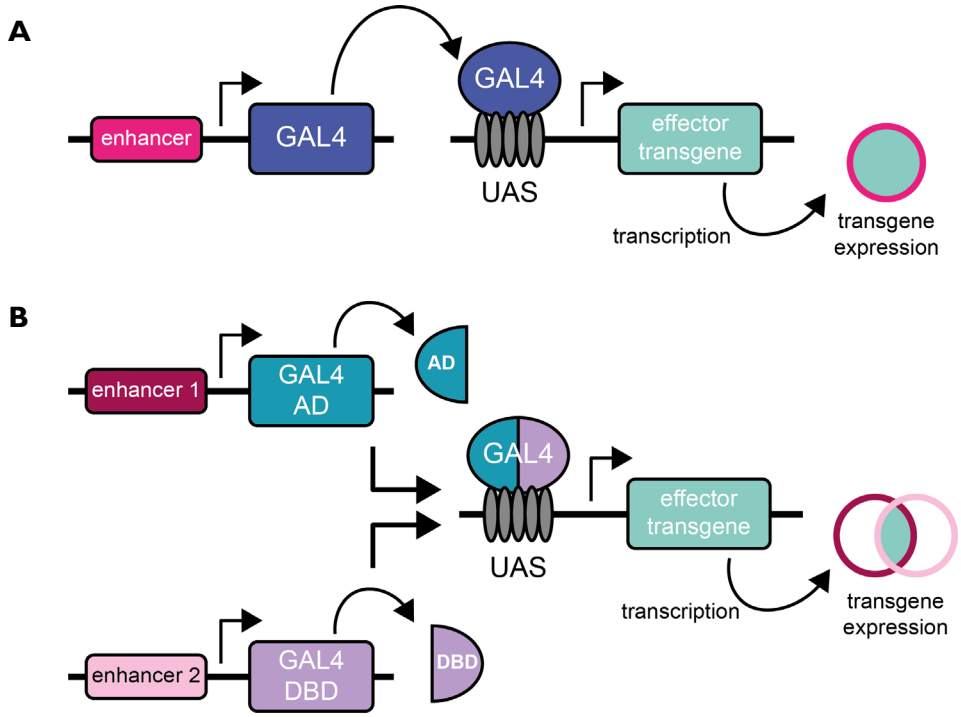

Figure 1 - The GAL4-UAS and split-GAL4-UAS expression systems

(A) The transcription factor GAL4 is expressed under the control of an endogenous enhancer. In cells expressing GAL4, GAL4 binds to the UAS and induces the transcription of the downstream effector transgene. **(B)** In the split-GAL4-UAS system, the activation domain (AD) and DNA-binding domain (DBD) of the GAL4 transcription factor are expressed under the control of different endogenous enhancers. Functional GAL4 is only reconstituted in cells where both AD and DBD are present, leading to a higher specificity of transgene expression.

Initially, a whole 'library' of driver lines expressing GAL4 in mostly broad patterns was generated by random genome insertions via P-element transposition. Further refinements of the GAL4-UAS system have aimed at increasing the temporal and spatial specificity of transgene expression. The transcriptional repressor GAL80 can inhibit the activity of GAL4

and can hence be used to limit expression of the target gene to cells that express only GAL4 (T. Lee et al., 1999). A temperature-sensitive version of GAL80 enables temporal control of transgene expression, as $GAL80^{ts}$ is unable to repress GAL4 activity at temperatures above 29°C (McGuire et al., 2003). Moreover, the GAL4-UAS system can be combined with other binary expression systems such as the LexA-lexAop (Lai et al., 2006) and the QF-QUAS-system (Potter et al., 2010) to simultaneously drive the expression of several effector transgenes in one fly. High spatial sensitivity up to the level of single cells can be achieved by the split-GAL4-UAS system, where the DNA-binding (DBD) and activation domains (AD) of the GAL4 transcription factor are expressed under the control of two different promoters (Luan et al., 2006). Functional GAL4 is only reconstituted in cells where both DBD and AD are expressed, leading to the transcription of the effector gene at the intersection of the two cell populations targeted by the two promoters (Figure 1B).

The different transcriptional activators can be combined with a large variety of effector and reporter transgenes to target and manipulate cells of interest. In the nervous system in particular, this provides researchers with an abundance of possibilities to visualize, activate or silence neurons, monitor neural activity or to express or downregulate specific genes, all from the level of single neurons through cell populations up to the whole brain. Visualizing cells is achieved by expressing a fluorescent reporter downstream of the binding sites of the transcriptional activator; and neuronal activity can be monitored *in vivo* via expression of genetically encoded calcium indicators such as GCaMP (T.-W. Chen et al., 2013). Neurons can be permanently silenced by overexpressing the inward-rectifying potassium channel Kir2.1, which prevents membrane depolarization, or tetanus toxin, which inhibits synaptic transmission by cleaving synaptobrevin (Sweeney et al., 1995; Baines et al., 2001; Paradis et al., 2001).

In addition, neurons can be transiently activated or silenced with heat or light via the expression of thermogenetic or optogenetic effectors, which is essential to avoid interference with development and allows for much more precisely timed behavioural experiments. One such thermogenetic effector is UAS-$shibire^{ts1}$. *$Shibire^{ts1}$* encodes a temperature-sensitive dominant-negative form of dynamin, which is required for synaptic vesicle recycling; higher temperatures thus cause a blockade of synaptic transmission by preventing vesicle endocytosis, thereby allowing the reversible silencing of neurons (Kitamoto, 2001). By

contrast, temperature-dependent increase of neural activity can be achieved by expressing the transient receptor potential (TRP) cation channel A1 (dTrpA1), which leads to the activation of neurons at temperatures above 29°C (Rosenzweig et al., 2005; Hamada et al., 2008; Rosenzweig et al., 2008). Similarly, researchers can transiently activate or silence neurons with light by expressing light-sensitive channelrhodopsins downstream of the transcriptional activator such as the red light-activated CsChrimson for neuronal activation (Klapoetke et al., 2014) or the green- and blue light-activated anion channelrhodopsins GtACR1 and GtACR2 for neuronal silencing (Mohammad et al., 2017).

Another invaluable tool that allows the systematic analysis of gene function is the use of RNA interference (RNAi) to downregulate gene expression and generate "knock-out" phenotypes (Clemens et al., 2000; Kennerdell et al., 2000; Lam et al., 2000). RNAi transgenes that are expressed downstream of the UAS enable the targeted inhibition of gene function in cells of interest. The establishment of genome-wide libraries of RNAi transgenes (Dietzl et al., 2007; Ni et al., 2008; Ni et al., 2011) has made it possible to target virtually any gene to investigate the phenotypic consequences of its downregulation.

Among the most recent technical advances in the fly field is the uncovering of the *Drosophila* larval and adult connectome (Eichler et al., 2017; Takemura et al., 2017; Zheng et al., 2018; Xu et al., 2020), which provide a detailed neuronal map of the circuitry as well as all neurons and cell types of the larval and adult *Drosophila* brain. The connectome of the adult *Drosophila* central brain constitutes the most complete wiring diagram of an animal with a complex brain to date and will be an immensely helpful foundational resource for functional studies.

The extensive toolkit for the cell-specific expression of a desired reporter or effector gene with high temporal and spatial specificity has made *Drosophila* a popular model not only in genetics, but also in immunology and in particular in neuroscience. The fruit fly is simple enough to be easily accessible for experimental manipulations, but likewise sufficiently complex to exhibit a variety of sophisticated behaviours. This enables the study of learned and innate behaviours at cellular resolution as well as from a holistic point of view that encompasses molecular, cellular and circuit levels to unravel how the brain combines information from peripheral organs and the environment and translates it into behaviour.

1.3 Chemosensory perception in *Drosophila*

For all animals, the odour and taste of a food source provide vital information about its quality. The chemical senses, i.e. smell and taste, are not only essential to find nutritious food, but also to detect the presence of hazardous substances such as toxins or pathogens before ingesting them and thereby risking intoxication or infection and endangering survival. *Drosophila* feeds on decaying and fermenting fruit where it is exposed to a large variety of nutritious as well as potentially harmful microbes and hence depends on chemosensory perception to evaluate its quality. For the fruit fly, odours serve as important long-distance cues and can elicit a wide range of different behaviours. They allow *Drosophila* to find food, a mating partner or potential oviposition sites as well as to avoid dangers, all without being in direct proximity to the odour source. By contrast, taste only functions in immediate contact, yet it is essential both to assess the nutritional value of a food source and to indicate potential contamination. The fundamental organization of the chemosensory system and the neurobiological principles governing odour and taste processing are conserved across species (Ache et al., 2005; Bargmann, 2006; Yarmolinsky et al., 2009). Thus, studying the fruit fly's response to detrimental food can provide valuable insights about the mechanisms underlying the perception and potential avoidance of spoiled food.

1.3.1 The olfactory pathway

Drosophila perceives odours via olfactory receptor neurons (ORNs) (also known as olfactory sensory neurons, OSNs) located in hair-like so-called sensilla in the two pairs of olfactory organs on the fly's head, i.e. in the third segment of the antennae and in the maxillary palps (Stocker, 1994). The fly can detect odorants via three different types of receptors: olfactory receptors (ORs) (Clyne et al., 1999; Vosshall et al., 1999), gustatory receptors (GRs) (W. D. Jones et al., 2007) and ionotropic receptors (IRs) (Benton et al., 2009). ORs are seven transmembrane domain proteins that primarily act as ligand-gated ion channels (Sato et al., 2008; Wicher et al., 2008), while IRs are related to ionotropic glutamate receptors and function as odour-gated ion channels (Abuin et al., 2011). The two GRs expressed in ORNs, *Gr21a* and *Gr63a*, are necessary for the olfactory detection of CO_2 (W. D. Jones et al., 2007). Each ORN commonly expresses only one ligand-specific receptor

(Vosshall et al., 2000). Additionally, all ORs are co-expressed with the obligate OR co-receptor ORCO (Or83b), which forms heteromeric complexes with the specific ORs and without which flies are rendered almost anosmic (Larsson et al., 2004; Benton et al., 2006). Even though *Drosophila* possesses only 60 OR, 60 GR and 61 IR genes (Robertson et al., 2003; Benton et al., 2009), the fly is able to cover the rich olfactory repertoire of its environment due to the combinatorial nature of odour recognition, as receptors in the ORNs usually respond to more than one odorant (J. W. Wang et al., 2003; Hallem et al., 2006).

Following odorant binding by the receptors in the ORNs, the ORNs as the primary olfactory afferents propagate the olfactory information to higher brain centres via only one synaptic relay in the antennal lobe (AL), the insect equivalent of the mammalian olfactory bulb (Figure 2).

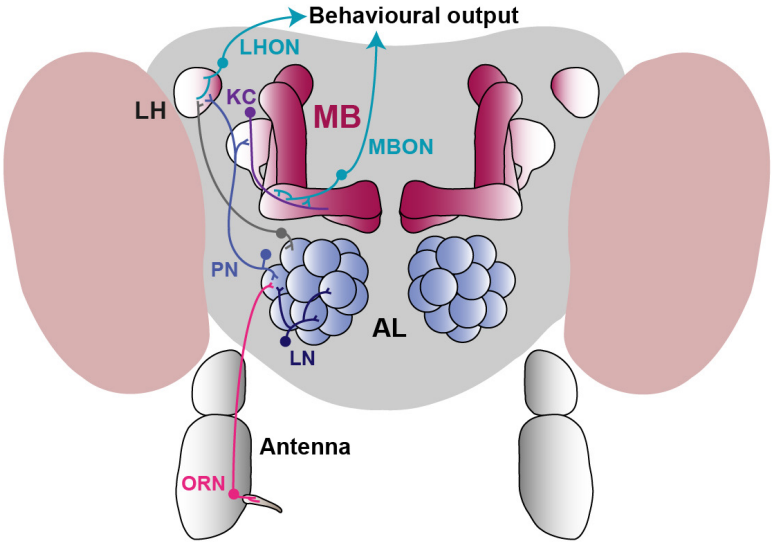

Figure 2 - The olfactory system in *Drosophila*

Odorants are recognized by receptors in olfactory receptor neurons (ORNs) on the antennae and maxillary palps. ORNs relay the olfactory information to the glomeruli of the antennal lobe (AL), where they form synapses with local interneurons (LNs) and projection neurons (PNs). PNs project to higher brain centres, the lateral horn (LH) and the Kenyon cells (KCs) of the mushroom body (MB), where behavioural output is generated via LH and MB output neurons (LHONs, MBONs).

ORNs expressing the same receptor converge primarily onto one glomerulus of the AL (Gao et al., 2000; Vosshall et al., 2000; Couto et al., 2005). Accordingly, the AL constitutes a topographic map of peripheral odorant recognition in the ORNs, as a particular odorant will bind to a distinct set of receptors and thus in turn activate a specific set of glomeruli. In the

glomeruli of the AL, ORNs form synapses with local interneurons (LNs) and the second-order neurons of the olfactory pathway, so-called projection neurons (PNs). LNs form lateral connections and link different glomeruli. They constitute a diverse population of neurons regarding their morphology, odour response properties or neurotransmitter types (Chou et al., 2010). Most LNs are GABAergic (γ-aminobutyric acid) and establish inhibitory connections with PNs and ORN afferents (Ng et al., 2002; Wilson et al., 2005). Lateral inhibition of ORNs has been suggested as a mechanism to prevent saturation of PNs, thus enabling the detection of a wider range of concentrations (Olsen et al., 2008; Root et al., 2008; Wilson, 2013). A smaller fraction of excitatory LNs releases acetylcholine, also innervates PNs and connects many glomeruli (Shang et al., 2007).

The majority of PNs in turn relays the olfactory information they receive from the ORNs of one AL glomerulus to two higher brain centres for further processing: the mushroom body (MB) and the lateral horn (LH) (Jefferis et al., 2001; Marin et al., 2002; Wong et al., 2002). Excitatory PNs project to both the MB and the LH, while another class of inhibitory GABAergic PNs bypasses the MB and directly innervates the LH (Jefferis et al., 2007; Lai et al., 2008; Okada et al., 2009; Liang et al., 2013). In both mammals and insects, this propagation of olfactory information via two divergent pathways has been traditionally thought to represent a hardwired circuit that mediates innate olfactory behaviours (*Drosophila* LH and rodent cortical amygdala) and a more unstructured circuit responsible for the formation of associate memories (*Drosophila* MB and rodent piriform cortex) (Heimbeck et al., 2001; Heisenberg, 2003; Sosulski et al., 2011; Aso et al., 2014a; Root et al., 2014). However, it has become more and more recognized that there is no sharp divide between innate and learned behaviour, but rather a continuum with interconnected neural circuits (Grunwald Kadow, 2019).

1.3.2 The gustatory system

Animals can detect potentially contaminated food not only via its smell, but also by tasting it. Being lured to a promising food source by its odour, they use taste to further evaluate it by detecting non-volatile compounds. In contrast to olfaction, gustation is more short-range and requires immediate contact with the food source. Taste is essential to both

identify beneficial, nutrient-rich food and to prevent ingesting harmful substances such as poisons or pathogens.

Due to its small body size, *Drosophila* usually walks on its food. Accordingly, its gustatory organs are dispersed over its entire body, from the labial palps and the pharynx of the proboscis (the fly's feeding organ) to the legs, wing margins and even the female ovipositor (Stocker, 1994). Tastants are detected in gustatory receptor neurons (GRNs) by GRs, IRs and receptors of the pickpocket and TRP family. IRs contribute to the gustatory detection of polyamines or salts (Y. V. Zhang et al., 2013; Hussain et al., 2016). Pickpocket 28 is required for water-sensing (Cameron et al., 2010; Z. Chen et al., 2010), while TRPA1 has for instance been implicated in the detection of electrophiles and the avoidance of harmful substances such as the plant antifeedant aristolochic acid or bacterial lipopolysaccharides (LPS) (S. H. Kim et al., 2010; Kang et al., 2012; Soldano et al., 2016).

GRs are seven transmembrane domain proteins encoded by 68 genes that are expressed in GRNs in sensilla of the gustatory organs (Clyne et al., 2000; Dunipace et al., 2001; Scott et al., 2001; Robertson et al., 2003). Similar to ORs, GRs have been suggested to be ligand-gated ion channels (Sato et al., 2011). In contrast to the olfactory system, GRNs can express multiple GRs, two of which, namely *Gr5a* and *Gr66a*, are broadly expressed in non-overlapping populations of GRNs and selectively respond to sugar (*Gr5a*) and bitter compounds (*Gr66a*) (Thorne et al., 2004; Z. Wang et al., 2004; Marella et al., 2006). In addition to sugar and bitter tasting, GRs are for instance involved in the detection of non-volatile pheromones (Bray et al., 2003; Miyamoto et al., 2008).

Furthermore, GRs are present in atypical locations outside the gustatory organs such as the already mentioned two GRs that detect CO_2 in the olfactory system (see 1.3.1). The fructose receptor *Gr43a* is for instance not only expressed on the legs or the proboscis, but also in neurons of the uterus, the proventricular ganglion of the gut and in neurons in the brain, which function as sensors of haemolymph fructose levels and regulate food consumption depending on the metabolic state (Miyamoto et al., 2012). Moreover, several genes of the *Gr28* cluster are expressed in neurons not associated with gustation such as hygroreceptive neurons of the arista or in neurons in the brain (Thorne et al., 2008). Interestingly, lack of Gr28b was moreover shown to cause anorexia in flies and thereby reduce resistance against a *Listeria* bacterial strain, while increasing survival after *Salmonella*

typhimurium infection (Ayres et al., 2008; Ayres et al., 2009). In mammals, taste receptors are also present in the intestine and enteroendocrine cells (e.g. Dyer et al., 2005; Mace et al., 2007). Enteroendocrine cells play a role in chemosensation, as they detect nutrients or chemicals in the gut lumen and release gut hormones in response and thereby modulate gut motility or appetite (Gribble et al., 2016). Interestingly, in *Drosophila*, too, a variety of GRs were found to be expressed in enteroendocrine cells in the gut that simultaneously express regulatory peptides (J.-H. Park et al., 2011). This points to additional functions of GRs beyond the perception of chemosensory cues in external gustatory organs, for example in energy metabolism via the post-ingestive sensing of nutrients or as a warning system upon ingestion of potentially detrimental compounds.

Further processing of gustatory information occurs in the central nervous system. Following the recognition of a tastant by GRs, gustatory information from the ovipositor, legs and wings is propagated to the ventral nerve chord, while GRNs from the proboscis and few from the legs project to the subesophageal zone (SEZ) in the central brain (Stocker, 1994). In contrast to the first relay centre of the olfactory system, the AL, the SEZ does not have a glomerular structure or clear morphological boundaries. Nevertheless, gustatory afferents are spatially segregated in the SEZ according to the taste organ, as GRN projections from the mouthparts are located more anteriorly in the SEZ than those of the proboscis (Stocker et al., 1981; Rajashekhar et al., 1994; Z. Wang et al., 2004). In addition, the SEZ exhibits a segregation by taste modality with different anatomical projection patterns for bitter- and sugar-sensing neurons (Thorne et al., 2004; Z. Wang et al., 2004).

Finally, it is important to note that gustation is typically embedded in the behavioural sequence underlying feeding behaviour. Upon detection of sugar, taste neurons in the legs for example suppress locomotion (Thoma et al., 2016). Thus, taste is essential in the early stages of the feeding programme for both evaluating the food source and initiating feeding, while simultaneously inhibiting competing behaviours (Thoma et al., 2017).

1.4 Learning to avoid harm

The fact that a food source is contaminated with harmful microbes can easily evade the detection by external sensory systems such as olfaction and gustation, causing an animal to ingest detrimental food. Once pathogens have entered the body via the digestive system, they elicit post-ingestive responses and trigger an immune response as well as behavioural adaptations to mitigate the impact of the infection. In addition, it is vital for an animal to remember food that made it sick to be able to avoid it in the future. Adapting behaviours in response to danger and learning to avoid harm in order to prevent or limit exposure to the potentially detrimental consequences are essential for well-being and survival. In the context of feeding on contaminated food, this requires the integration of post-ingestive signals in the brain and potentially an associative learning mechanism to induce lasting behavioural adaptations.

1.4.1 The *Drosophila* mushroom body

The major centre for learning and associative memory formation in the fly brain is the MB (Heisenberg, 2003; Owald et al., 2015). However, even though most of what we know about the MB is due to its role in olfactory memory, it is involved in a wide range of different behaviours such as associative visual or taste memories, sleep, courtship conditioning or sensory integration and the modulation of innate behaviours (McBride et al., 1999; Masek et al., 2010; Vogt et al., 2014; Lewis et al., 2015; Sitaraman et al., 2015). Together with the LH, the MB forms the higher brain centres of the olfactory pathway (see 1.3.1). The dendrites of the approximately 2000 intrinsic neurons of the MB, the Kenyon cells (KCs), primarily receive random input from olfactory PNs in the calyx (Caron et al., 2013; Aso et al., 2014a). The cholinergic KCs form three classes: the α/β and the α'/β' neurons, which extend parallel axons that bifurcate to form vertical (α, α') and horizontal (β, β') lobes as well as the γ neurons constituting the horizontal γ lobe. The KCs can be further subdivided into seven cell types, whose axons are located in spatially segregated layers within the MB lobes. KCs form synapses (including *en passant* synapses) both with other KCs and with only 34 MB output neurons (MBONs) of 21 different cell types, which target a variety of downstream brain regions and whose dendrites divide the MB lobes into 15 distinct compartments (Aso

et al., 2014a; Takemura et al., 2017). This convergence of KCs onto a small number of output neurons corresponds to a conversion of the high-dimensional representation of odour identity into a low-dimensional output. MBONs thus do not represent odour identity, but collectively influence behavioural responses and encode valence, with glutamatergic neurons driving avoidance behaviours and cholinergic and GABAergic MBONs promoting approach (Aso et al., 2014a; Aso et al., 2014b). Moreover, the MB receives input from 20 different types of dopaminergic neurons, which mainly reside in two clusters, PPL1 (protocerebral posterior lateral) and PAM (protocerebral anterior medial). Just as MBONs, the axons of dopaminergic neurons project to distinct compartments of the MB lobes, where they form synapses with MBONs and KCs, with PPL1 dopaminergic neurons primarily innervating the vertical lobes and neurons of the PAM cluster targeting the horizontal lobe (Aso et al., 2014a; Takemura et al., 2017).

The *Drosophila* MB hence represents a vastly interconnected neural circuitry with recurrent connections between KCs, MBONs and dopaminergic neurons. For example, KCs also form synaptic connections with dopaminergic neurons, and some feedforward MBONs project to the MB lobes, where they synapse onto KCs and form axo-axonic synapses with other feedforward MBONs (Aso et al., 2014a; Takemura et al., 2017). This interconnected and reciprocal circuitry of the MB constitutes the foundation for associative memory formation and the modulation of behaviours depending on context and internal state.

1.4.2 Associative learning mechanisms

In its broadest sense, associative learning can be described as remembering the relationship between two items, stimuli or events and hence covers most forms of learning except for habituation after repeated exposure to a stimulus. In the context of avoidance (or reward) learning, it designates the association between the feelings of pain (or pleasure) and the stimulus that has caused said feeling. A fundamental form of associative learning is classical Pavlovian conditioning (Pavlov, 1906, 1927), where an originally neutral stimulus such as a sound or smell, the conditioned stimulus (CS), is associated with an innately attractive or aversive stimulus such as food or electric shock, i.e. the unconditioned stimulus (US). Following repeated pairing of the US and the CS, the CS alone will be sufficient to elicit the behavioural response normally provoked by the US, which is consequently called a

conditioned reflex. In rodents for example, fear conditioning is a common experimental learning paradigm to study the neural basis of fear, in which animals form an association between a sound or a specific place and a highly aversive stimulus such as an electric shock so that the sound or place alone elicits fear and avoidance responses (Maren, 2001). In *Drosophila*, conditioning paradigms are often used to investigate olfactory learning and comprise the repeated pairing of an odour with a sugar reward or a punitive electric shock (Quinn et al., 1974; Tempel et al., 1983; Tully et al., 1985).

Shock- or reward learning has been employed to decipher the role of the MB in associative memory formation. The MB is the major learning and memory centre in the fly brain, as disrupting the MB causes a deficiency in associative olfactory learning (Heisenberg et al., 1985; de Belle et al., 1994; McGuire et al., 2001). In the *Drosophila* brain, the synaptic connections between KCs and MBONs are highly plastic and constitute the fundamental sites of associative learning. Memory formation requires synaptic plasticity, i.e. the strengthening or weakening of synaptic connections depending on increased or decreased activity of the involved neurons, a process regulated by neuromodulators such as dopamine or octopamine, the insect equivalent of vertebrate norepinephrine.

During associative learning, the CS, i.e. the odour, is conveyed by a distinct KC activity pattern, while the US, i.e. the information about reward or punishment, is primarily mediated by dopamine, but also by octopamine. Dopamine release by dopaminergic neurons can alter the strength of KC-MBON synapses and thereby biases behavioural responses towards approach or avoidance (Hige et al., 2015; Owald et al., 2015). Interestingly, valence categories during learning correspond to distinct sites within the MB lobes. MBONs promoting attraction are located in the vertical lobes, and MBONs driving aversive behaviours predominantly reside in the horizontal lobes (Aso et al., 2014a; Aso et al., 2014b). The two dopaminergic neurons clusters, too, split according to valence and innervation pattern. While PPL1 dopaminergic neurons innervating the vertical lobe have been implicated in negative reinforcement during learning, dopaminergic neurons of the PAM cluster convey reward (Claridge-Chang et al., 2009; Aso et al., 2010; Aso et al., 2012; Burke et al., 2012; Liu et al., 2012; Siju et al., 2019). Octopamine can mediate olfactory associative learning by influencing the dopaminergic neuron circuits of the MB, for example via the anterior paired lateral neuron innervating the whole MB (Burke et al., 2012; Wu et al.,

2013). In both *Drosophila* and the honeybee, octopamine has mostly been linked with mediating reward in appetitive olfactory conditioning paradigms (Hammer et al., 1998; Schwaerzel et al., 2003; Schroll et al., 2006); however, some reports have also implicated octopamine in avoidance learning (Agarwal et al., 2011; Iliadi et al., 2017).

On the molecular level, synaptic plasticity in both vertebrates and invertebrates requires cAMP signalling. Long-term potentiation and depression, i.e. the long-term increase or decrease in synaptic strength, of mossy fibre synapses on CA3 pyramidal cells in the mammalian hippocampus were shown to be mediated by cAMP (Huang et al., 1994; Weisskopf et al., 1994; Nicoll et al., 1995; Tzounopoulos et al., 1998). The first memory mutants discovered in the fruit fly, *dunce* and *rutabaga*, both encode components of the cAMP pathway. Rutabaga is a Ca^{2+}/calmodulin-stimulated adenylyl cyclase necessary for cAMP synthesis and is activated by G-protein coupled receptors, while dunce is a cAMP-specific phosphodiesterase that degrades cAMP (Dudai et al., 1976; Byers et al., 1981; Davis et al., 1981; Livingstone et al., 1984; Levin et al., 1992).

Dunce and rutabaga (as well as other genes implicated in olfactory memory) are primarily expressed in the MB, thus further emphasizing the role of the MB as the major learning and memory centre in the fly brain (Nighorn et al., 1991; Han et al., 1992). The short-term memory defect of *rutabaga*-deficient flies can be rescued by re-expressing *rutabaga* exclusively in the MB, in particular in the γ lobe (Zars et al., 2000; McGuire et al., 2003; Mao et al., 2004), while rescue of rutabaga in the α/β lobe of *rutabaga*-mutants restored long-term memory (A. L. Blum et al., 2009; Trannoy et al., 2011). During associative olfactory memory formation, the CS, e.g. an odour, is conveyed by a specific KC activity pattern, while the US, i.e. reward or punishment, activates MB-innervating dopaminergic neurons that can modulate the strength of KC-MBON synapses. In this scenario, rutabaga is thought to function as a coincidence detector in the MB, which initiates the molecular changes underlying synaptic plasticity (Tomchik et al., 2009; Gervasi et al., 2010).

1.4.3 Adaptive behaviour and the mushroom body

The MB is not only crucial for associative learning processes and memory formation, but has a much wider role in the control of internal states and the adaptation of behaviours depending on context as well as internal state. For example, the MB has been reported to be

involved in regulating locomotor activity (Martin et al., 1998). In addition, the MB mediates sleep, as different manipulations of the MB can increase or reduce sleep (Joiner et al., 2006; Pitman et al., 2006). Specific glutamatergic MBONs were found to suppress sleep, while MBONs promoting sleep were GABAergic or cholinergic (Aso et al., 2014b). One innate, adaptive behaviour relying on the MB is the innate temperature preference of flies. *Drosophila* cannot control its body temperature endogenously and thus has to move to reach areas of its preferred temperature of ~24°C. This so-called temperature preference behaviour was found to rely on cAMP signalling in the MB as well as on dopaminergic neurons innervating the MB (Hong et al., 2008; Bang et al., 2011; Tomchik, 2013); and the MB was shown to be an important part of the neural circuits mediating hot and cold avoidance (Frank et al., 2015).

More generally, internal states such as hunger, but for instance also reproductive state greatly influence a fly's behaviour, and in many cases, the integration of internal states and the subsequent modulation of behaviours happens at the level of the MB (Sayin et al., 2018). These internal states are often conveyed to the MB via dopamine signalling (Bräcker et al., 2013; Siju et al., 2014; Cohn et al., 2015; Lewis et al., 2015; Tsao et al., 2018). The MB has been suggested to serve as a switchboard, as dopaminergic neurons modulate transmission between KCs and MBONs depending on external context and internal state so that the same odour representation can lead to a different net output from MBONs to enable adaptive behavioural responses (Cohn et al., 2015). One internal state that affects many different behaviours due to its high urgency is hunger. The MB circuitry mediates innate, hunger-driven food search behaviour, as five MBONs were reported to be necessary for food odour attraction in hungry flies, with specific dopaminergic neurons potentially conveying hunger and satiety signals to the MB (Tsao et al., 2018). Moreover, the MB integrates conflicting sensory information to modify the innate aversion of flies to CO_2 depending on the hunger state: hungry flies were shown to overcome their aversion to CO_2 to approach attractive vinegar odour, a behaviour that required PAM dopaminergic input to the MB and glutamatergic output from the β'2 region (Bräcker et al., 2013; Siju et al., 2014; Lewis et al., 2015). Furthermore, the persistent food odour-tracking behaviour of hungry flies in the absence of reward was reported to depend on dopaminergic neurons and Dop1R2 signalling as well as on two specific MBONs, while octopaminergic neurons of the VPM4 cluster, which directly inhibit one of the MBONs (MBON-γ1pedc>αβ), disrupted odour tracking to enable

feeding-related behaviours (Sayin et al., 2019). Apart from hunger, MB circuits also mediate water-seeking behaviours in thirsty flies via specific dopaminergic neurons (Lin et al., 2014). In addition to the integration of sensory information and internal states such as hunger or thirst, the MB receives post-ingestive signals, as the long-term association between an odour and energy content of ingested food was for instance reported to rely on dopaminergic signalling to the MB (Musso et al., 2015). Thus, in addition to its role in learning, the MB is crucial for the integration of internal states and modulates innate behaviours, which allows the fly to adjust its behaviours to changing needs in consideration of the external circumstances.

1.4.4 Acquired aversions to detrimental food

Inadvertent ingestion of contaminated food can cause infection, illness, and damage to internal organs. Hence, it is vital that animals realize that they are ingesting something potentially dangerous to stop feeding on it as soon as possible. Moreover, animals have to remember food sources that have previously caused illness to ensure health and survival. The adaptation of behaviour and the learned aversion of a food source after its pairing with intestinal malaise are known as conditioned taste aversion (CTA) or conditioned food aversions. This involves forming an association between a particular character of a food source, such as a specific smell or taste, and the negative post-ingestive effects, e.g. gastrointestinal malaise, which occur minutes up to hours after feeding. This behavioural adaptation differs from learning during classical conditioning in several important aspects. In classical conditioning paradigms, the innately aversive or attractive US has to occur at the same time as the CS, e.g. a sound or smell, and both have to be paired repeatedly to ensure learning and memory formation. Even if there is a delay between CS and US, as is the case in trace or delay learning paradigms, this time span has to be shorter than 30 s for aversive associative memory formation to occur (Galili et al., 2011). By contrast, conditioned food aversions are already formed after a single trial and do not require immediate pairing of the two stimuli. Thus, it is sufficient for an animal to ingest contaminated food and experience the ensuing malaise only once to adapt its behaviour and avoid the food source. Moreover, the onset of negative post-ingestive effects occurs minutes or even hours after feeding, but is nevertheless associated with the food source (Garcia et al., 1966; Etscorn et al., 1973).

The ability to adapt behaviours following the ingestion of contaminated food or to remember said food source is essential for any animal and hence widespread across the animal kingdom. In rodents, CTA is commonly used to investigate learning and memory and involves the learned avoidance of sweet, non-nutritive saccharin-flavoured drinking water due to its pairing with a later occurring, artificially induced malaise (originally by radiation, later typically by injection of lithium chloride) (Garcia et al., 1955; Welzl et al., 2001). Invertebrates, too, avoid odours or tastes due to an association with negative post-ingestive effects. The nematode *C. elegans* was shown to avoid the odour of pathogenic bacteria following oral infection, a behaviour that was mediated by an increase of serotonin expression in chemosensory neurons, suggesting that serotonin acts as a negative reinforcing stimulus during infection (Y. Zhang et al., 2005). Interestingly, while this aversion was only transient in adults, exposing *C. elegans* larvae to pathogenic bacteria induced persistent avoidance of the odour of those bacteria and an aversive memory that was maintained into adulthood (Jin et al., 2016). Insects such as the honeybee also form conditioned food aversions. Honeybees can learn to associate odours with the taste of a toxin as well as the malaise induced by toxin ingestion, indicating that they can remember negative post-ingestive effects (Wright et al., 2010). While dopamine mediated the associative, pre-ingestive memory of an aversive taste, post-ingestive learning required serotonin, similar to the acquired pathogen avoidance seen in *C. elegans*. This puts forward serotonin as a neuromodulator of the circuits integrating negative post-ingestive effects within circuits governing olfactory or gustatory learning.

In *Drosophila*, too, acquired aversions to pathogens following feeding have been observed. *Drosophila* larvae develop a lasting aversion of a food source contaminated with highly virulent, but not to one with harmless bacteria; an aversion that was diminished in starved flies and depended on the neuropeptide hugin (Surendran et al., 2017). Adult fruit flies were reported to decrease their attraction to an odour after prior exposure to a food source scented with said odour and contaminated with pathogenic bacteria (Babin et al., 2014). Similar to the honeybee, dopamine has been implicated in the formation of pre-ingestive aversive taste memories in the fly, more specifically dopaminergic neurons of the PPL cluster that innervate the MB (Masek et al., 2015). However, research in *Drosophila*, but also in other organisms has so far not elucidated the precise mechanisms underlying the

acquired avoidance to pathogen-contaminated food. For example, it is not known which signals from the body are detected by the brain to elicit avoidance, how the information about the negative post-ingestive effects of pathogen uptake reaches the nervous system or which neural circuits guide these adaptive behaviours.

1.5 Fighting pathogens

Animals have developed a variety of strategies to protect themselves from pathogens ingested with food. In addition to physical barriers such as epithelial tissues, they depend on their immune system to detect and combat microorganisms and evade diseases. Moreover, there are many reciprocal interactions between pathogens and animal behaviour. While some harmful microbes can manipulate their host's behaviour, animals also exhibit a range of specific behaviours in response to pathogens that allow them to either avoid infection or reduce its impact, a concept known as behavioural immunity.

1.5.1 Innate immunity in *Drosophila*

Mounting an efficient immune response relies on the recognition of patterns that are specific to the infectious agent, thereby enabling animals to distinguish between self and non-self and avoid targeting their own healthy tissues. There are two main subcomponents of the immune response, namely the innate and the adaptive immune response. The innate immune response is triggered immediately upon detection of extraneous microbes, but is mostly non-specific. By contrast, vertebrates additionally possess an adaptive immune system, which entails the formation of an immunological memory by adapting the response to specific microorganisms. The adaptive immune system hence is slow upon first contact with a particular pathogen, but highly efficient upon re-exposure to the same pathogen.

1.5.1.1 Immune effectors

Invertebrates such as *Drosophila* only have an innate immune system, which consists of a cellular and a humoral response. The cellular response is mounted by cells circulating in the haemolymph and comprises phagocytosis of intruding microbes by macrophage-like plasmatocytes, melanisation induced by crystal cells in larvae and encapsulation of large pathogens by lamellocytes (Vlisidou et al., 2015). Additionally, the cellular response involves

coagulation of the haemolymph at the site of infection to block the spread of pathogens (Vlisidou et al., 2015).

The humoral response mainly relies on the inducible expression of antimicrobial peptides (AMPs), which are primarily produced by the fat body and secreted into the haemolymph, and which constitute the key effectors of the systemic immune response. The existence of an inducible component of the innate immune response in insects was originally discovered in *Drosophila* (Boman et al., 1972), with the first AMPs being isolated from silkmoths (Hultmark et al., 1980). Yet AMPs are not only present in invertebrates, but are employed as immediate, first-line defence mechanisms against pathogens across the animal kingdom as well as by various fungi or plants (G. Wang et al., 2015). Due to their additional immunomodulatory functions, they have become known as host defence peptides in higher vertebrates to incorporate their role in immune signalling beyond mere antimicrobial activity (Hancock et al., 2016). AMPs are induced in many different tissues and are for instance also produced in the central nervous system of higher vertebrates (e.g. Hao et al., 2001).

The term AMP refers to a broad class of peptides with antimicrobial activity and considerable diversity in sequence and structure between different species. Nevertheless, AMPs from insects to mammals have highly conserved sequences: they are small, cationic peptides that have both hydrophilic and hydrophobic sequences and can hence adopt an amphiphilic structure, allowing them to interact with both aqueous and lipid environments such as membranes (Bulet et al., 1999; Zasloff, 2002, 2019). Put briefly, AMPs combat intruding pathogens by disrupting their membranes, which frequently display anionic compounds on the outer surface, meaning that AMPs are electrostatically attracted to microbial membranes (Zasloff, 2002, 2019).

1.5.1.2 Signalling pathways of the *Drosophila* immune response

Much of what is known today about the signalling cascades regulating innate immunity traces back to the observation that AMPs as inducible effectors of the innate immune response are regulated by conserved NF-κB signalling pathways in both insects and mammals (Silverman et al., 2001). In *Drosophila*, the inducible humoral immune response is activated by two distinct signalling pathways, namely the Toll and the immune deficiency (Imd) pathways, which regulate the response to Gram-positive bacteria or fungi and Gram-negative bacteria, respectively. The specificity of the signalling pathways for different classes

of pathogens arises from the recognition of different types of peptidoglycans (PGN) in bacterial cell walls by so-called peptidoglycan-recognition proteins (PGRPs) (Leulier et al., 2003). First isolated from the silkworm (Yoshida et al., 1996), PGRPs are pattern recognition receptors that are conserved from insects to mammals. While mammalian PGRPs are a family of four secreted proteins, the *Drosophila* PGRPs constitute a more diverse group of 19 proteins encoded by 13 genes (Werner et al., 2000; Dziarski et al., 2010; Royet et al., 2011). *Drosophila* PGRPs can have amidase activity or activate downstream signalling cascades. Amidase PGRPs such as the secreted PGRP-LB or PGRP-SC1 and -SC2 degrade PGN and thus act as negative regulators of the Imd pathway to prevent an excess, detrimental activation of the immune response (Bischoff et al., 2006; Zaidman-Rémy et al., 2006). PGRPs specific for the lysine-type PGN of Gram-positive bacteria trigger downstream signalling via the Toll pathway, while the Imd pathway is activated upon recognition of diaminopimelic acid (DAP)-type PGN found in the cell wall of Gram-negative bacteria and *Bacillus* sp. (Leulier et al., 2003).

The Toll pathway was originally discovered due to its role in the establishment of the dorso-ventral axis in *Drosophila* (Nüsslein-Volhard et al., 1980; Anderson et al., 1985). Later, it was found that the Toll pathway is required for the innate immune response towards fungi (Lemaitre et al., 1996) and Gram-positive bacteria (Rutschmann et al., 2002). Genes coding for Toll-like receptors (TLRs) are also present in humans, where they induce the expression of inflammatory cytokines essential for the innate immune response (Medzhitov et al., 1997; Rock et al., 1998), thus pointing to similarities between the *Drosophila* and vertebrate innate immune system. The recognition of fungal cell wall components or the lysine-type PGN of Gram-positive bacteria by PGRP-SA and further pattern recognition receptors as well as sensing of microbial proteases or other danger signals (Michel et al., 2001; Gobert et al., 2003; Leulier et al., 2003; Gottar et al., 2006; Chamy et al., 2008) induces cleavage of the cytokine Spätzle. Spätzle in turn initiates the immune response upon binding to the Toll transmembrane receptor (Lemaitre et al., 1996; Weber et al., 2003; Hu et al., 2004). The Toll pathway culminates in the nuclear translocation of the NF-κB-like transcription factor DIF, which in turn induces the transcription of antifungal AMPs such as drosomycin to combat the intruding pathogens (Rutschmann et al., 2000a).

By contrast, the Imd pathway is the primary regulator of the immune response towards Gram-negative bacteria (Figure 3) and shares similarities with the mammalian tumour necrosis factor receptor (TNFR) signalling cascade (Myllymäki et al., 2014). It was discovered because *Imd* mutant flies were found to be highly susceptible to infection with Gram-negative bacteria, while the antifungal immune response remained mostly unaffected (Lemaitre et al., 1995; Corbo et al., 1996). The immune response is activated upon recognition of the DAP-type PGN of Gram-negative bacteria by the two main receptors of the Imd pathway, PGRP-LC and PGRP-LE (Figure 3). PGRP-LC is a transmembrane receptor that serves both as a pattern recognition receptor by recognizing bacterial PGN and as the major signal-transducing receptor of the Imd pathway (Choe et al., 2002; Gottar et al., 2002; Rämet et al., 2002). Heterodimers of the PGRP-LC isoforms bind monomeric PGN, while homodimers of the signal-transducing isoform PGRP-LCx bind polymeric PGN (Kaneko et al., 2004; Kaneko et al., 2005; Mellroth et al., 2005). The second receptor of the Imd pathway, PGRP-LE, has a dual role in sensing bacterial PGN in that it functions both upstream and in parallel with PGRP-LC: an extracellular form of PGRP-LE consisting only of the PGRP domain assists PGRP-LC in recognizing DAP-type PGN, while a full-length cytoplasmic version of PGRP-LE binds monomeric PGN and activates downstream Imd signalling (Takehana et al., 2002; Takehana et al., 2004; Kaneko et al., 2006; Neyen et al., 2012).

Binding of PGN to the PGRP receptors leads to the activation of a downstream signalling cascade that culminates in the nuclear translocation of the NF-κB-like transcription factor Relish and the transcription of AMPs and other immune effectors (Hedengren et al., 1999; Choe et al., 2005) (Figure 3). Initially, this entails the recruitment of a complex comprising the death domain protein Imd, the adaptor protein dFadd, which is structurally similar to the mammalian Fas-associated death domain-containing protein (FADD), and the caspase Dredd (Leulier et al., 2000; Georgel et al., 2001; Leulier et al., 2002; Naitza et al., 2002). Dredd then cleaves Imd, which in turn leads to the activation of TAK1 (Paquette et al., 2010; C.-H. Kim et al., 2014). TAK1 is a MAPKKK (mitogen-activated protein kinase kinase kinase) (Vidal et al., 2001; Silverman et al., 2003), which phosphorylates and activates the *Drosophila* IκB kinase (IKK) complex (Rutschmann et al., 2000b; Silverman et al., 2000; Lu et al., 2001).

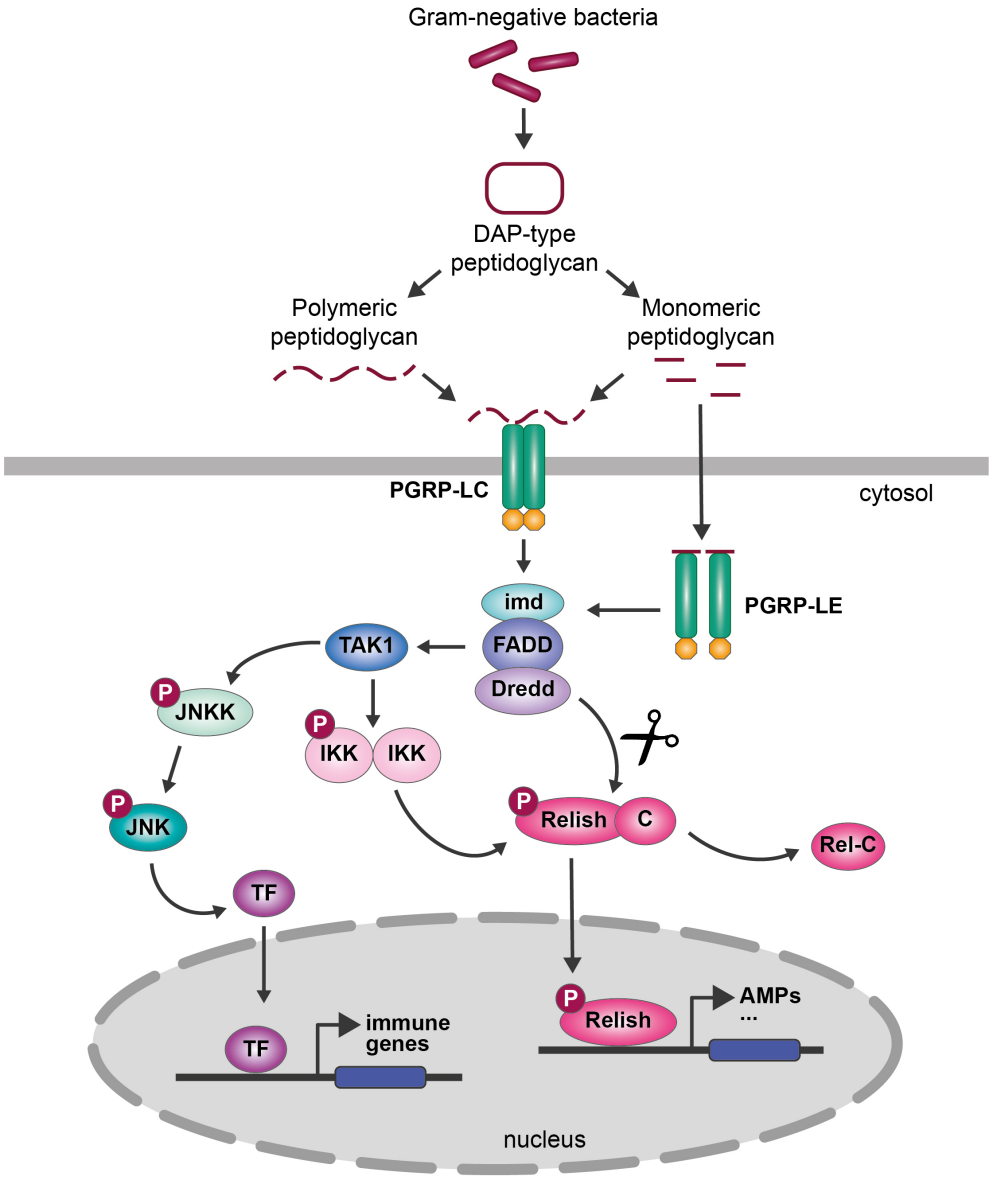

Figure 3 - The Imd pathway

Diaminopimelic acid (DAP)-type peptidoglycan (PGN) from the cell wall of Gram-negative bacteria is recognized by the transmembrane peptidoglycan recognition protein (PGRP) PGRP-LC and by PGRP-LE, which binds monomeric PGN in the cytosol. This leads to the recruitment and activation of a signalling complex comprising the death domain protein Imd, the adaptor protein FADD and the caspase Dredd. Following cleavage by Dredd and phosphorylation by the IKK complex, which is activated by TAK1, the N-terminal fragment of the NF-κB-like transcription factor Relish translocates into the nucleus and initiates the transcription of antimicrobial peptides (AMPs) and other immune effector genes. TAK1 additionally activates JNK-signalling (TF, transcription factor).

Relish, the NF-κB-like transcription factor of the Imd pathway, is located in the cytoplasm in unstimulated conditions. Following the initiation of the immune response, Relish is activated via phosphorylation by the IKK complex and cleavage of its C-terminal part by the caspase

Dredd (Silverman et al., 2000; Stöven et al., 2000; Stöven et al., 2003; Ertürk-Hasdemir et al., 2009). While the C-terminal fragment remains in the cytoplasm, the N-terminal fragment translocates into the nucleus, where it initiates the transcription of AMP genes such as *diptericin* and other immune effectors (Stöven et al., 2000). However, the role of TAK1 in Imd signalling remains controversial, as other studies have reported TAK1 to be dispensable for Relish activation (Delaney et al., 2006).

The *Drosophila* Imd pathway splits at the level of TAK1, as TAK1 not only functions in the NF-κB/Relish-mediated activation of AMP expression, but also triggers JNK signalling (Silverman et al., 2003; Kallio et al., 2005). JNK pathway activation leads to the transcription of cytokines and components for cytoskeletal remodelling and has been implicated in the reciprocal downregulation of NF-κB signalling, but could likewise be essential for the transcription of immune effector genes such as AMPs (Stronach et al., 1999; Boutros et al., 2002; J. M. Park et al., 2004; T. Kim et al., 2005; Delaney et al., 2006; L. K. Kim et al., 2007).

1.5.1.3 Local immune defence in the gut

The epithelial tissues of the skin, respiratory organs and the digestive system constitute important physical barriers against intruding pathogens. The gut epithelium is the first line of defence against intestinal microbes and pathogens taken up with the food (Sansonetti, 2004). In the *Drosophila* gut, the persistence of pathogens is impeded due to peristaltic movements, regions with low acidity and the presence of lysozymes (Miguel-Aliaga et al., 2018). Another physical and biochemical layer of protection against digestive enzymes as well as oral pathogenic infection is the peritrophic matrix, a non-cellular layer of chitin and glycoproteins that coats the gut epithelium (Hegedus et al., 2009; Kuraishi et al., 2011).

The local protection against pathogens moreover relies on two defence mechanisms of the inducible innate immune response, i.e. the local production of AMPs and the generation of reactive oxygen species (ROS). Similar to the systemic immune response, the local synthesis of AMPs is triggered via the Imd pathway upon recognition of bacterial PGN (Buchon et al., 2009b). Interestingly, sensing of PGN in the gut is regionalized, with recognition of PGN depending primarily on PGRP-LC in the proventriculus in the anterior part of the gut and on PGRP-LE in the midgut (Bosco-Drayon et al., 2012; Neyen et al., 2012). The second local defence mechanism is the production of microbicidal ROS by the NADPH enzyme dual oxidase (Duox) or the NADPH oxidase Nox (Ha et al., 2005; Ha et al., 2009; R.

M. Jones et al., 2013). Bacteria-derived uracil of pathogenic bacteria was shown to activate Duox and generate ROS, with the uracil/Duox pathway moreover increasing defecation upon oral infection with pathogens (K.-A. Lee et al., 2013; Du et al., 2016).

Yet the generation of ROS also inflicts damage upon the gut epithelium. In order to ensure recovery from the infection, the local immune response in the gut is thus accompanied by epithelial repair mechanisms. Infection with pathogenic bacteria triggers stem cell proliferation and epithelium renewal, with the oxidative burst inducing stem cell activation via JAK-STAT and JNK signalling (Buchon et al., 2009a; Buchon et al., 2009b; Jiang et al., 2009). However, despite all these protective measures, PGN fragments are able to cross the epithelial barrier in the gut and can thereby elicit a systemic immune response via the fat body (Zaidman-Rémy et al., 2006; Neyen et al., 2012; Charroux et al., 2018).

1.5.1.4 Immune signalling and metabolism

Infection with pathogenic microbes not only activates the immune response, but also disturbs metabolic processes, as the animal needs to allocate more energy to the fight against intruding pathogens. Hence, the host typically undergoes metabolic adaptations in response to an infection to inhibit survival of the pathogen and prevent the depletion of nutrients (e.g. Eisenreich et al., 2013).

In *Drosophila*, the interplay between the immune system and metabolism is already visible on the macroscopic level, as the fat body is essential for both energy storage and the systemic humoral immune response. At the transcriptional level, this link is for example illustrated by the transcription factor Mef2 in the fat body, which functions as an immune-metabolic switch. In healthy flies, Mef2 is phosphorylated and promotes the expression of anabolic enzymes, whereas infection causes dephosphorylation of Mef2, which thereupon triggers AMP expression (Clark et al., 2013). Another mechanism mediating the systemic metabolic switch necessary to efficiently combat bacteria relies on adenosine signalling, which induces the release of glucose from glycogen during pathogenic infections (Bajgar et al., 2018). Interestingly, AMP expression can be induced independently of the NF-κB signalling pathways of the innate immune system. In non-infected, starved flies, the forkhead transcription factor FOXO, which is essential for adjusting metabolism to nutrient conditions, can directly activate AMP expression, thus pointing to a cross-regulation of innate immune responses and metabolism (Becker et al., 2010). Accordingly, *Foxo* mutant

flies also exhibit higher bacterial load and reduced survival upon oral infection with pathogenic bacteria (Fink et al., 2016).

In *Drosophila*, in particular the Imd pathway has been linked to metabolic processes and the regulation of metabolic homeostasis. For example, the NF-κB transcription factor Relish has been suggested to regulate the expression of metabolic genes that are induced by the microbiota in the midgut, which indicates a role for Relish in the response to both beneficial microbiota and harmful pathogens (Combe et al., 2014). Moreover, the microbial metabolite acetate from intestinal bacteria triggers Imd pathway activation in enteroendocrine cells via PGRP-LC, which in turn increases tachykinin transcription, a neuropeptide involved in glucose and lipid metabolism, and thus prevents excess accumulation of lipid droplets in enterocytes (Kamareddine et al., 2018). Pathogenic infection was also shown to induce physiological disorders, as chronic infection with *Ecc15* leads to lipid droplet reduction and autophagy in the fat body as well as organ wasting due to an excess of circulating PGN and intracellular PGN-dependent signalling (Charroux et al., 2018). Continuous activation of the Imd pathway in the fat body triggers depletion of fat reserves, induces hyperglycemia and delays development, while the disruption of Imd signalling in *imd* mutants is accompanied by weight gain and impaired glucose tolerance and increased lipid and glucose storage (Davoodi et al., 2019). Interestingly, however, loss of Imd signalling does not seem to have an effect on total food consumption (Davoodi et al., 2019). Thus, there is more and more evidence that the Imd pathway controls metabolic homeostasis in addition to its role in the response to pathogenic bacteria, pointing to the close connections between immune and metabolic processes.

1.5.2 *Drosophila* pathogens: *Erwinia carotovora* and *Pseudomonas entomophila*

In its natural environment, *Drosophila* is constantly exposed to a variety of beneficial as well as potentially detrimental microbes. Two common entomopathogenic microorganisms that have been widely studied and moreover infect wild *Drosophila* populations are *Erwinia carotovora carotovora 15* (*Ecc15*) and *Pseudomonas entomophila* (*Pe*).

Erwinia carotovora are phytopathogenic Gram-negative enterobacteria that cause soft rot in plants such as potatoes (Pérombelon et al., 1980; Toth et al., 2003). These bacteria can exploit insects like *Drosophila melanogaster* as vectors for plant-to-plant transmissions

(Kloepper et al., 1981; Nadarasah et al., 2011). However, *Erwinia carotovora* also use fruit flies as a host, as natural infection by one *Erwinia* strain, *Ecc15*, induces both a local and a systemic immune response leading to the upregulation of AMPs via the Imd pathway (Basset et al., 2000; Tzou et al., 2000). *Ecc15* can infect *Drosophila* due to the effects of two genes, *homologue of Rap* (*hor*) and *Erwinia* virulence factor (*evf*). Hor has a regulatory function in phytopathogenicity and additionally controls *evf* expression (Thomson et al., 1997; Basset et al., 2003). By contrast, *evf* is required for the infectious properties of *Ecc15*: *evf* allows bacteria to persist in the gut of *Drosophila* larvae and triggers the activation of the immune response, while *Ecc15* lacking *evf* are quickly cleared from the gut (Basset et al., 2003). Evf is not a toxin and does not directly counteract the Imd pathway or provide protection against AMPs, but is thought to confer infectious properties by promoting persistence in the anterior midgut, even though *Ecc15* does not destroy the peritrophic matrix (Muniz et al., 2007). Feeding on wild-type *Ecc15* is usually not lethal for *Drosophila* (e.g. Zaidman-Rémy et al., 2006; Chakrabarti et al., 2012). However, an *Ecc15* strain that overexpresses *evf* by expressing it under the control of a constitutive promoter in a pSC101 plasmid derivative called *pOM1* allows *Ecc15* to persist in the gut for longer periods of time and thus increases lethality among infected flies (Espéli et al., 2001; Basset et al., 2003; Muniz et al., 2007).

While *Ecc15* is only mildly virulent even when overexpressing *evf*, a second Gram-negative bacterial strain that naturally infects *Drosophila*, the soil bacterium *Pe*, is highly pathogenic and kills a large fraction of infected flies. *Pe* was originally isolated from a fly in Guadeloupe and triggers a strong local and systemic immune response via the Imd pathway following oral infection (Vodovar et al., 2005; Mulet et al., 2012). As opposed to *Ecc15*, *Pe* virulence is multi-factorial. One way in which *Pe* can evade the immune response of *Drosophila* and avoid clearance from the gut is via the secretion of the zinc metalloprotease AprA that directly counteracts the antimicrobial action of AMPs in the gut (Liehl et al., 2006). The expression of *aprA* depends on the GacS/GacA system. The GacS/GacA two-component system comprises the GacS transmembrane sensor kinase and the response regulator GacA and is involved in the regulation of a variety of processes such as secondary metabolite production and is essential for *Pseudomonas* virulence (Heeb et al., 2001). While *Pe aprA* deficiency only attenuates pathogenicity and nevertheless triggers AMP expression, *Pe gacA* mutant bacteria are completely avirulent and do not induce a systemic immune response

after oral infection (Vodovar et al., 2005; Liehl et al., 2006). Another factor that contributes to *Pe* virulence independently from the GacS/GacA system is the *Pe* virulence factor (*pvf*) gene cluster, whose products are involved in secondary metabolite synthesis required for *Pe* virulence, meaning that *Pe pvf* mutant bacteria do not persist in the gut of *Drosophila* or induce immune response activation (Vallet-Gely et al., 2010).

The main reason why fruit flies succumb so quickly to an infection with *Pe* is that *Pe* infection leads to a massive destruction of the gut. *Pe* produces a pore-forming toxin regulated by the GacS/GacA two-component system that disrupts membrane permeability and induces cell death (Opota et al., 2011). In addition, the AprA protease secreted by *Pe* can degrade a specific peritrophic matrix protein, thereby facilitating the activity of pore-forming toxins (Shibata et al., 2015). At the same time, *Pe* infection leads to an overshooting stress response in its host manifested in a massive production of ROS and a global translational arrest, meaning that epithelium renewal is disrupted and the damage inflicted upon the gut by the oxidative burst is not repaired (Buchon et al., 2009a; Chakrabarti et al., 2012).

1.5.3 Modulation of behaviour by pathogens and the immune system

Much is known about the signalling components and the mechanisms underlying the innate immune response upon infection. However, the protection against pathogens taken up with food has many facets beyond the activation of the immune response. Animals adapt their behaviour to avert exposure to pathogens or alleviate the impact of an infection once bacteria are ingested; a range of behaviours subsumed under the term behavioural immunity (Pacheco-López et al., 2011). Furthermore, they can learn to avoid future infections. Conversely, pathogens can also manipulate their host's behaviour to increase dissemination and their own fitness.

Innately aversive cues are danger signals that can indicate the presence of pathogens in a food source and elicit avoidance reactions. Prior to ingestion, animals can detect pathogens by their smell or taste and thereby adjust their behaviour and refrain from feeding. *Drosophila* can for instance sense geosmin, an odorant produced by specific toxic fungi and bacteria, which activates a functionally segregated olfactory circuit and elicits feeding aversion and a reduction in egg-laying (Stensmyr et al., 2012). Flies can moreover detect highly dangerous parasites via their olfactory system, as larvae and ovipositing flies are innately repelled by

food or oviposition sites scented with the odour of parasitic wasps that frequently attack *Drosophila* larvae (Ebrahim et al., 2015).

However, olfactory cues can be deceptive and trick flies into approaching bacteria-contaminated sites, as can be seen from the pathogenic bacterial strain *Pe*, which can manipulate the social communication of *Drosophila*. While healthy flies avoid both feeding and egg-laying on pathogenic *Pe* compared to LB medium, they are attracted to the odour of infected flies or their frass as opposed to that of uninfected flies; a behaviour that was shown to be due to an increased fatty-acid-derived pheromone release in infected flies (Keesey et al., 2017). This increase required Imd immune signalling as well as the insulin receptor pathway and thus facilitated dispersal of the bacteria by attracting healthy flies to sites of high bacterial load.

Moreover, flies can recognize toxins or harmful microbes via their gustatory system. A narrowly tuned GR, Gr8a, is for example required to detect the plant insecticide L-canavanine and induce avoidance (Y. Lee et al., 2012). In the case of bacteria, LPS from the membrane of Gram-negative bacteria serves as a crucial indicator of food contamination and can elicit innate avoidance or otherwise adaptive behavioural responses in *Drosophila*. For instance, flies avoid walking on a substrate containing LPS, and bacteria as well as LPS induced grooming in decapitated flies, an important hygienic measure to reduce the possibility of infection, via contact chemoreceptors on the wings (Yanagawa et al., 2014). Grooming induction in decapitated flies upon exposure to LPS or monomeric PGN was suggested to be mediated by the transmembrane immune receptor PGRP-LC and required taste receptors such as the bitter receptors Gr66a and Gr33a or Ir76b (Yanagawa et al., 2017; Yanagawa et al., 2019). In addition, flies evade food supplemented with LPS both during feeding and oviposition due to the detection of LPS by TRPA1 in GRNs expressing the bitter receptor Gr66a (Soldano et al., 2016). *Drosophila* hence relies on its chemosensory system to detect components of potentially harmful microbes such as LPS that indicate the contamination of a food source.

Nevertheless, if innately aversive cues are absent from a food source, animals will ingest pathogen-infested food and experience the corresponding negative post-ingestive consequences such as infection and intestinal malaise. Apart from the activation of the innate immune response, ingestion of harmful microbes has been linked to a range of behaviours

that aim at alleviating the negative impact of the infection. In *Drosophila*, oral infection with pathogenic bacteria was shown to inhibit further food uptake. The highly virulent Gram-negative *Pe* strain for example induces food-uptake cessation (Liehl et al., 2006). A similar phenotype was observed in the case of the less pathogenic *Ecc15* strain: *Drosophila* larvae significantly decreased their food-intake on *Ecc15*-contaminated food (Keita et al., 2017). This decrease was not mediated by the Imd pathway or sensing of bacterial uracil, but required TRPA1 and the obligate olfactory co-receptor ORCO. Hence, similar to the gustatory avoidance of LPS also mediated by TRPA1 (Soldano et al., 2016), the reduction of feeding following exposure to pathogenic bacteria relied on chemosensory rather than post-ingestive mechanisms. In addition, infection with *Listeria monocytogenes* and *Salmonella typhimurium* – as well as a mutation in the taste receptor Gr28b – was shown to cause anorexia, which aided or impeded survival dependent on the bacteria strain (Ayres et al., 2009). Another way in which *Drosophila* adapts its behaviour in response to the uptake of harmful microbes is by increasing defecation to accelerate the expulsion of pathogens. Ingestion of uracil-producing *Ecc15* or pure uracil increased defecation via TRPA1 and the activation of Duox signalling, with TRPA1 being required in a subset of enteroendocrine cells (Du et al., 2016).

Furthermore, an infection with pathogens usually affects an animal's overall activity patterns and sleep. Sleep has been suggested to serve as an adaptive response during an immune challenge to facilitate elimination of the pathogen (Besedovsky et al., 2019). For instance, a study comparing sleep duration and white blood cell count across several mammalian species found that increased sleep was associated with a higher number of circulating immune cells as well as lower parasitic load (Preston et al., 2009). Moreover, components of the innate immune response in mammals such as TNF-α are known to promote sleep (Rockstrom et al., 2018). In *Drosophila*, many of the genes that are upregulated following sleep deprivation are implicated in the innate immune response, like the NF-κB transcription factor Relish (Williams et al., 2007). Infecting flies by injecting Gram-negative bacteria increased sleep, with Relish being required to promote sleep in the course of the immune response (Kuo et al., 2010). In addition, flies that exhibited enhanced sleep due to decreased neuronal excitability in the MB had a higher probability of surviving an infection with Gram-negative bacteria (Kuo et al., 2014), suggesting that sleep supports fighting off pathogens. Interestingly, a recently identified AMP called nemuri has been shown to induce

sleep. Flies lacking nemuri slept less after infection with pathogenic bacteria, while overexpression in neurons promoted survival upon infection, pointing to a crucial role for an AMP in the regulation of sleep during periods of high sleep need such as infection (Toda et al., 2019).

However, while the interactions between the immune response and sleep are slowly being unravelled, the detailed mechanisms underlying other behavioural adaptations to an infection with detrimental bacteria regarding for example feeding or oviposition aversions are largely unknown. Some evidence points to a contribution of immune signalling directly in the brain: infecting flies with *E. coli* induced a decrease in egg-laying that was elicited by PGN-mediated NF-κB pathway activation in a group of octopaminergic neurons in the brain (Kurz et al., 2017; Masuzzo et al., 2019). More support for a role of octopamine in bacteria-modulated behaviour comes from the observation that a specific enzyme of a commensal bacterial strain of the *Drosophila* microbiome influences locomotor activity via octopamine signalling (Schretter et al., 2018). Other components of the innate immune system can be found in the nervous system, too. The transmembrane receptor of the Imd pathway, PGRP-LC, regulates homeostatic synaptic plasticity in the nervous system (Harris et al., 2015). Some AMPs may also have been repurposed in the central nervous system, since two specific AMPs are required for forming both long-term appetitive associative memories and memories of an unsuccessful mating experience (Barajas-Azpeleta et al., 2018).

An important aspect of the behavioural defence mechanisms against pathogens are the behavioural adaptations following the ingestion of contaminated food. Conditioned food aversions (or CTA, see 1.4.4), i.e. associating a food source with the negative post-ingestive consequences caused by pathogen uptake, are essential to avoid repeated infections and ensure survival. Moreover, avoidance reactions following the ingestion of pathogens can considerably reduce the impact of an infection. Not surprisingly, these adaptive behaviours and 'negative food memories' are wide-spread across the animal kingdom. Nevertheless, little is known about the neural mechanisms guiding adaptive behaviour and memory formation in response to pathogen ingestion. Studies in the honeybee and *C. elegans* implicate serotonin in integrating negative post-ingestive signals during the formation of conditioned food aversions (Y. Zhang et al., 2005; Wright et al., 2010), while evidence from

pathogen-modulated behaviour in *Drosophila* points to a role of octopamine and immune signalling in the brain (Kurz et al., 2017; Masuzzo et al., 2019).

1.6 Aims of this thesis

The fruit fly *Drosophila melanogaster* feeds on rotten and fermenting fruit, where it is constantly exposed to an abundance of beneficial as well as harmful microbes. Some of the pathogenic microbes elicit innate avoidance via their smell or taste, allowing the fly to refrain from feeding. However, if pathogens evade detection by the external sensory systems, they are taken up with the food, trigger an immune response and potentially damage internal organs or jeopardise survival. Hence, animals have to be able to adapt their behaviours once ingested pathogens are detected and ideally remember the chemosensory perception of a food source that made them sick in order to avoid it and ensure survival. There are many open questions surrounding this avoidance of food that caused malaise, or so-called conditioned food aversion, as it requires the interaction between the chemical senses, the gut, the immune system and the brain to guide adaptive behaviour. What is the nature of the signals transmitting the information about an infection from the body to the brain? How does the brain integrate signals from the chemosensory systems, the gut and the immune system to trigger lasting behavioural changes? What are the neural circuits required for the adaptation of behaviour in response to pathogenic infection or for the formation of memories of past infections?

In this thesis, I aimed at investigating the behavioural responses of the fruit fly *Drosophila melanogaster* to an infection with pathogenic bacteria in order to unravel the neural and immune mechanisms that underlie acquired food aversions following the ingestion of pathogens.

A food source can be contaminated with pathogenic bacteria. Establishing an oral infection paradigm using Gram-negative bacteria allows investigating the consequences of a natural pathogenic infection and its corresponding post-ingestive effects. How virulent are the different bacterial strains used? Moreover, does infection with pathogenic bacteria induce any kind of obvious, immediate 'sickness behaviours' which are detectable as a change in locomotor activity or circadian rhythm?

Odours can be important indicators for the contamination of a food source. Thus, do flies innately prefer or avoid the smell of pathogenic bacteria? Decreased attraction to or avoidance of the odour of pathogenic bacteria after oral infection with those bacteria could indicate the formation of an associative olfactory memory. Accordingly, can flies associate bacterial odours with the negative post-ingestive consequences of pathogen infection and thus learn to avoid that odour?

A detrimental food source can elicit avoidance reactions mediated via taste, or, in the case of food uptake, potentially via post-ingestive mechanisms. Thus, does *Drosophila* display feeding aversions to pathogenic bacteria and can flies distinguish between food sources containing harmless or pathogenic bacteria, respectively? Immediate rejection of pathogenic bacteria could indicate a contribution of taste, while a delayed avoidance would point to a contribution of post-ingestive effects.

If flies can adapt their behaviour and avoid food contaminated with pathogenic bacteria following ingestion, what are the mechanisms underlying this behaviour? Associative memory formation and learning requires the mushroom body and the involvement of synaptic plasticity. If flies indeed exhibit an acquired feeding aversion to pathogenic bacteria, this behaviour should be mediated by the mushroom body.

Ingestion of pathogens activates the innate immune system. Thus, what is the contribution of the Imd pathway, the immune signalling pathway responding to Gram-negative bacteria, in the behavioural adaptation to pathogenic bacteria? Are specific components of the Imd pathway involved in triggering avoidance to pathogens and where exactly are they required? Understanding if and how the immune system interacts with the nervous system as a consequence of pathogen ingestion could provide an insight into a mechanism guiding adaptive behaviour and memory formation following the uptake of detrimental food to avert repeated infections in the future.

2. Methods

2.1 Flies

2.1.1 Fly husbandry

Drosophila melanogaster flies were raised and stored in a 60% humidified incubator with a 12 h / 12 h light-dark cycle. Experimental fly lines were kept at 25°C; fly stocks at 18°C. All flies were reared on standard cornmeal medium (per 100 L: 1170 g agar, 10 kg corn flour, 1 kg soya flour, 1850 g brewers' yeast, 4 kg diamalt, 4 kg sugar beet syrup, 250 g methyl paraben and 1 L 10% phosphoric acid) and flipped onto fresh food twice a week (at 25°C) or every three weeks (at 18°C). Experimental flies were generally collected directly after hatching, sorted on ice the following day and aged 3-7 days prior to experiments. Unless stated otherwise, all experiments were conducted using mated female flies, i.e. females that had been housed with males for a minimum of 24 h.

2.1.2 Fly lines and crosses

Table 1 provides an overview of all fly lines used in this study. Experiments investigating wild-type behaviour were conducted with the Canton-Special (CS) and Oregon-R (OrR) fly lines. In all other cases, either flies carrying a mutation or transgenic fly lines relying on the Split-GAL4 > UAS or the GAL4 > UAS expression systems were used to study the effects of a certain mutation on behaviour or to drive the expression of RNAi constructs or thermogenetic effector genes, respectively. Crosses were typically set up between 20-30 males and ~60 virgin females and kept at 25°C until eclosion.

For flies where a large number of virgins was needed on a regular basis (w^+, w^-, UAS-shibire^{ts1}), a virginated version of the respective line was used. In these virginator fly lines, males carry a heat-shock–inducible conditional lethal *hid* gene on the Y chromosome, which eliminates all male larvae from a culture upon 2-4 h heat-shock at 37°C, hence leaving only females to develop (Starz-Gaiano et al., 2001; Venema, 2006).

Table 1 - Fly lines

Name	Origin	FlyBase ID
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
diptericin-mCherry	Gift from François Leulier	N/A
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
actin-GAL4	Bloomington DSC	FBst0004414
Lpp-GAL4	Gift from Irene Miguel-Aliaga	N/A
takeout-GAL4	Gift from Carla Margulies	N/A
mex-GAL4	Gift from François Leulier	N/A
nSyb-GAL4	Bloomington DSC	FBst0051635
pBDP-GAL4U	Bloomington DSC	FBst0068384
MB10B-GAL4	Janelia Farm Research Campus	FBst0068293
rutabaga^{2080}	Bloomington DSC	FBst0009405
Tdc2-GAL4	Bloomington DSC	FBst0009313

2.1.3 Starvation

The present study revolved around the behavioural responses prompted by contact with pathogenic bacteria. Hence, nearly all of the experiments carried out for this thesis required flies to ingest bacteria and/or show olfactory responses to bacterial odours. To increase the motivation to track odours or to feed on bacterial substrates, flies were starved prior to the experiments using different protocols. For wet starvation, experimental flies were placed in bottles containing only tissue paper moistened with filtered water. Depending on the fly strain and the behavioural assay, wet starvation lasted between 12 and 42 h at 25°C and 60% humidity. Dry starved flies were kept in entirely empty bottles for a maximum of 5 h.

Since different fly strains are prone to starvation to varying degrees, the survival during wet starvation was determined for some of the frequently used fly lines. Flies were flipped into wet starvation bottles in groups of 100 flies/bottle and kept at 25°C and 60% humidity for up to 72 h. The number of dead flies was counted and recorded at various time points (8,

24, 32, 48 and 72 h) (appendix, Figure 45). The wet starvation period where ~5% of flies were dead was then considered suitable for feeding experiments.

2.2 Pathogenic infection

2.2.1 Bacterial strains

The bacteria used to infect *Drosophila* were two different Gram-negative pathogenic strains as well as harmless versions of the same strains that served as controls: *Erwinia carotovora* strains *Ecc15 pOM1-evf* (abbreviated as *Ecc15 pOM1*, pathogenic) and *Ecc15 evf* (avirulent) (provided by François Leulier) and the *Pseudomonas entomophila* (*Pe*) wild-type (pathogenic) and *gacA* (avirulent) strains (provided by Bruno Lemaitre). Bacteria plates (LB agar, for 1 L: 10 g NaCl, 10 g tryptone, 5 g yeast, 15 g agar for bacteriology) were inoculated once per week and otherwise stored at 4°C. For experiments, liquid cultures were commonly prepared from 400 ml lysogeny broth (LB) medium (for 1 L: 10 g NaCl, 10 g tryptone, 5 g yeast) in order to grow a sufficient amount of bacteria.

The *Ecc15* strains were streaked onto LB agar plates containing 100 μg/ml rifampicin (*evf*) (AppliChem, Darmstadt, Germany) or 100 μg/ml spectinomycin (*pOM1*) (#S4014, Sigma-Aldrich, St. Louis, MO USA) and incubated at 29°C for approximately 24 h. For the preparation of concentrated bacteria for experiments, single bacterial colonies were picked from LB plates and inoculated in liquid LB medium with 100 μg/ml rifampicin (*Ecc15 evf*) or 100 μg/ml spectinomycin (*Ecc15 pOM1*) overnight at 220 rpm and 29°C. After measuring the optical density (OD) at 600 nm, bacterial cultures were spun down (3500 rpm, 4°C, 20 min), washed with PBS and pelleted again (4000 rpm, 4°C, 20 min). The pellets were then resuspended in PBS, adjusted to the desired $OD_{600} \approx 200$ and stored at 4°C for a maximum of 24 h until usage.

The *Pe* strains were streaked onto LB agar plates containing 100 μg/ml rifampicin and 1% skim milk (#70166, Sigma-Aldrich), respectively, which were subsequently incubated at 30°C for a minimum of 24 h (*Pe* WT) or 30 h (*Pe gacA*). Skimmed milk serves as an indicator of pathogenicity, as pathogenic clones of *Pe* WT have proteolytic activity and hence form clear colonies on the otherwise turbid LB/milk agar plates. By contrast, *Pe gacA* lacks this protease;

meaning that plates remain hazy. For the preparation of a concentrated bacterial pellet, 40 ml LB medium containing 100 µg/ml rifampicin was inoculated with a protease-positive *Pe* WT clone (i.e. a clear colony) or a single colony of *Pe gacA*, respectively, for 8 to 12 h at 250 rpm and 30°C. An overnight culture was then prepared by diluting the pre-culture 1:16 in LB medium containing 100 µg/ml rifampicin and incubated for a minimum of 16 h at 250 rpm and 30°C. After measuring the OD at 600 nm, the two bacterial cultures were spun down (2500 g, 4°C, 15 min), most of the supernatant was removed and the pellets were quickly resuspended in the remaining medium. The concentrated bacterial suspension was adjusted to the desired $OD_{600} \approx 200$ with PBS and stored at 4°C for a maximum of 24 h until usage.

In some experiments, heat killed pathogenic bacteria served as a control instead of the harmless strains. For that purpose, the concentrated bacteria suspension was kept shaking at 95°C for 15 min, followed by a cold shock at -20°C for 5 min.

2.2.2 Natural bacterial infection

Since throughout this study, I sought to understand if and how pathogen infection caused by ingesting contaminated food induces behavioural adaptations and memory formation, it was crucial to emulate a natural bacterial infection in a laboratory setting. Thus, contrary to the injection of bacteria via pricking (i.e., septic injury) frequently used to study *Drosophila* immunity (Neyen et al., 2014), I established a protocol for the natural oral infection of flies by letting them directly feed on bacteria. Several measures had to be taken to ensure that flies were indeed ingesting the bacteria: first, flies were dry starved for 3-5 h before bacteria feeding to deprive them of both water and food and hence considerably increase their motivation to feed and drink. For the same reason and to moreover mask the bitter taste of the bacterial strains, the bacteria solution was mixed with sucrose. Finally, bacteria were fed to the flies at high concentrations to allow for a reliable pathogenic infection.

For the oral infection, the respective bacterial solution with an $OD_{600} \approx 200$ (see 2.2.1) was mixed in equal parts with a 10% sucrose solution to achieve a final concentration of $OD_{600} \approx 100$ and 5% sucrose. Flies were then placed into a standard fly bottle containing 1.5% agarose to provide humidity and a filter paper soaked with bacteria-sucrose solution.

Depending on the specific experimental paradigm, flies were allowed to feed on the bacteria for 3 to a maximum of 24 h at 25°C and 60% humidity.

2.2.3 Survival analysis

One measure to validate successful infection after bacteria feeding was to conduct a survival analysis. For that purpose, flies were fed either the pathogenic bacterial strain (*Ecc15 pOM1*, *Pe* WT) or the respective harmless strain (*Ecc15 evf*, *Pe gacA*) as a control. Groups of 80 flies per bottle were orally infected with bacteria according to the protocol described in 2.2.2. Flies were incubated on the bacteria solutions for 24 h and then transferred back onto standard fly food. Dead flies were counted after overnight feeding (~17 h), 20 h, 24 h, 26 h, 40 and 48 h post infection (Figure 4).

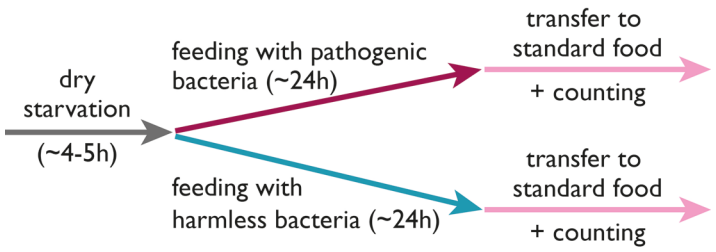

Figure 4 - Protocol for survival analysis

Following 4-5 h of dry starvation, flies were placed into standard fly bottles (80 flies/bottle) containing a mixture of sucrose and either the pathogenic or the harmless bacterial strain. After 24 h of bacteria feeding, flies were transferred back onto standard fly food. Dead flies were counted at different time points (17/20/24/26/40/48 h).

2.3 Behavioural assays

2.3.1 *Drosophila* Activity Monitor

The *Drosophila* Activity Monitor (DAM) system (TriKinetics Inc, Waltham, MA USA) allows the recording of a fly's locomotor activity over the course of several days and hence provides insights into its circadian rhythm and general motor behaviour. Infected flies were tested in the DAM to determine if pathogen infection leads to changes in overall locomotor activity or circadian rhythm. For the purpose of this study, the MB5 Multibeam Activity Monitor was used, which comprises 16 separate tubes to simultaneously record the activity

of 16 individual flies. During an experiment, each tube is crossed by 17 infrared beams, which are interrupted as the fly walks along the tube and thereby record activity counts. Combined with the information about light-on and -off periods, this assay provides a detailed picture of a fly's movements, activity peaks and rest phases over several days.

For DAM experiments, virgin females had to be used, as egg-laying and the subsequent hatching of larvae would have interfered with activity recordings. Female OrR virgins were either infected with pathogenic (*Ecc15 pOM1* or *Pe* WT) or harmless bacteria (*Ecc15 evf* or *Pe gacA*) according to the standard infection protocol described in 2.2.2. Following a period of dry starvation, flies were allowed to feed on the bacteria for approximately 3 h before being transferred into DAM tubes to start the recording. Experiments were conducted in a 12 h / 12 h light-dark cycle at 60% humidity and 25°C and stopped after roughly three days.

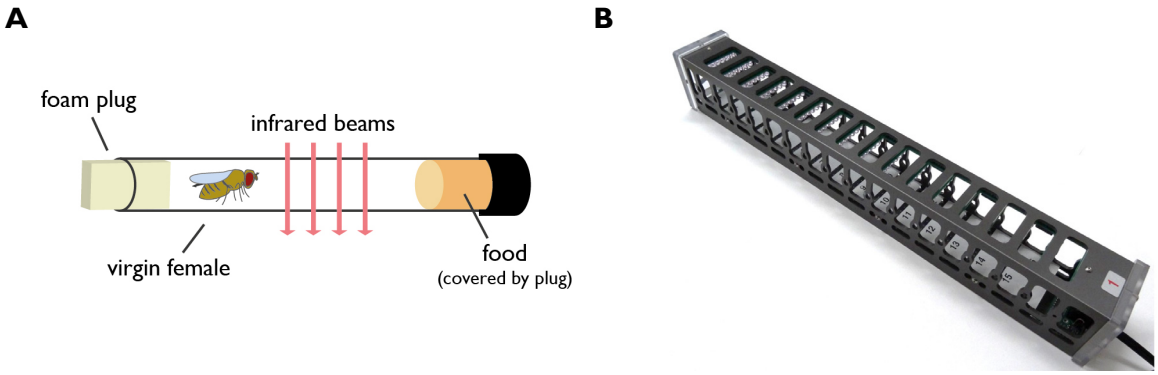

Figure 5 - The *Drosophila* Activity Monitor

(A) Illustration of an activity monitor tube. 17 infrared beams bisect each tube, which allows for the monitoring of a virgin fly's movements along the tube by recording beam crossings. Each tube is enclosed by standard fly food and a foam plug. **(B)** Picture of the activity monitor setup.

The raw data was processed using *DAM File Scan* (TriKinetics Inc), which produces new output files within a specified date/time range and at a desired bin length and ensures that the data records are complete, i.e. without gaps or duplicate readings. For the purpose of this study, only activity counts, namely beam crossings, were analysed to compare overall locomotor activity between flies infected with pathogenic bacteria and those infected with the harmless strains. Since a fly's activity counts over time exhibit bursts and are of an intermittent, i.e. irregular nature, the so-called burstiness parameter was calculated as a measure for the distribution of the interevent time τ between two successive events (Goh et al., 2008) by Dr. Sophie Aimon.

Burstiness was computed according to the following formula, with m_τ and σ_τ being the mean and the standard deviation of the Poisson distribution $P(\tau)$, respectively:

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

In addition, Dr. Sophie Aimon quantified the rhythmicity of the flies' locomotor activity patterns as the maximum of the activity autocorrelation (considering only flies living more than 24 h). Accordingly, flies could be divided in three groups: flies with an autocorrelation peak at 24 h, flies with a peak at 12 h, and flies with an autocorrelation peak inferior to 12 h that suggested no periodicity. To determine whether the number of flies in the 24 h or the 12 h and less group was affected by infection with the specific bacterial strain, we employed the chi-squared test of independence.

2.3.2 The 4-field olfactory choice arena

The 4-field arena is a custom-made behavioural assay to determine the preference behaviour of freely moving adult *Drosophila* upon olfactory stimulation. While it is possible to use the 4-field arena for mere optogenetic stimulation as well as a combination of olfactory and optogenetic stimuli, only the olfactory paradigm was used for the purpose of this study, i.e. to investigate innate or learned behavioural responses towards bacterial odours. The 4-field arena was originally developed at Janelia Farm Research Campus following an earlier olfactometer design (Vet et al., 1983; Aso et al., 2014b; Aso et al., 2016). It was built in our laboratory by Dr. Laurence Lewis and Christian Schmid (Lewis et al., 2015), and refined for olfactory stimuli by Dr. Laurence Lewis, Christian Schmid and myself (see also Sayin et al., 2019).

The 4-field olfactory choice assay consists of the main body of the arena including an LED array and odour inputs and outputs as well as a camera, which are situated in a dark compartment, and an odour delivery system, which is placed on top of the enclosure (Figure 6A). The main body of the arena comprises various layers, most importantly the LED array and Arduino, a light guide, the arena floor comprising the odour output connector, the 4-arm odour input layer which also confines the space for the flies, an upper lid frame including the odour input connectors, a fly containment ring and a glass lid as the top layer. The circular behavioural arena itself has a diameter of 10 cm, is 3 mm high and is divided

into four equally sized quadrants underneath the arena floor level. These can be independently illuminated in the case of optogenetic experiments or flooded with odours for olfactory choice assays.

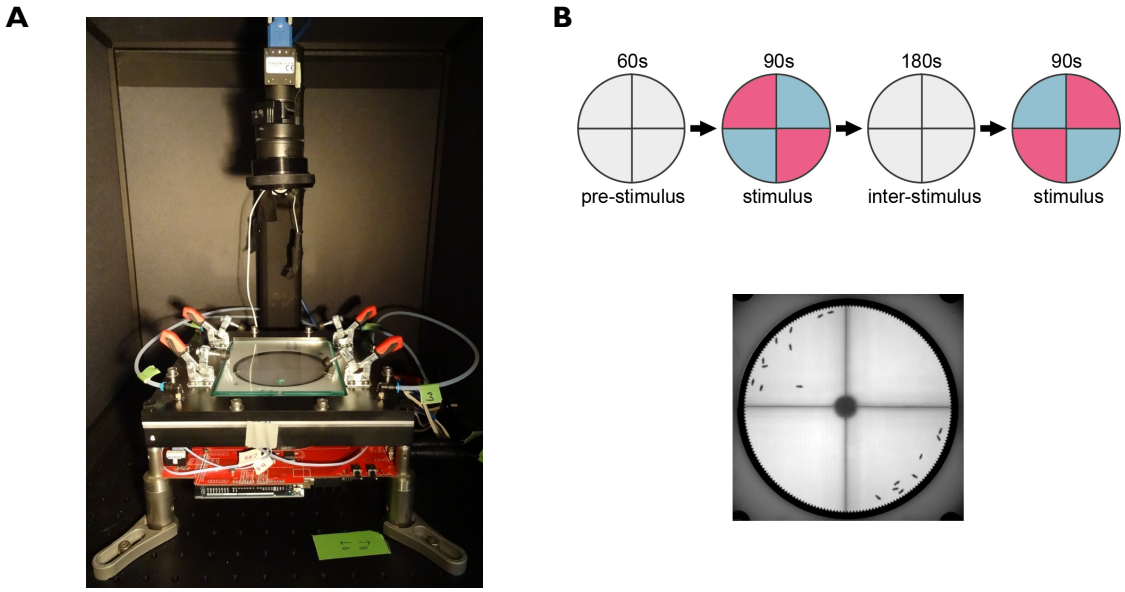

Figure 6 - Olfactory choice arena

(A) Picture of the main body of the arena with the IR-camera on top. **(B)** General experimental protocol for two-odour olfactory choice assays (top), top view of the arena with flies (bottom).

Experiments were conducted at 22-25°C and approximately 40-60% humidity. 15-25 flies were placed into the arena per experimental run, with a full experiment requiring 8-16 individual runs. During one run, flies were able to choose between four identical quadrants with different olfactory stimuli, which were introduced into the arena according to a pre-defined experimental protocol. A custom-made Matlab script was used both for the specification of the experimental paradigm and timing as well as for the execution of the experiments. The general protocol for olfactory experiments comprised a pre-stimulus phase for acclimatization (60 s) and a first stimulus phase (90 s) with the same stimulus being presented in two opposing quadrants (vs. air or another odour in the remaining two quadrants) (Figure 6). The subsequent inter-stimulus phase (typically 180 s) without odour and/or light was intended to allow the flies to return to baseline and was followed by a second stimulus phase (90 s), where the stimulus was reverted and presented in the remaining two quadrants to control for potential spatial bias (again vs. air or another odour in the other two quadrants) (Figure 6B). At the beginning of the pre-stimulus as well as the

inter-stimulus phase, the arena was flushed with air at the highest pump speed possible (air flow ~1000 ml/min).

The odour delivery system allows introducing the desired odour(s) into selected quadrants in a timed manner and at the preferred flow rate (Figure 7). It works via passive suction by a rotary vane pump (G12/01-4 EB, Gardner Denver Thomas GmbH, Fürstenfeldbruck, Germany), which is connected to the centre of the arena. Set to a flow rate of 200 ml/min, it creates negative pressure that sucks in air from the odour input connectors located at each corner of the arena, i.e. at each quadrant. Each input is connected to a valve (MFH-3-MF, Festo, Esslingen, Germany) with polytetrafluoroethylene (PTFE) tubing. These four valves can select between two possible inputs, which in turn select between two pairs of Schott bottles via additional valves. The bottles used for olfactory stimulation contain soluble odours, while the bottles for the blank 'air only' stimulus contain filtered water.

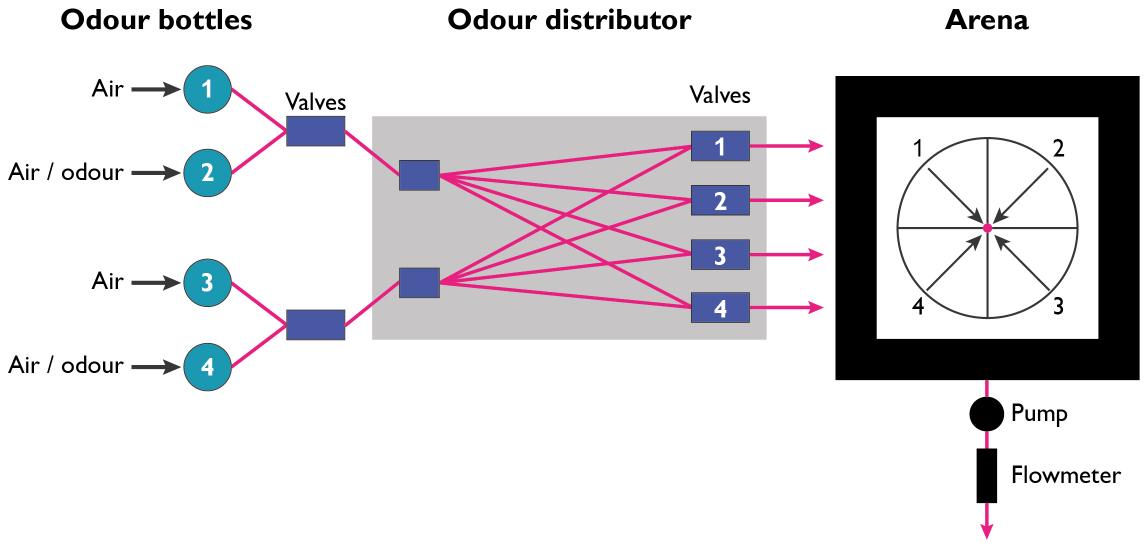

Figure 7 - The odour delivery system for the 4-field arena

A rotary vane pump connected to the centre of the arena and set to a flow rate of ~200 ml/min creates negative pressure and sucks in air from each of the quadrants, which are in turn connected to valves (1-4). These select between two possible inputs and via a further set of valves choose between water/air and a soluble odorant, respectively. In this way, the 4-field arena allows olfactory choice assays between a single soluble odorant and air or between two different odorants.

In addition, the 4-field arena is equipped with infrared LEDs, which provide background illumination. An infrared camera placed above the arena (Flea3 USB3 FL3-U3-13Y3M-C, FLIR Systems Inc., Wilsonville, OR USA) captured the behaviour of the flies throughout the

experiment (Figure 6). The video files were analysed using a custom-made Matlab script that extracted the flies' positions at every time point recorded (i.e. 15 frames per second) and calculated a preference index (PI) for each n as a measure for olfactory attraction or aversion according to the following formula:

$$Preference\ index = \frac{\#\ flies\ in\ quadrants\ 1\ and\ 3 - \#\ flies\ in\ quadrants\ 2\ and\ 4}{total\ \#\ flies}$$

For the PI calculation, the number of flies was averaged during the last five seconds of each stimulus period. The values for the PI range from -1 until 1, with -1 representing total aversion, 0 being neutral behaviour and 1 signifying total attraction.

During the adaptation of the 4-field arena for olfactory stimuli, wild-type CS female flies were starved for ~40 h and then tested for their preference to 1% balsamic vinegar (Alnatura, Germany) as a potent appetitive odour. For the present study, the 4-field olfactory choice arena was primarily used to determine the innate responses of *Drosophila* to the odour of pathogenic bacteria (*Ecc15 pOM1* and *Pe* WT, respectively) as well as to other olfactory choices involving bacteria (harmless vs. pathogenic bacteria, pathogenic bacteria vs. LB medium, pathogenic bacteria + yeast vs. yeast only). The bacterial odours were obtained from the supernatant that was left after pelleting the respective bacterial ON culture and stored for a maximum of 48 h at 4°C until usage. For choices involving yeast, 1.5 g of dry yeast was added to 40 ml of filtered water or bacterial solution in the odour bottles. Finally, flies were also tested in the 4-field arena to investigate a potential change in preference behaviour towards these bacterial odours after subjecting them to a variety of conditioning paradigms (see 2.4).

2.3.3 Capillary feeding assay

To examine feeding behaviour and a potential feeding aversion to pathogenic bacteria in *Drosophila*, we employed the Capillary Feeder (CAFE) assay. This assay was developed as a method to study food intake in *Drosophila* in a precise manner and in real-time (Ja et al., 2007). In the CAFE, flies feed on a liquid food source that is provided in a graduated glass capillary fixed at the top of a vial, with liquid decrease over time representing food intake (Figure 8). One criticism frequently directed at the CAFE assay is its unnatural setting, with

flies having to feed upside down from a capillary containing liquid food. However, food intake in flies was shown to be unaffected by the location of the food (Deshpande et al., 2014). The CAFE hence allows the direct, quantitative measurement of ingestion of individual or groups of flies in real-time. Since the capillaries can be exchanged with minimal disturbance to the flies, it is possible to monitor feeding behaviour over the course of minutes up to days or even the entire lifespan. For the purpose of the present study, food intake was typically measured for a duration of 9 h.

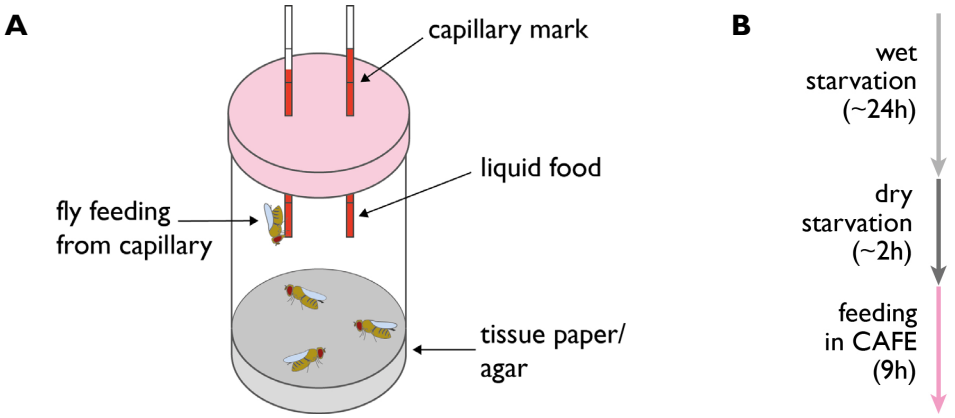

Figure 8 - Capillary Feeder

(A) Illustration of a CAFE chamber. Flies feed upside down from graduated capillaries containing liquid food. Tissue paper or agarose at the bottom of the vial provide humidity. **(B)** Standard protocol for CAFE experiments. Following ~24 h of wet starvation and 1-2 h of dry starvation, liquid food intake in the CAFE was measured every hour for a total of 9 h.

The CAFE chambers consisted of standard fly vials (85 mm x 25 mm) with tissue paper soaked in filtered water at the bottom of the vial to provide water and humidity. Disposable graduated glass microcapillaries (# 022.7142, CAMAG, Muttenz, Switzerland) filled with 5 µl of liquid food were poked through soft foam plugs and thus held in place at the top of the vials during experiments. For the present study, the CAFE assay was used to measure food intake in flies that were given a choice between two different food sources. Hence, two capillaries per chamber were filled with the respective test solution, i.e. sucrose or bacteria + sucrose solutions. To facilitate visualizing the descent of the meniscus, standard red food dye was added to all liquid food sources (0.05% Allura Red AC, also known as FD&C Red 40, colour index 16035, # 458848 Sigma-Aldrich).

For each experiment, female flies were put into wet starvation for approximately 24 h before the experiment (protocol Figure 8B). However, this also depended on the individual

fitness of each fly line, meaning that starvation times could range from 12 h to a maximum of 40 h (see also appendix, Figure 45). The next morning, flies were counted and put into dry starvation in numbers of 10 per vial for 1-2 h. While the flies were in dry starvation, CAFE chambers were prepared and the capillaries filled with the test solutions. Typically, 8-10 vials were tested per experimental group and day, and an n = 16 was required for a full set. The three different feeding choices tested for each fly strain were 5% sucrose vs. harmless bacteria (*Ecc15 evf* or *Pe gacA*), 5% sucrose vs. pathogenic bacteria (*Ecc15 pOM1* or *Pe* WT) or harmless vs. pathogenic bacteria (*Ecc15 evf* vs. *pOM1* or *Pe gacA* vs. WT). All bacterial solutions were $OD_{600} \approx 100$ and additionally contained 5% sucrose (for preparation of bacteria, see 2.2.1). For each feeding choice, a CAFE chamber containing the respective test solutions but no flies served as an evaporation control. After flipping the flies into the CAFE chambers, they were returned to a standard incubator (25°C, 60% humidity) and liquid decrease was measured on an hourly basis for 8-10 h. To determine the cumulative consumption per fly, the liquid decrease in the evaporation controls was subtracted from the decrease in experimental CAFE chambers, and this value was then divided by the total number of flies (i.e. 10, or less in case dead flies had to be excluded). Vials in which the cumulative consumption per fly for both capillaries combined was less than 0.03 µl after 9 h as well as vials in which flies did not consume food at all were excluded from further analysis. Data were obtained from a minimum of two independent experiments using different batches of flies and bacteria.

2.3.4 flyPAD

Another assay that was used to investigate feeding decisions regarding pathogenic bacteria was the fly Proboscis and Activity Detector (flyPAD) (Figure 9). The flyPAD is an automated behavioural assay based on capacitive measurements to quantify an individual fly's physical interaction with food and was developed by Pavel Itskov and colleagues at the Champalimaud Centre for the Unknown in Lisbon, Portugal (Itskov et al., 2014). The measurement of *Drosophila* feeding behaviour in the flyPAD relies on capacitive proximity sensors that detect capacitance changes across two electrodes. Within one flyPAD arena, the food is located on one electrode, which is surrounded by a second, circular electrode. In case a fly interacts with the food, it will stand on the second electrode and touch the food on the first electrode with its proboscis or legs, hence causing a change in capacitance that can be

measured (Figure 9A). Two independent channels can be recorded per arena, i.e. from two different food sources, which allows for the investigation of food choice behaviour in individual flies (Figure 9B). One flyPAD setup comprises up to 48 arenas for single fly experiments.

After the signals from each arena have been sent to a computer via a capacitance-to-digital converter and a FPGA-based multiplexing board, specifically developed algorithms for activity and feeding detection extract periods of activity and sips, respectively, since contact with the food or rhythmic proboscis extensions both generate characteristic patterns in the capacitance signal (Itskov et al., 2014). The flyPAD relies on the open-source software Bonsai (Lopes et al., 2015) for data acquisition via a custom-made script.

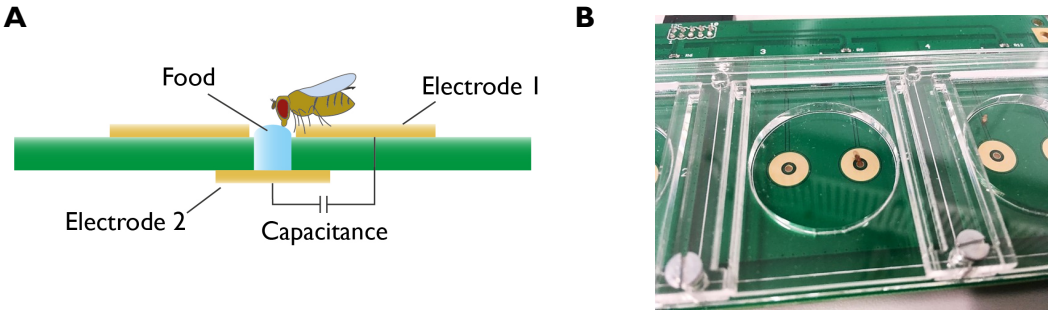

Figure 9 - fly Proboscis and Activity Detector

(A) Illustration of the concept of capacitive-based measurements in the flyPAD. A fly standing on electrode 1 causes a detectable change in capacitance once it physically interacts with the food placed on electrode 2 with its leg(s) or proboscis. **(B)** Picture of an individual flyPAD arena. One arena can record from two channels, i.e. from two different food sources.

The extracted behavioural metrics that correlate with actual ingestion are the number of activity bouts, the total duration of activity bouts and the number of sips. While the number of activity bouts had a weak correlation with food intake, the total duration of all activity bouts and in particular the number of sips correlated best with food intake (Itskov et al., 2014). In the present study, we hence primarily used the cumulative number of sips as a measure for ingestion. Other relevant parameters extracted are feeding bursts (number and duration), sip duration as well as a quadratic coefficient representing the strength of the satiation signal and a linear coefficient as a proxy for the motivation to feed (Itskov et al., 2014).

Due to the high-resolution measurement of feeding behaviour, preferences for one food source over the other can commonly be observed within one hour. One factor restricting the

duration of the experiment is the drying up of the food source, making it harder for the flies to feed. flyPAD experiments were thus limited to a maximum duration of 70 min. All experiments were performed in a 25°C and 60% humidified climate chamber. Prior to the experiment, flies were dry starved for 4-6 h.

The flyPAD was employed as a more high-resolution feeding assay and to corroborate findings from the CAFE regarding feeding behaviour towards pathogenic bacteria as well as to test additional feeding choices. The following choices were tested (preparation of bacteria and heat-killed bacteria see 2.2.1):

- 1% sucrose vs. 10% sucrose
- harmless vs. pathogenic bacteria (*Ecc15 evf* vs. *pOM1* by myself and *Pe gacA* vs. *Pe* WT under my supervision by my master student Irina Petcu)
- pathogenic *Ecc15 pOM1* vs. LB medium
- pathogenic *Ecc15 pOM1* vs. heat-shock inactivated *Ecc15 pOM1*

The food sources in the flyPAD had to be gelatinous, which is why food substrates were dissolved in 1% agarose (sucrose choice) or mixed 1:1 with 2% agarose (low gelling temperature, # A9414, Sigma-Aldrich; all choices involving bacteria). Apart from the mere sucrose feeding choice, all food substrates additionally contained 5% sucrose to motivate feeding. After filling each electrode with 5 µl of the respective food mix, individual flies were quickly aspirated into each arena and the experiment started.

The post-analysis was conducted via a custom-made Matlab script provided by Pavel Itskov, which was used to calculate the parameters specified above. Overall non-eating flies were excluded from further analysis, as were arenas where spillage had prevented the proper recording of capacitance changes.

2.4 Behavioural protocols for olfactory conditioning experiments

To investigate whether flies can learn to associate an odour with the negative postingestive effects induced by pathogenic infection, I tested a variety of different conditioning protocols. The conditioning phase, where flies fed on pathogenic bacteria ON and presumably experienced the negative post-ingestive consequences of the infection, was followed by a test phase the next morning to determine potential memory formation, i.e. avoidance of the odour that was present during infection.

In general, after a period of dry starvation, experimental flies were orally infected with pathogenic bacteria (*Ecc15 pOM1* or *Pe* WT) according to the protocol described in 2.2.2 and left to feed on the bacteria overnight. Flies that were fed the corresponding harmless strain (*Ecc15 evf* or *Pe gacA*) or, for some protocols, the heat-killed pathogenic strain (*Pe* WT), served as controls. In one case, flies that had been fed 5% sucrose only served as an additional control. To assist potential associative olfactory memory formation, filter paper soaked in the supernatant of the respective bacterial strain was added to the bottles during overnight feeding. The next morning, olfactory preferences towards bacterial odours were assessed using the 4-field olfactory choice arena (see 2.3.2). The following olfactory choices were examined as part of the different behavioural protocols:

- bacterial odour vs. air:

  Ecc15 evf vs. air, following infection with *Ecc15 evf*

  Ecc15 pOM1 vs. air, following infection with *Ecc15 pOM1*

  Pe gacA vs. air, following infection with *Pe gacA*

  Pe WT vs. air, following infection with *Pe* WT or dead *Pe* WT
- pathogenic vs. harmless bacterial odour:

  Ecc15 pOM1 vs. *Ecc15 evf*, following infection with *Ecc15 pOM1* or *Ecc15 evf*

  Pe WT vs. *Pe gacA*, following infection with *Pe* WT or *Pe gacA*

  Pe WT vs. *Ecc15 evf*, following infection with *Pe* WT

  Pe gacA vs. *Ecc15 evf*, following infection with *Pe gacA*

For some conditioning protocols, additional groups of flies were kept on the respective bacterial strain or transferred to standard food after ON feeding for 24 h before testing. The specific conditioning protocols can also be found next to the corresponding data in 3.4.

2.5 Imaging

The diptericin-mCherry fluorescent immune reporter line was infected with pathogenic or harmless *Ecc15* bacteria (see 2.2.2) to study the upregulation of the AMP *diptericin* upon infection. After 48 h of feeding on the bacteria or 5% sucrose only, guts were dissected according to standard protocols previously described (Micchelli, 2014). The guts were directly mounted and imaged using a Leica M205 FA microscope; images were processed via ImageJ.

2.6 Statistical analysis

All behavioural data from the DAM, 4-field arena, CAFE and flyPAD assays was initially tabulated and stored in Microsoft Excel. All further statistical analyses were conducted using the GraphPad Prism8 software (GraphPad Software Inc., San Diego, CA USA). Data sets were tested for normality prior to further statistical testing via the Anderson-Darling and the D'Agostino & Pearson test.

The activity counts during specific time windows and the burstiness parameter from DAM experiments as well as the PIs obtained from 4-field arena experiments were compared using the unpaired t-test with Welch's correction for unequal variances in the case of two groups, and by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons test in the case of three or more groups. Rhythmicity in the DAM was compared using the Chi-Square test. Survival and CAFE assay data were analysed with a repeated-measures two-way ANOVA followed by the Bonferroni's post hoc test for multiple comparisons. In cases where the data was compared to a hypothetical value (0 for PIs or 1 for ratios), the one sample t-test was used for normally distributed data and the Wilcoxon signed-rank test for non-parametric distributions to calculate p-values. Feeding choice in the flyPAD was analysed by calculating the ratio between the number of sips of the two feeding substrates at specific time points and comparing the ratios to 1 via the Wilcoxon signed-rank test. Flies that had not fed on one of the substrates yet at the time point analysed were excluded from this analysis. The remaining flyPAD parameters were compared using the Wilcoxon matched-pairs signed rank test for paired non-parametric distributions.

The significance threshold (α) was set to 0.05 according to standard statistical conventions; the statistical notations were as follows: 'ns' $p > 0.05$, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$. Error bars in the graphs denote the standard error of the mean (SEM). For box plots, the bisecting line represents the median, and the box extends from the 25^{th} to the 75^{th} percentiles (the so-called inter-quartile range (IQR)). The whiskers are drawn according to Tukey, i.e. correspond to 1.5 x IQR. All values outside of this range are shown as outliers.

3. Results

3.1 Validation of successful natural pathogenic infection

3.1.1 Survival after pathogen feeding

The first step on the way to unravelling the mechanisms underlying *Drosophila*'s response to contaminated food was to establish a reproducible protocol for pathogenic infection by feeding bacteria in the laboratory (see 2.2.2). The most apparent and reliable method to validate successful oral infection with pathogenic bacteria and to assess the negative effects of pathogen ingestion was to determine survival after bacteria feeding (Figure 10).

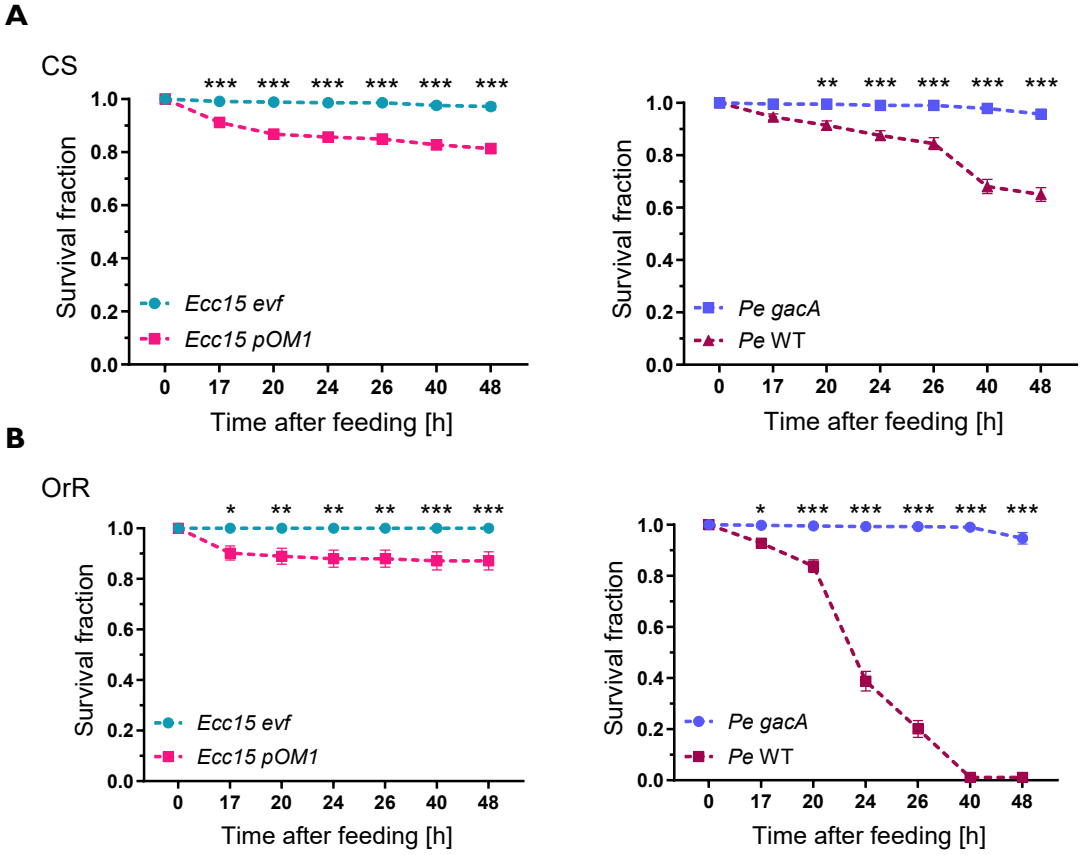

Figure 10 - Survival of wild-type flies after oral infection with *Ecc15* and *Pe*

(A) Survival of wild-type CS flies after feeding with pathogenic *Ecc15 pOM1* or harmless *Ecc15 evf* (left panel, n = 9) and after *Pe* WT (pathogenic) or *Pe gacA* (harmless) feeding, (right panel, n = 12 (*gacA*), n = 14 (WT)). **(B)** Survival of wild-type OrR flies after *Ecc15* feeding (left panel, n = 8) and after *Pe* feeding (right panel, n = 12). 1 n represents one bottle with 80 female flies. Bacteria suspensions are $OD_{600} \approx 100$ + 5% sucrose. Error bars denote SEM, p-values calculated via repeated-measures two-way ANOVA followed by Bonferroni's post hoc test for multiple comparisons.

For that purpose, I fed groups of wild-type flies (1n = 80 flies / bottle) either the pathogenic *Pe* or *Ecc15* strain or the corresponding harmless strains at an $OD_{600} \approx 100$ (supplemented with 5% sucrose) and monitored survival at different time points over the course of two days. From the wild-type CS female flies that had been infected with pathogenic *Ecc15 pOM1-evf* (abbreviated as *Ecc15 pOM1* in this thesis), which overexpresses the *Erwinia* virulence factor, on average 91.2% survived overnight feeding (confidence interval (CI) 87.4 - 94.9%), and 81.4% were still alive after 48 h (CI 77.3 - 85.5%) (Figure 10A, left panel). By contrast, the avirulent mutant *Ecc15 evf* control strain, which lacks the Erwinia virulence factor, did not kill a relevant fraction of flies (97.2% mean survival after 48 h, CI 94.3 - 100%). As expected, less flies survived when infected with the highly pathogenic *Pe* WT strain. The time course of *Pe* WT infection differed slightly from the *Ecc15 pOM1* infection, with a slower onset of pathogen-induced deaths. A significant divergence from flies that had fed on avirulent *Pe gacA* bacteria was visible after 20 h (91.4% mean survival, CI 87.8 - 95.1%). After 48 h, on average only 65% of *Pe* WT-fed flies were still alive (CI 59.4 - 70.6%) compared to 95.7% in the *Pe gacA* control (CI 94.0 - 97.4%) (Figure 10A, right panel).

A similar pattern was observed for wild-type OrR female flies. Regarding the infection with *Ecc15 pOM1*, on average 87.1% (CI 78.7 - 95.5%) had survived the infection at 48 h post-feeding (Figure 10B, left panel). Wild-type OrR flies are generally smaller and weaker than the CS strain, and hence almost no flies had survived *Pe* WT feeding after two days (mean 1.2%, CI 0.2-2.1% Figure 10B, right panel).

Thus, both *Ecc15* and *Pe* reliably infected *Drosophila* using a protocol for a natural pathogenic infection via feeding, confirming previous reports (Basset et al., 2000; Liehl et al., 2006). While the *Ecc15 pOM1* infection was potentially harmful, yet only lethal for a small fraction of flies, infection with *Pe* WT proved to be much more severe, in particular in OrR flies, which did not survive.

3.1.2 AMP expression after pathogenic infection

Besides monitoring survival, another way to validate successful infection was to investigate the upregulation of the immune response upon feeding with pathogenic bacteria. To that aim, I used diptericin-mCherry flies, where the AMP *diptericin* is coupled to the

fluorescent reporter mCherry. Infection with *Ecc15* is known to induce the expression of AMPs such as *diptericin* in the cardia and the midgut (Basset et al., 2000; Tzou et al., 2000). Diptericin-mCherry flies that were fed with pathogenic *Ecc15 pOM1* showed strong expression of *diptericin* in the cardia and the anterior midgut 48 h after feeding, while *Ecc15 evf*-fed flies only weakly expressed *diptericin* and sucrose-fed control flies did not show notable fluorescence (Figure 11). Strongest fluorescent reporter expression was observed in a fly that had died as a consequence of the infection with *Ecc15 pOM1*. Hence, feeding with pathogenic, but not harmless *Ecc15* activated the immune response, which led to the expression of AMPs such as *diptericin*.

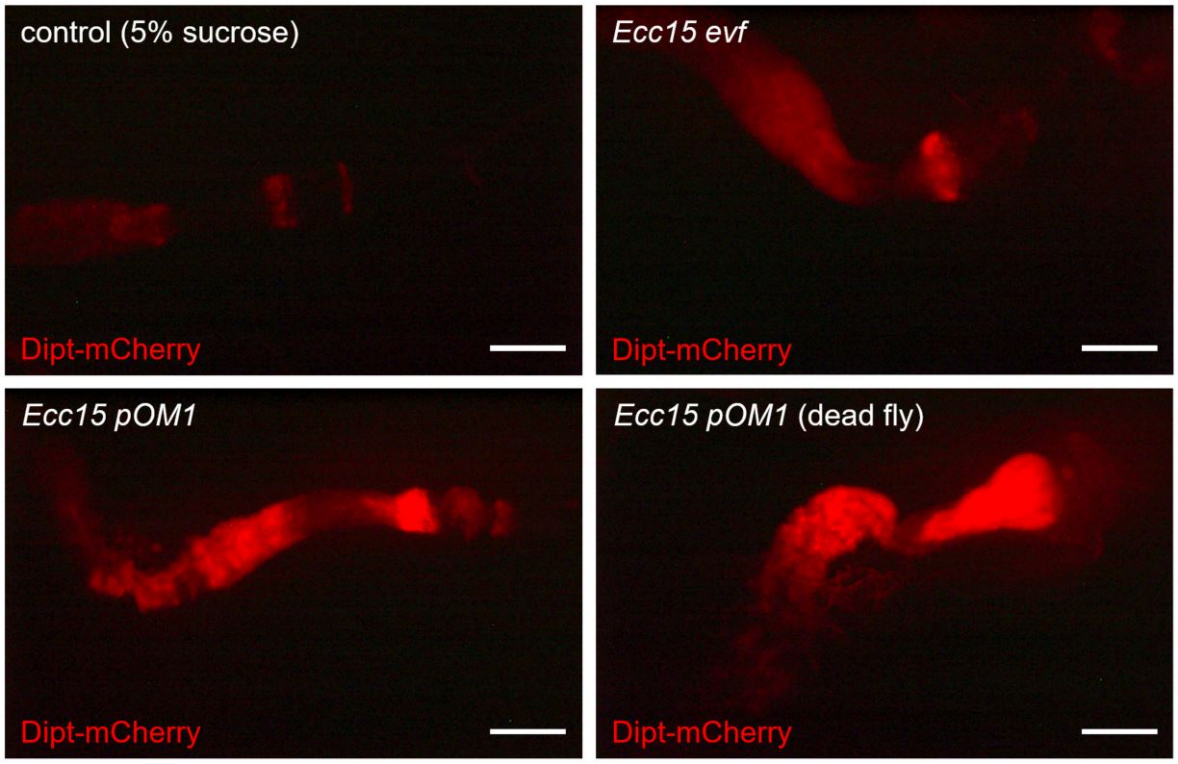

Figure 11 - *Diptericin* expression after bacteria feeding

Expression of the AMP *diptericin* in the cardia and anterior midgut of Diptericin-mCherry fluorescent reporter flies 48 h after feeding with harmless *Ecc15 evf*, pathogenic *Ecc15 pOM1* or 5% sucrose (control). Guts were dissected, mounted and directly imaged without prior staining. Scale bar = 200µm.

As infection with *Pe* WT heavily damages the gut epithelium (see also 1.5.2) and hence impairs the dissection of the gut, this experiment was only conducted for the infection with *Ecc15*.

3.2 Circadian rhythm and locomotor activity of infected flies

Infection with pathogenic bacteria or changes to the microbiome have been reported to increase sleep and modulate locomotion, respectively (Kuo et al., 2010; Schretter et al., 2018). Thus, it was crucial to determine potential changes in general motor behaviour upon infection, since heavily impaired flies might not perform well in subsequent behavioural experiments such as the 4-field arena or the CAFE assay. Moreover, assessing locomotion following pathogen feeding would provide additional information about the time course of bacterial infection. Having validated that both *Ecc15* and *Pe* can be reliably used to infect *Drosophila* in a natural oral infection paradigm, I recorded the activity of infected flies over several days to establish whether feeding with pathogenic *Ecc15* or *Pe* leads to changes in general locomotor activity or circadian rhythm.

Following feeding with either pathogenic or harmless *Pe* or *Ecc15* according to the standard oral infection protocol described in 2.2.2, the locomotor activity of infected wild-type OrR flies was recorded for three days in the *Drosophila* activity monitor (Figure 12A). Overall, flies that had fed on pathogenic bacteria showed the same regular activity patterns as flies fed with harmless bacteria in the case of both *Pe* and *Ecc15*, with activity peaking at light onset and in particular around the time the light was switched off (Figure 12B). In addition, I analysed locomotor activities for the 5 h-time windows comprising the first (5-10 h post-infection) and the second (29-34 h post-infection) light-off activity peak after bacteria feeding by summing up all activity counts during that period in order to get a more fine-grained analysis of locomotor activity following infection. *Pe* WT-infected flies did not differ from *Pe gacA*-infected control flies, neither directly after bacteria feeding nor more than 24 h later (Figure 12C, however, note that $p = 0.0530$ for the 29-34 h period, also see appendix Figure 46). By contrast, locomotor activity was significantly decreased in pathogenic *Ecc15 pOM1*-infected flies as opposed to *Ecc15 evf*-fed control flies shortly after bacteria feeding (5-10 h post-infection), but had returned to control levels in the second light-off period (Figure 12C). This decrease in activity extended into the first dark phase after infection (i.e. up to 17 h post-infection, see appendix Figure 46). Thus, while prior feeding with highly virulent *Pe* bacteria left flies unaffected, an infection with pathogenic *Ecc15 pOM1* led to a short and transient reduction in locomotor activity directly after bacteria feeding.

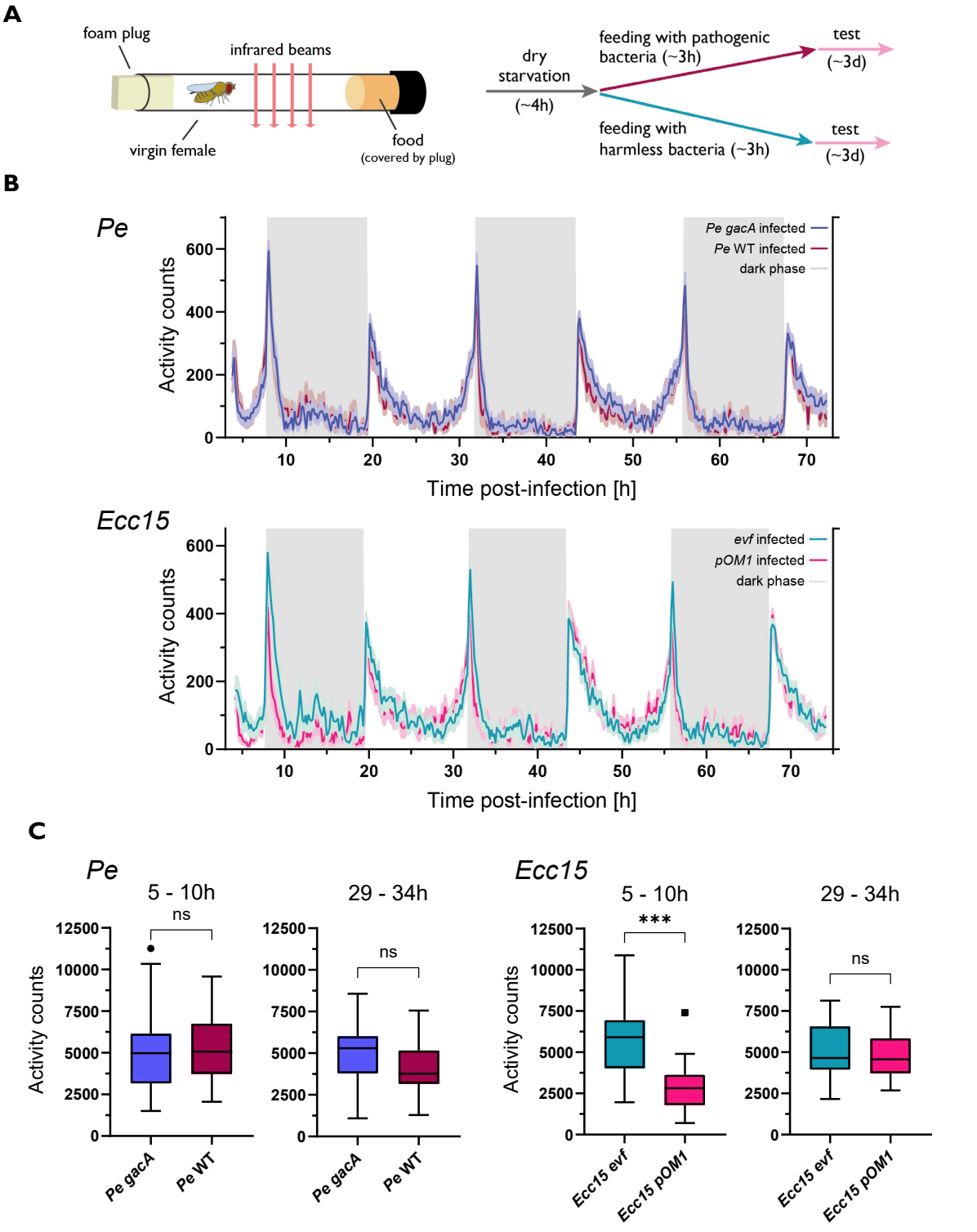

Figure 12 - Activity monitoring after bacteria feeding

(A) Illustration of an activity monitoring tube and behavioural protocol. **(B)** Activity counts over time of wild-type OrR flies infected with *Pe gacA* or *Pe* WT (top) and of flies infected with *Ecc15 evf* or *Ecc15 pOM1* (bottom), n = 23 (*Pe gacA*), 17-23 (*Pe* WT), 24 (*Ecc15 evf*), 17-24 (*Ecc15 pOM1*). Infected flies were excluded from the time of death onwards, mean ± SEM. **(C)** Total activity counts for two 5 h-long periods comprising the first two light-off phases after infection. Infected flies were excluded from the time of death onwards. *Pe*: n = 23 (5-10 h), 23/21 (*gacA*/WT 29-34 h). *Ecc15*: n = 24 (5-10 h), 24/18 (*evf*/*pOM1* 29-34 h); p-values calculated via unpaired t-test with Welch's correction.

The examination of the activity patterns of individual flies allowed getting a more detailed picture of the general motor behaviour following pathogenic infection, in particular with respect to the time course of the infection and the regularity of activity patterns (exemplary traces, Figure 13). Interestingly, flies that were fed with the pathogenic *Ecc15 pOM1* strain in general died much earlier than *Pe* WT-fed flies. While oral infection with *Ecc15 pOM1* was lethal between 10 and 27 h after the bacteria feeding period, flies infected with *Pe* WT died between 22 and 56 h post-infection.

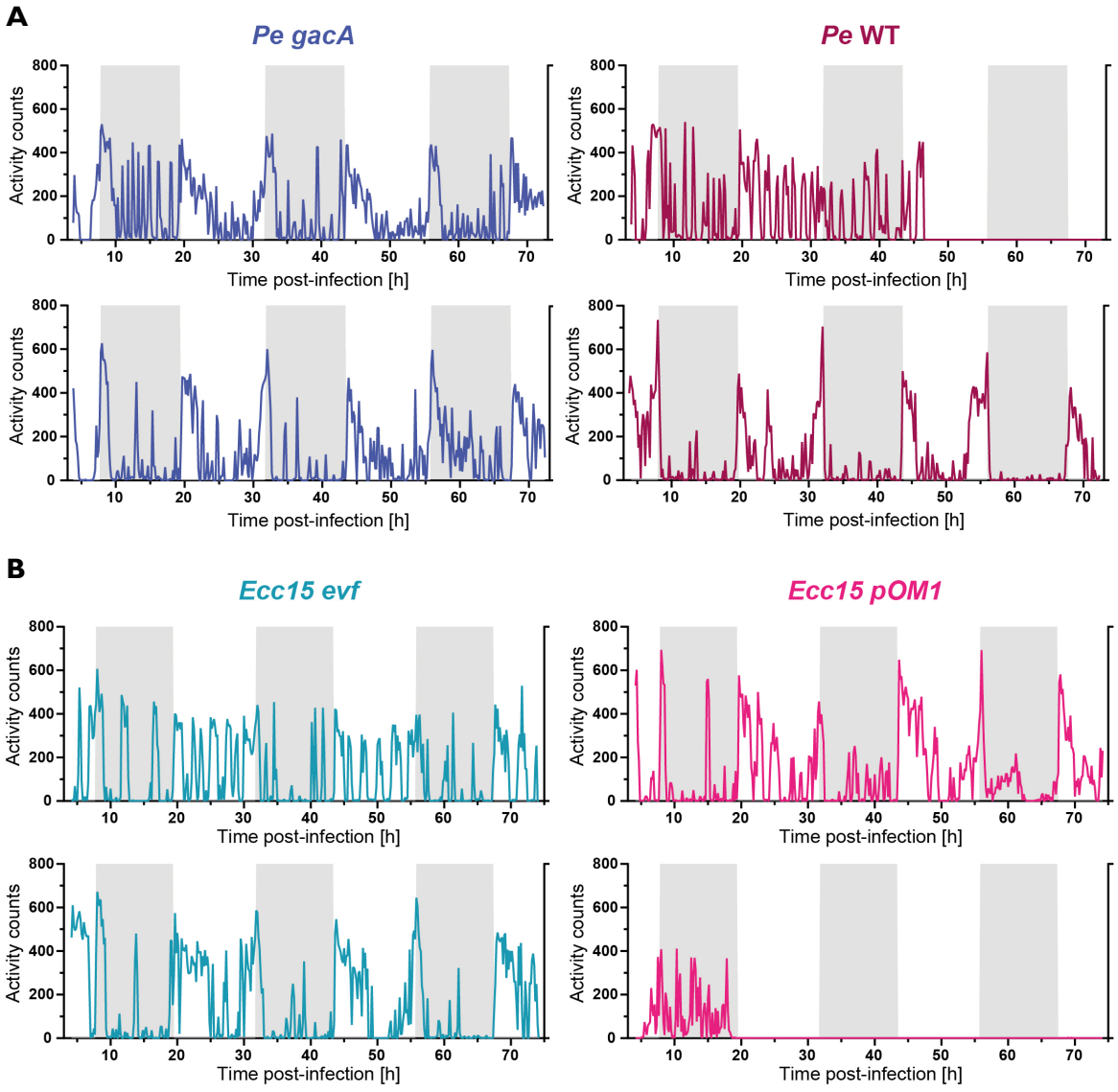

Figure 13 - Locomotor activity patterns of individual flies after bacteria feeding

(A) Activity counts over time of two harmless *Pe gacA*- and two pathogenic *Pe* WT-infected flies, one of which died ~46 h after bacteria feeding. **(B)** Activity counts over time of two harmless *Ecc15 evf*- and two pathogenic *Ecc15 pOM1*-infected flies, one of which died ~19 h after bacteria feeding.

Flies seemingly exhibited inter-individual differences as to the regularity of their locomotor activity over time, with some flies showing highly rhythmic behaviours and others apparently being mostly arrhythmic. Hence, I collaborated with Dr. Sophie Aimon, who determined the burstiness and periodicity for the different conditions. Burstiness describes the distribution of the interevent time between two successive events for patterns with an intermittent, i.e. irregular nature (Goh et al., 2008). The burstiness of locomotor activity patterns of pathogenic *Pe* or *Ecc15*-infected flies did not differ from that of flies infected with the respective harmless strains (Figure 14A). In addition, the analysis of the periodicity of individual flies showed that the majority of flies had regular 12 h- or 24 h-activity cycles regardless of prior feeding on pathogenic or harmless *Pe* or *Ecc15* (Figure 12B).

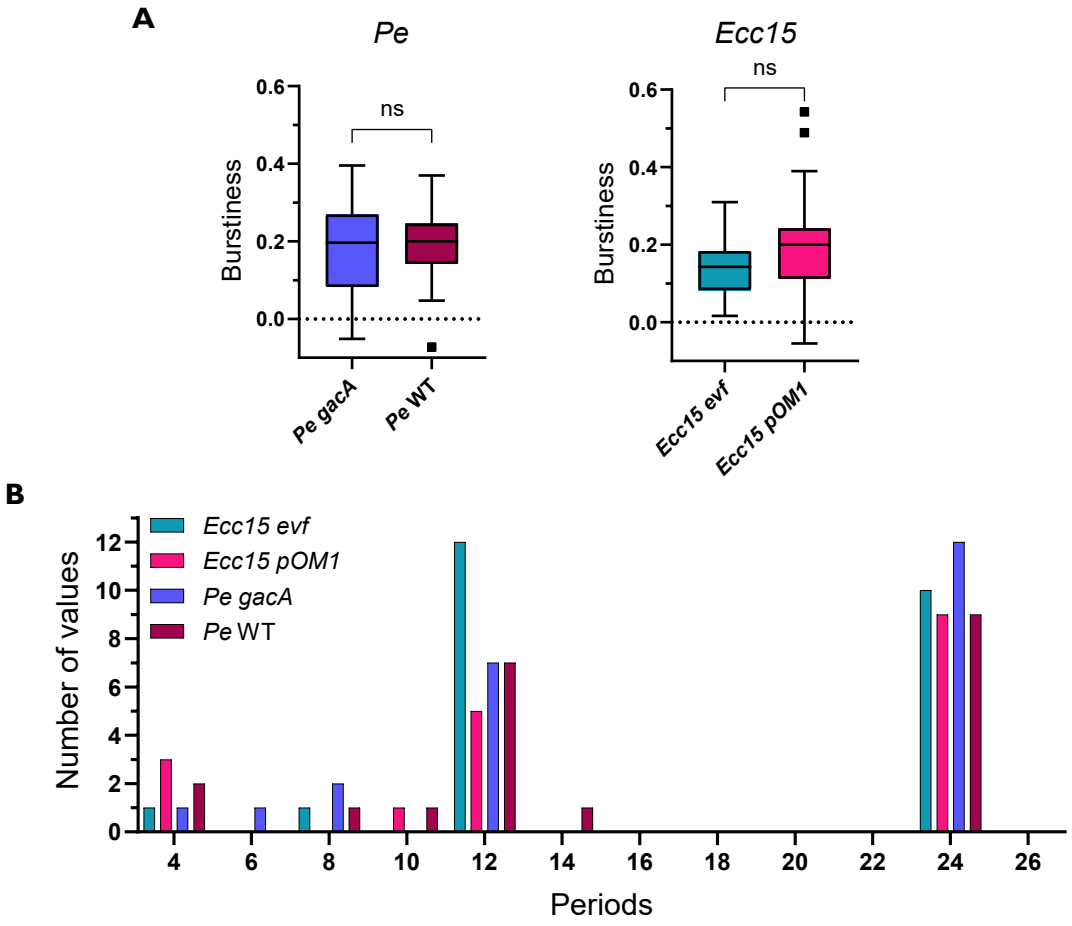

Figure 14 - Quantification of burstiness and rhythmicity after bacteria feeding

(A) Burstiness of locomotor activity patterns following pathogenic infection. n = 23 (*Pe*), 24 (*Ecc15*), p-values calculated via unpaired t-test with Welch's correction, p = 0.0861 for *Ecc15*. **(B)** Histogram of rhythmicity. Most flies exhibit a rhythmic 12 h- or 24 h- activity cycle regardless of bacterial strain. Flies dying before a full 24 h-cycle was recorded were excluded from this analysis. Chi-square test of independence showed no significant differences for periodicity of flies fed with different bacterial strains (p = 0.8676). Analysis performed by Sophie Aimon.

Thus, even though feeding with pathogenic bacteria was lethal for some wild-type OrR flies in the case of *Ecc15* and for most or even all flies in the case of *Pe* (see Figure 10), flies were only transiently impaired in their general locomotor activity after *Ecc15* infection and unaffected after *Pe* infection. Therefore, infection with pathogenic bacteria left flies fit enough for the subsequent analysis of preference behaviour in assays such as the arena or the CAFE. Interestingly, *Ecc15* infection had a dampening effect on locomotor activity in the relatively short time frame up to 17 h after bacteria feeding, which roughly corresponds to the time frame in which *Ecc15 pOM1*-infected flies died. By contrast, *Pe* WT-induced deaths extended over a much longer period of time. Yet, as seen from the periodicity analysis, both *Ecc15* and *Pe* infection did not have an effect on circadian rhythm.

3.3 Innate preferences towards bacterial odours

Odours are important indicators not only of the presence of a food source but also of its quality. *Drosophila* heavily relies on olfactory cues to find food during foraging and to identify potential contaminants before feeding. For instance, one such microbial odour, geosmin, acts as a warning signal for flies and elicits innate avoidance (Stensmyr et al., 2012). One of the questions I tried to answer in this study was if flies could learn to associate the negative post-ingestive consequences of pathogenic infection with the odour present at feeding, i.e. form a memory of the olfactory perception of spoiled food. However, before investigating if flies would avoid bacterial odours following infection, it was crucial to determine the innate behaviour towards these bacterial odours in order to then be able to compare innate and potentially learned behaviours. Yet first, the 4-field arena (see 2.3.2) had to be established as a reliable olfactory choice assay.

3.3.1 Establishment of the 4-field arena for olfactory stimuli

The 4-field arena allows monitoring the behavioural responses of freely moving adult *Drosophila* upon optogenetic and/or olfactory stimulation. During the process of refining the 4-field arena for olfactory stimuli, ~40 h-starved wild-type CS females were tested for their preference behaviour towards 1% balsamic vinegar. As *Drosophila* is known to be attracted to vinegar as a food odour, in particular following starvation (e.g. Semmelhack et al., 2009; Root et al., 2011; Ko et al., 2015), using this odour enabled us to determine the functionality of the odour delivery system and develop suitable protocols for subsequent olfactory choice assays. In contrast to optogenetic experiments, which require only light on- and offset, protocols for olfactory experiments had to be much longer for odours to flood the arena during stimulus phases as well as to clear the arena of odour and allow flies to return to baseline in between stimulus phases. After testing a variety of different time courses for olfactory experiments in the 4-field arena, the protocol that yielded the best results comprised one minute of acclimatization, 90 s-long stimulus phases and 3-4 minutes for the inter-stimulus phase. Subjecting starved wild-type CS flies to this protocol in the 4-field arena yielded the expected high attraction to 1% vinegar (Figure 15).

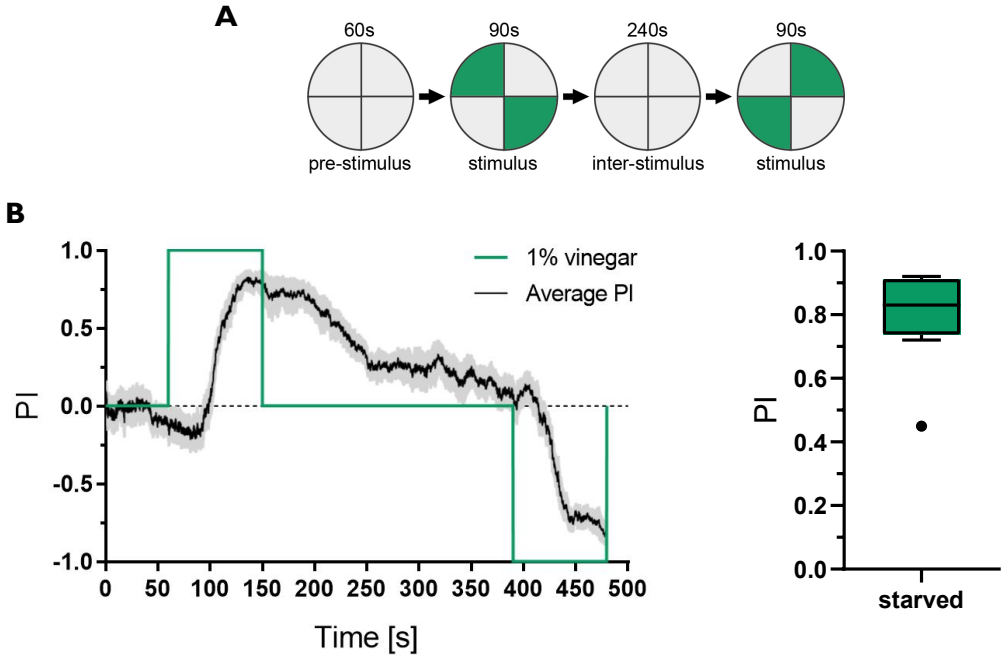

Figure 15 - Vinegar attraction in the 4-field arena

(A) Experimental protocol in the 4-field arena with stimulus-, pre- and inter-stimulus phases. **(B)** 40 h-starved, female CS flies are highly attracted towards 1% vinegar by the end of both stimulus phases as indicated by PI values over time (left panel, mean ± SEM) and by the PI averaged across the last 5 seconds of both stimulus phases (right panel), n = 8.

Upon stimulus onset in two opposing quadrants, it took 20-30 s before flies started reacting to the odour, and highest average PI values were reached ~70 s into the first stimulus phase. In the stimulus-off phase, preference behaviour again reached baseline values after around 180 s, demonstrating that flies had redistributed equally across all quadrants. Vinegar attraction in the second stimulus phase mirrored the first stimulus phase, which indicated the absence of spatial bias (Figure 15, left panel). The PI for vinegar as calculated and averaged from the last 5 s of each stimulus period was 0.796 (CI 0.665-0.926) (Figure 15, right panel). Thus, the 4-field arena is a reliable experimental assay to determine olfactory preferences in *Drosophila*.

3.3.2 Preferences for the olfactory choice between bacteria and air or LB medium

To identify innate olfactory preferences towards bacterial odours, I subjected wild-type OrR flies to a variety of olfactory choices in the 4-field arena using the previously established protocol (Figure 16A). I started by offering either fed or starved wild-type OrR flies the choice between *Pe* WT odour and humidified air only (Figure 16B, left panel). Interestingly,

not only were starved flies highly attracted to the odour of pathogenic *Pe*, but fed flies were also slightly attracted by *Pe* WT odour, even though to a significantly lesser extent than starved flies. However, as fed flies were usually less agile in the 4-field arena and in order to more easily identify attraction or aversion phenotypes, I conducted all further experiments using starved flies.

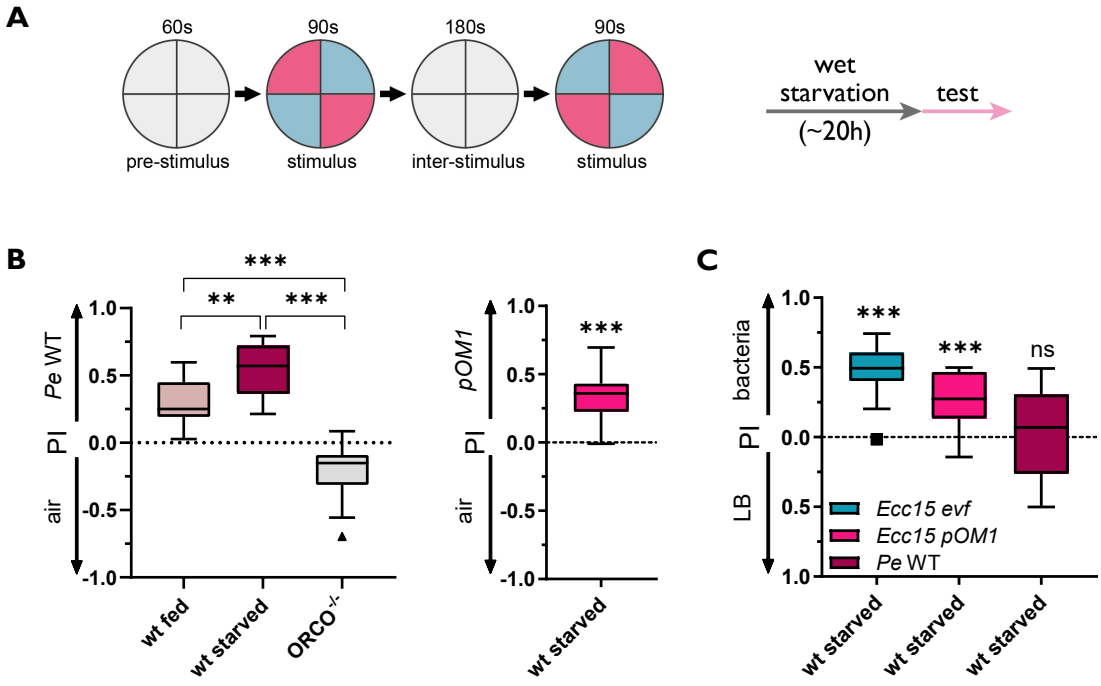

Figure 16 - Olfactory choice between pathogenic bacteria and air or LB

(A) General experimental paradigm for testing preferences for bacterial odours in the 4-field arena. **(B)** Left panel: Olfactory preferences of fed and starved wild-type OrR flies and of starved ORCO null mutants for the choice between *Pe* WT and air. n = 16, p-values calculated via one-way ANOVA and Tukey's post hoc test for multiple comparisons. Right panel: Olfactory preference of starved wild-type OrR flies for the choice between *Ecc15 pOM1* and air. n = 16, p-value calculated via one-sample t-test comparing to 0 as the theoretical mean. **(C)** Olfactory preferences of starved wild-type OrR flies for the choices *Ecc15 evf* vs. LB (n = 16), *Ecc15 pOM1* vs. LB (n = 16) and *Pe* WT vs. LB (n = 21). p-values indicate significant differences from 0 and are calculated via one-sample t-tests comparing to 0 as the theoretical mean.

Since I was moreover interested if this attraction to pathogenic bacteria was olfaction-dependent, I tested flies deficient for the obligate co-receptor for insect ORs, the odorant receptor co-receptor (ORCO / Or83b), which are mostly anosmic (Larsson et al., 2004). Starved ORCO$^{-/-}$ flies did not show the previously observed preference for *Pe* WT odour over air but instead were even slightly repelled by pathogenic bacteria (Figure 16B, left panel), indicating that the innate attraction to *Pe* WT was dependent on ORCO-mediated olfaction.

Similar to pathogenic *Pe*, starved wild-type flies preferred the odour of the pathogenic *Ecc15 pOM1* strain to humidified air only (Figure 16B, right panel). Thus, if the only other option is air, hungry flies are innately attracted to pathogenic *Pe* or *Ecc15* bacterial odours, which could indicate putative nutritive food sources, even though these odours could signify the presence of spoiled food.

Another question was whether *Drosophila* would prefer bacterial odours not only to air but also to the odour of the respective growth medium, i.e. LB. This was indeed the case for the two *Ecc15* strains tested: starved wild-type OrR flies were attracted to harmless *Ecc15 evf* or pathogenic *Ecc15 pOM1*, respectively, if the other option was LB medium (Figure 16C). By contrast, flies were indifferent to the choice between *Pe* WT and LB medium (Figure 16C). However, it should be noted that the variability for the *Pe* WT vs. LB choice was fairly high (PI = 0.0286(0.3071), mean(SD)). Hence, if the only other option was the low-nutritive LB medium, flies still preferred *Ecc15* odour regardless of pathogenicity and were indifferent in the case of pathogenic *Pe* WT vs. LB medium.

3.3.3 Preferences for the olfactory choice between bacteria and yeast

One could argue that flies preferred the odour of pathogenic bacteria to humidified air or LB medium because they were starved and hence motivated to track the only or the most promising nutritive food source available regardless of pathogenicity. To evaluate how flies would decide if offered an olfactory choice between a protein source as opposed to pathogenic bacteria, I tested starved wild-type OrR flies for their preference in a yeast versus pathogenic bacteria choice. Starved flies were clearly attracted to yeast odour when compared to the odour of pathogenic *Pe* WT (Figure 17A). Even when *Pe* WT odour was presented in a mixture with yeast, wild-type flies were able to detect the difference and still preferred pure yeast odour. This behaviour was olfaction-dependent, as starved $ORCO^{-/-}$ flies did not distinguish between *Pe* WT + yeast and yeast only (Figure 17B).

By contrast, when flies were tested for the same olfactory choice behaviour using the pathogenic *Ecc15* strain *pOM1*, they exhibited a weak preference for the pathogenic bacteria/yeast mixture over pure yeast (PI = 0.1219, CI 0.0233 - 0.2205, Figure 17C). This indicates that flies were either not able to properly detect a difference between the *Ecc15*

pOM1/yeast mixture and yeast or that they were indifferent to such a choice and considered both odours to be almost equally attractive or repulsive.

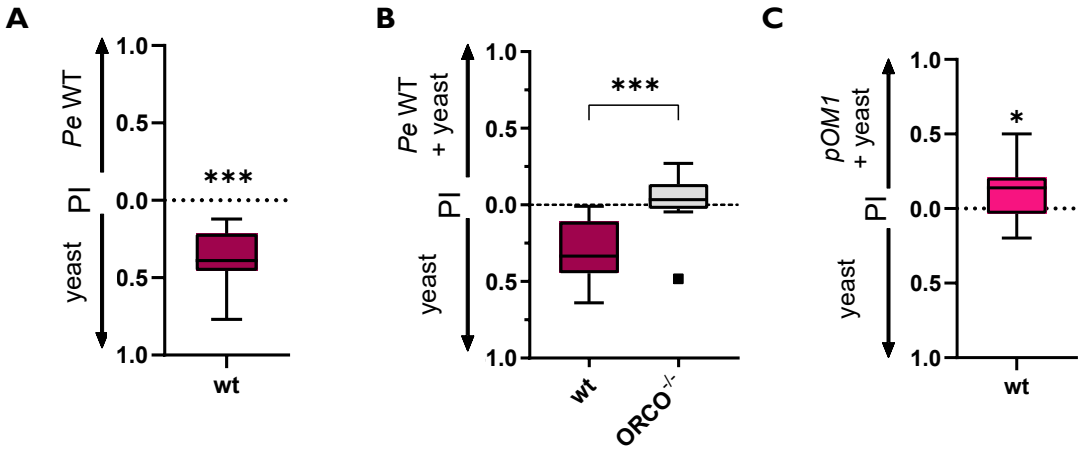

Figure 17 - Olfactory choice between pathogenic bacteria and yeast

(A) Olfactory preference of starved wild-type OrR flies for the choice between yeast and *Pe* WT. n = 16, p-value calculated via one-sample t-test comparing to 0 as the theoretical mean. **(B)** Olfactory preferences of starved wild-type OrR and starved ORCO$^{-/-}$ flies for the choice between a *Pe* WT/yeast mixture and yeast only. n = 16, p-values calculated via the non-parametric Mann-Whitney test. **(C)** Olfactory preference of starved wild-type OrR flies for the choice between an *Ecc15 pOM1*/yeast mixture and yeast only. n = 16, p-value calculated via one-sample t-test comparing to 0 as the theoretical mean.

3.3.4 Olfactory preferences for pathogenic compared to harmless bacteria

Having established that *Drosophila* is not repelled by the odour of pathogenic *Ecc15* and *Pe* per se and moreover chooses yeast over a mixture of pathogenic *Pe* and yeast while slightly preferring an *Ecc15 pOM1*-yeast mixture to pure yeast, I was further interested in the direct olfactory choice between pathogenic and harmless bacteria. The two supernatants from *Ecc15* and *Pe*, respectively, are similar with regard to bacterial growing conditions and nutritive value, hence their only relevant difference being their pathogenicity. It was unclear whether flies could differentiate harmless from pathogenic strains at all. If so, given the importance of olfactory cues for evaluating the quality of a food source, one might expect a clear preference for the harmless over the respective pathogenic bacterial strains. I hence set out to determine if *Drosophila* prefers the odour of the two *Ecc15* strains to *Pe* WT with regard to potential olfactory choices for later conditioning experiments (see 3.4). Yet when subjected to an olfactory choice between *Pe* WT and harmless *Ecc15 evf* or *Pe* WT and pathogenic *Ecc15 pOM1*, starved wild-type OrR flies were completely indifferent (Figure 18).

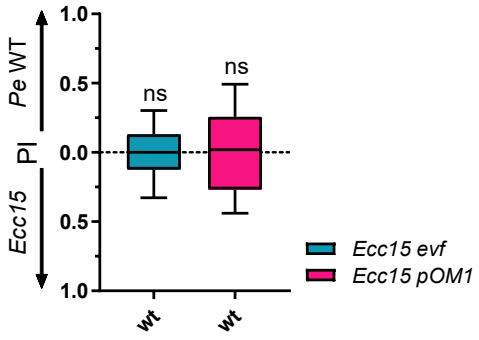

Figure 18 - Olfactory choice between pathogenic *Pe* and *Ecc15*

Preferences of starved wild-type OrR flies for the olfactory choice between *Pe* WT and harmless *Ecc15 evf* as well as for the choice between *Pe* WT and pathogenic *Ecc15 pOM1*. n = 16, p-values calculated via one-sample t-test comparing to 0 as the theoretical mean.

However, when I tested an olfactory choice between the harmless and pathogenic versions of the same strain, I found that starved wild-type OrR flies clearly preferred the odour of pathogenic *Pe* WT to harmless *Pe gacA* odour (Figure 19A) as well as pathogenic *Ecc15 pOM1* to harmless *Ecc15 evf* odour (Figure 19B).

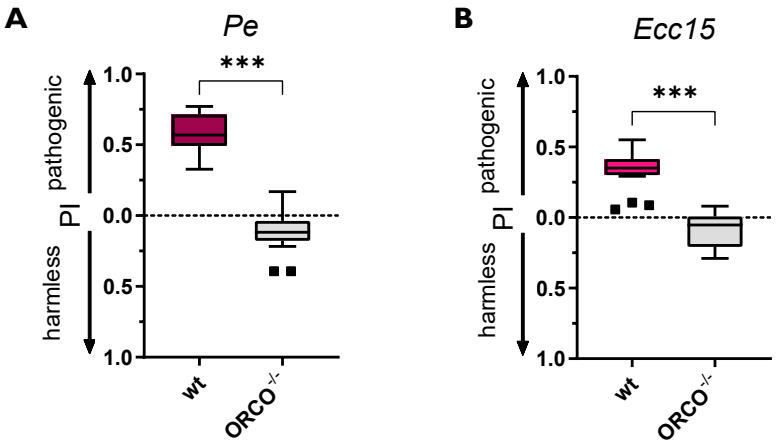

Figure 19 - Olfactory preferences for pathogenic over harmless bacteria

(A) Preferences of starved wild-type OrR and ORCO null mutant flies for the olfactory choice between pathogenic *Pe* WT and harmless *Pe gacA* **(B)** Preferences of starved wild-type OrR and ORCO null mutant flies for the olfactory choice between pathogenic *Ecc15 pOM1* and harmless *Ecc15 evf*. n = 16 for all groups, p-values calculated via unpaired t-test with Welch's correction.

Again, this preference behaviour for pathogenic over harmless bacterial odours relied on olfactory input, as ORCO null mutant flies did not distinguish between the respective pathogenic and harmless strains (Figure 19).

Taken together, the data presented so far show that *Drosophila* is not innately repelled by the odour of pathogenic *Ecc15* or *Pe*, but instead is attracted to it. Interestingly, wild-type but not smell-blind flies even chose the odour of pathogenic bacteria over the respective harmless mutant counterparts. This indicates not only that flies can differentiate pathogenic from the harmless strains via their olfactory system, but also that pathogenic *Ecc15* and *Pe* bacterial odours by themselves are not sufficient to elicit innate avoidance behaviour even though they could be indicators of contaminated food.

3.4 Olfactory preferences following pathogenic infection

The data presented so far show that flies that have never before come into contact with pathogenic *Pe* or *Ecc15* are attracted by their odour. However, assuming that *Drosophila* can remember spoiled food would mean that infection with those pathogens could reduce this innate olfactory attraction or even turn it into aversion. I hence tested a variety of behavioural protocols in order to condition flies to avoid bacterial odours. To that aim, flies were fed with either pathogenic or harmless bacterial strains overnight according to the protocol described in 2.2.2. The next morning, they were subjected to an olfactory choice that involved the odour of the bacteria they had been infected with to determine potential memory formation manifested as an avoidance of the odour present at feeding.

3.4.1 Olfactory preferences for bacteria versus air after pathogenic infection

In a first effort to investigate potential memory formation following infection with pathogenic bacteria, I fed wild-type flies with either pathogenic bacteria (*Ecc15 pOM1* or *Pe* WT), harmless bacteria (*Ecc15 evf* or *Pe gacA*) or 5% sucrose only. After overnight feeding, I used the 4-field arena to determine their preferences regarding the choice between the odour of the bacteria they had been infected with and humidified air (Figure 20A). Flies from the sucrose-fed group served as further controls and were tested for their olfactory preferences towards pathogenic or harmless bacterial odours, respectively, as opposed to air only. In the case of *Pe* conditioning, additional groups of flies were transferred back to standard fly food after overnight bacteria or sucrose feeding, allowed to recover for 24 h and then tested in the 4-field arena to investigate the possibility of long-lasting memory formation (Figure 20A).

Following feeding with *Ecc15*, neither pathogenic *Ecc15 pOM1*- nor harmless *Ecc15 evf*-infected wild-type CS flies differed from the corresponding sucrose-fed controls in that they did not show attraction to or aversion of the respective bacterial odour (Figure 20B). Interestingly, *evf*-infected flies showed significantly higher attraction to *evf* odour than *pOM1*-infected flies to *pOM1* odour, suggesting a difference in the reaction to bacterial odours depending on the pathogenicity of the strain the flies had fed on prior to the choice assay. However, these data were acquired from three independent experiments, with one experiment yielding aversion in *pOM1*-infected flies and the other two experiments failing to replicate this phenotype. Combined with the missing difference to sucrose-fed controls, it hence cannot be concluded from this conditioning paradigm whether infection with pathogenic *Ecc15 pOM1* indeed induced learned aversion of *pOM1* odour.

Similarly, ON feeding with highly pathogenic *Pe* WT or the avirulent *Pe gacA* control strain did not lead to changes in olfactory preferences between bacteria- and sucrose-fed wild-type CS flies; and all experimental groups including sucrose-fed flies were indifferent towards the respective bacterial odour (Figure 20C). Testing flies for a potential acquired avoidance of bacterial odours after an additional 24 h recovery period yielded the same neutral behaviour (Figure 20C).

For both *Ecc15* and *Pe*, this conditioning approach abolished the high attraction to pathogenic bacterial odours vs. air seen in naïve flies (see Figure 16). However, it should be noted that while the olfactory choice was the same for both innate behaviour and conditioning, the behavioural paradigm beforehand was slightly different. Innate preferences were tested on wet-starved flies, whereas conditioned flies were tested directly after sucrose or bacteria feeding. The reduced attraction to bacterial odours seen across all groups after conditioning was hence most likely due to flies being less starved than in naïve testing paradigms.

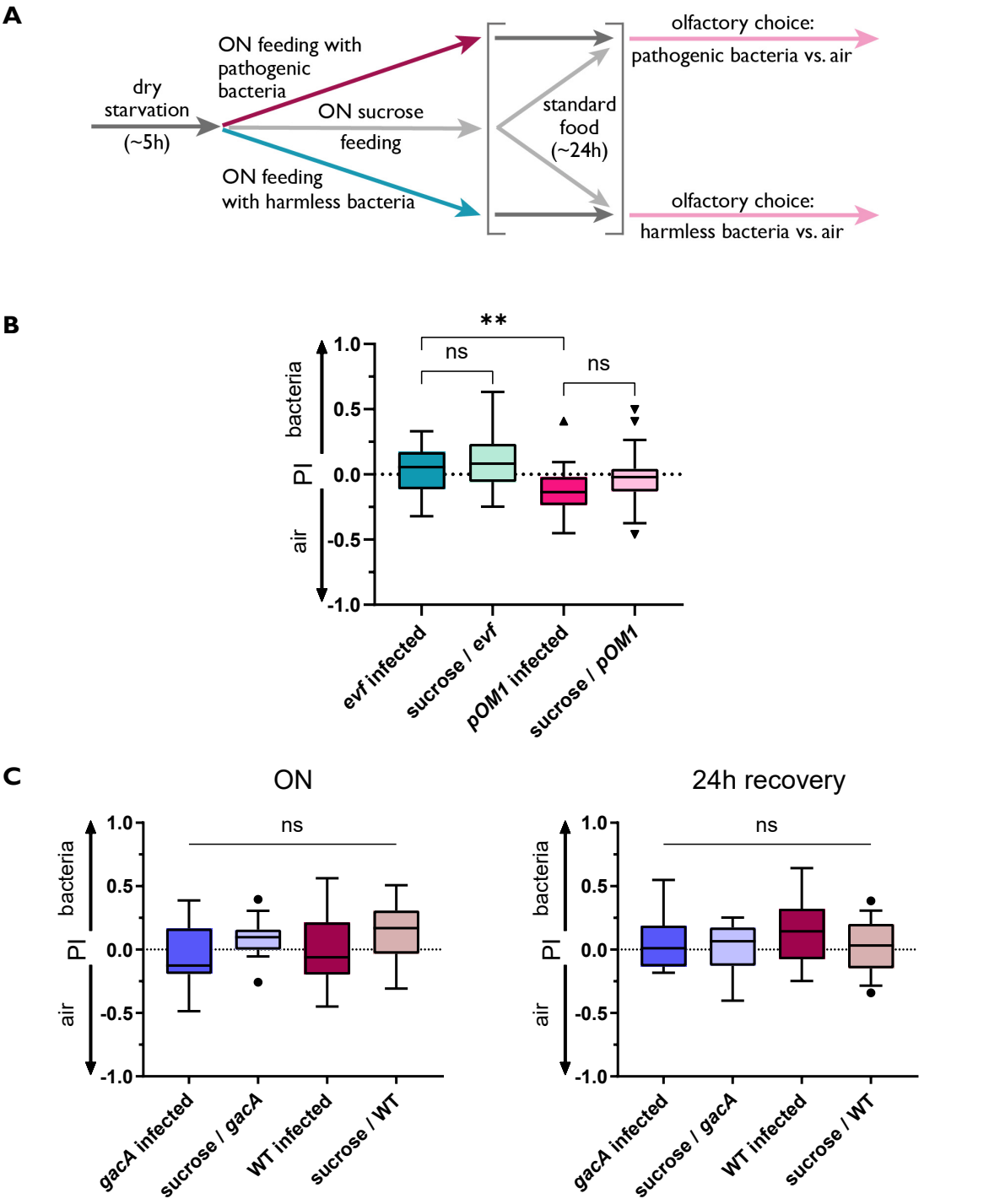

Figure 20 - Olfactory preferences for bacteria vs. air following pathogenic infection

(A) Conditioning protocol for bacteria feeding and olfactory choice in the 4-field arena. **(B)** Olfactory preferences of wild-type CS flies for the choice between *Ecc15 evf* or *Ecc15 pOM1* and air, respectively, following ON feeding on *Ecc15 evf*, *Ecc15 pOM1* or sucrose. n = 24 (*evf* infected) / 26 (sucrose/*evf*) / 30 (*pOM1* infected) / 27 (sucrose/*pOM1*). **(C)** Olfactory preferences of wild-type CS flies for the choice between *Pe gacA* or *Pe* WT and air, respectively, following ON feeding (left panel) on *Pe* WT, *Pe gacA* or sucrose and after additional 24 h of recovery (right panel). n = 16 for all groups except for n = 17 in *Pe* WT-infected 24 h recovery group. All p-values calculated via one-way ANOVA followed by Tukey's post hoc test for multiple comparisons.

Since feeding with pathogenic or harmless *Pe* and *Ecc15* had not yielded acquired aversion in an olfactory choice between bacteria and air, I next tried a different approach for conditioning with *Pe* using heat-killed, i.e. avirulent *Pe* WT as a control instead of the harmless strain. To that aim, two groups of wild-type CS flies were fed overnight with either pathogenic or heat-killed and hence harmless *Pe* WT and tested for their olfactory preferences towards *Pe* WT odour the next day (Figure 21A). For both live and dead *Pe* WT feeding, additional groups of flies were kept on the bacteria for another 24 h before testing to investigate potential long-term memory formation, but without introducing the odour of standard fly food as a potential confounding factor as was the case in the previous conditioning paradigm. Again, memory of the odour associated with the negative post-ingestive consequences of the infection should then manifest as an avoidance of pathogenic bacterial odour.

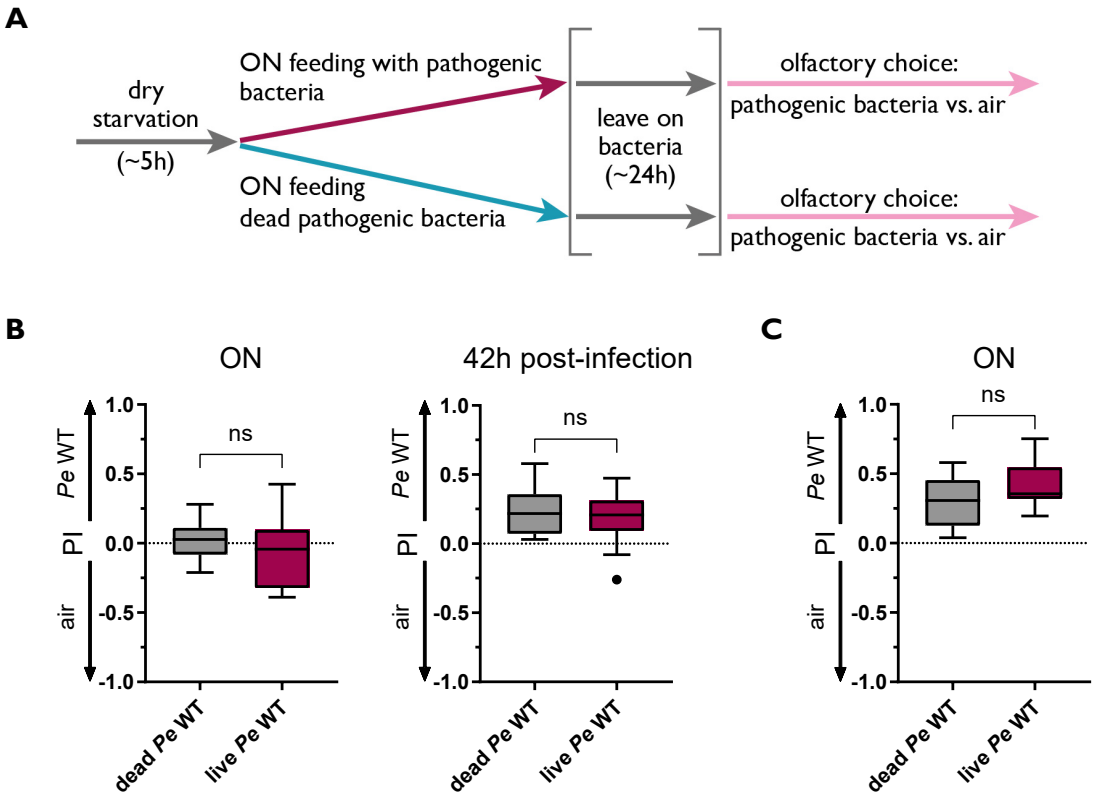

Figure 21 - Olfactory preferences for dead vs. live *Pe* following pathogenic infection

(A) Conditioning protocol for bacteria feeding and olfactory choice in the 4-field arena. **(B)** Olfactory preferences of wild-type CS flies for the choice between *Pe* WT and air after ON feeding with dead or live *Pe* as well as 42 h post-infection; n = 16. **(C)** Olfactory preferences of wild-type OrR flies for the choice between *Pe* WT and air following ON feeding with dead or live *Pe*; n = 16 (dead *Pe* WT), n = 15 (live *Pe* WT). All p-values calculated via unpaired t-test with Welch's correction.

Yet similar to the previous approach for *Pe* conditioning, flies were indifferent to *Pe* WT odour regardless of earlier feeding on live or dead *Pe* WT, both if tested directly after ON feeding and more than 40 h after feeding onset (Figure 21B). At 42 h post-infection, flies were even attracted by *Pe* WT odour, again irrespective of prior feeding on dead or live *Pe* (Figure 21B). However, this preference resembles the attraction to *Pe* WT (vs. air) seen in naïve, starved flies (see Figure 16B), and should hence most likely be regarded as an effect of starvation due to the drying out of the food source.

Furthermore, I subjected a second wild-type strain (OrR) to the same protocol, as OrR flies are much more seriously affected by *Pe* infection (see Figure 10B). The 42 h post-infection group was not tested in this case, as *Pe* is lethal for OrR after approximately 30 h (see Figure 10B). Again, dead and live *Pe* WT-fed flies did not differ and were both attracted by *Pe* WT odour after ON feeding (Figure 21C), resembling the behaviour observed in naïve flies (Figure 16).

3.4.2 Preferences for pathogenic versus harmless bacterial odours after infection

One of the reasons why the previous conditioning protocols were not successful in establishing whether *Drosophila* can learn to avoid bacterial odours after pathogenic infection could have been the olfactory choice itself. Since all paradigms relied on flies choosing between bacterial odours and air, starvation effects due to the prolonged feeding on the desiccating bacteria might have caused attraction to the bacterial odours as the only potential food source available, thereby masking any avoidance due to memory formation. For the next set of conditioning experiments, I hence decided to use an olfactory choice between pathogenic and harmless bacteria, which are similar as to their nutritive value, to identify potential changes in preferences towards bacterial odours after infection (Figure 22A). I started out by testing *Pe gacA*- and *Pe* WT-infected flies for their olfactory preferences towards a choice between the odour of the bacteria they had fed on (*Pe* WT or *Pe gacA*) and *Ecc15 evf* odour as a dissimilar and harmless second option. Yet flies infected with pathogenic *Pe* WT were indifferent to this olfactory choice and did not avoid *Pe* WT odour and moreover did not differ from harmless *Pe gacA*-infected control flies (Figure 22B); a behaviour also seen in naïve flies (Figure 18).

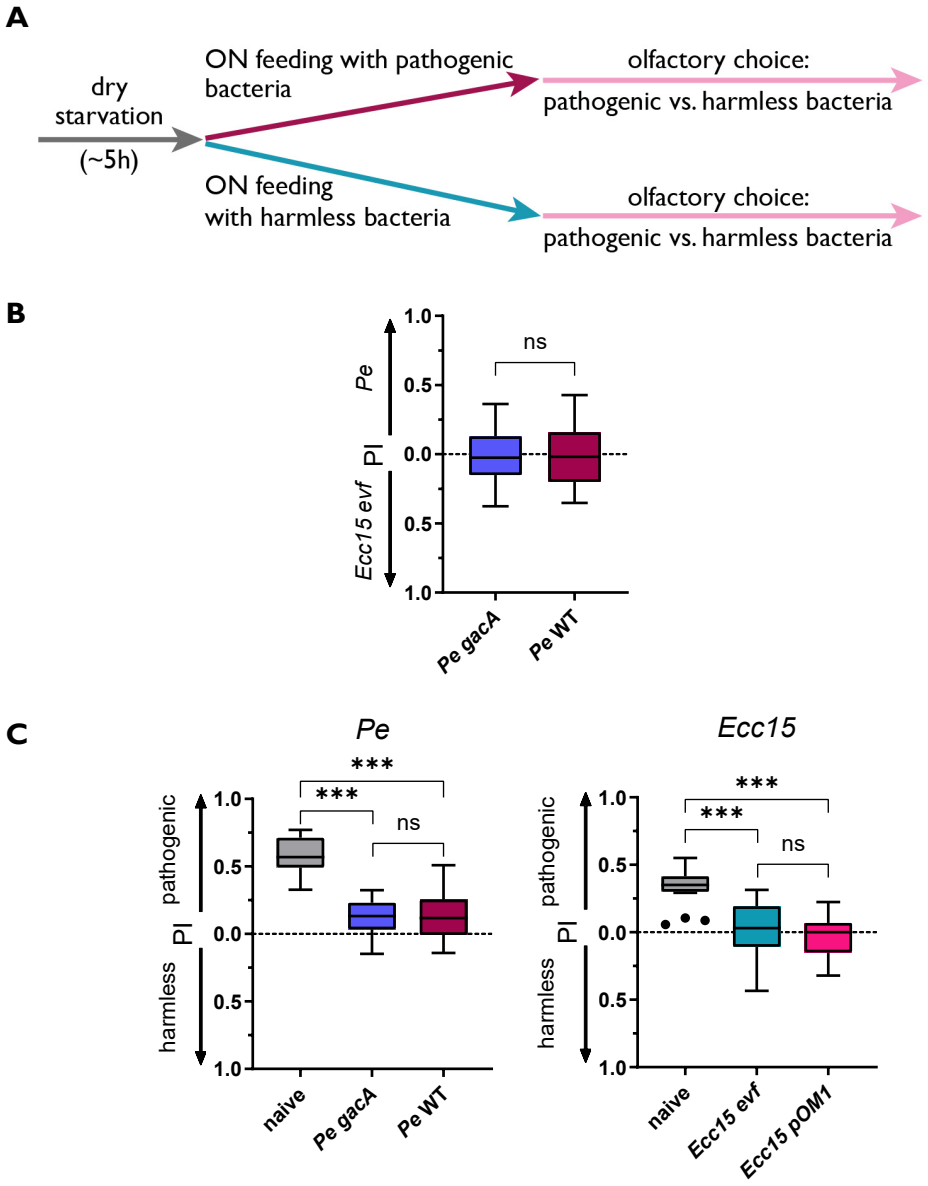

Figure 22 - Olfactory preferences for pathogenic vs. harmless bacteria after pathogenic infection

(A) Conditioning protocol for bacteria feeding and olfactory choice in the 4-field arena. **(B)** Olfactory preferences of wild-type OrR flies for the choice between *Pe* WT or *Pe gacA* and *Ecc15 evf*, respectively, following ON feeding on *Pe* WT or *Pe gacA*. p-value calculated via unpaired t-test with Welch's correction, n = 16. **(C)** Olfactory preferences of wild-type OrR flies for the choice between pathogenic and harmless *Pe* or *Ecc15*, respectively, following ON feeding on the corresponding bacteria. *Pe* all n = 16, *Ecc15* n = 16 (naïve) / 24 (*evf* infected) / 25 (*pOM1* infected). Naïve starved flies in C are from innate behaviour experiments also shown in Figure 19. p-values calculated via one-way ANOVA followed by Tukey's post hoc test for multiple comparisons.

Knowing that wild-type flies innately prefer the odour of pathogenic *Ecc15* or *Pe* over that of the respective harmless strain (see Figure 19), prior feeding with *Ecc15 pOM1* or *Pe* WT should induce a shift in preference in this olfactory choice in direction of the harmless strain, provided that flies form an olfactory memory of pathogen infection. For the next conditioning approach, I hence fed wild-type flies with either *Ecc15 pOM1* or *Ecc15 evf* as well as *Pe* WT or *Pe gacA* and subjected them to an olfactory choice between *Ecc15 evf* and *Ecc15 pOM1* or *Pe* WT and *Pe gacA* in the 4-field arena the next morning. However, for both *Ecc15* and *Pe*, flies that had been infected with the pathogenic strains did not differ from flies fed with harmless control strains in that all groups were neutral regarding the choice between pathogenic and harmless bacterial odours (Figure 22C). This behaviour significantly deviated from the attraction to pathogenic bacteria seen in naïve flies (Figure 22C, data for innate behaviour also shown in Figure 19). Regarding the difference between naïve and bacteria-fed flies, it should be noted that naïve flies were tested after ~20 h of wet starvation, while bacteria-fed flies were only slightly starved due to the bacteria-sucrose mixture drying out overnight.

To conclude, contrary to the attraction to pathogenic bacterial odours seen in naïve flies, prior bacteria feeding significantly reduced this attraction, as bacteria-fed flies were mostly indifferent to bacterial odours. However, this behaviour was irrespective of the pathogenicity of the bacterial strain flies had fed on. Accordingly, infecting flies with either pathogenic or harmless bacterial strains did not induce differences in their olfactory preference behaviour towards these bacteria for any of the conditioning paradigms tested. Thus, while showing that prior bacteria feeding does not seem to induce specific olfactory avoidance behaviours, these experiments also emphasize the need for considering hunger state and nutritive value when investigating choice behaviour towards pathogens.

3.5 Preferences for bacteria-contaminated food

Apart from potential olfactory aversions after pathogenic infection, another way to determine if *Drosophila* can avoid detrimental bacteria due to the post-ingestive consequences of pathogenic infection was to investigate feeding behaviour. For that purpose, wild-type flies that had never before been exposed to bacteria were offered different feeding choices involving pathogenic bacteria. Assuming that flies can detect pathogenic bacteria and adapt their behaviour in response to the potential negative post-ingestive consequences of feeding on them, they should, after some time, start avoiding the food source containing pathogenic bacteria. For all CAFE assays, I tested three different feeding choices: sucrose vs. harmless bacteria (+sucrose), sucrose vs. pathogenic bacteria (+sucrose) and harmless vs. pathogenic bacteria (both with sucrose). In the flyPAD, I only tested the latter choice. All bacteria preparations contained the same concentration of sucrose as the sucrose-only solution, i.e. 5%. For matters of simplicity and readability, the sucrose content in the bacteria preparations will henceforth not be mentioned separately.

3.5.1 Lasting avoidance of pathogen-contaminated food in the CAFE

3.5.1.1 *Pe* feeding preferences

To determine whether *Drosophila* would avoid feeding on pathogenic *Pe*, I used the CAFE assay to test feeding preferences for food containing harmless and/or pathogenic *Pe*. Following a period of ~24 h of wet starvation and ~2 h of dry starvation, wild-type OrR flies that had never before been exposed to bacteria were put into CAFE chambers, where they were offered a choice between two liquid food sources. Consumption was measured as the liquid decrease in the capillaries on an hourly basis for a total of 8-9 h (Figure 23A).

When given a choice between sucrose and the harmless *Pe gacA* strain, flies readily fed on both (Figure 23C, left). By contrast, flies consumed significantly more sucrose as compared to *Pe* WT a few hours after feeding onset, with sucrose and bacteria consumption again converging towards the end of the experiment (Figure 23C, centre). Interestingly, wild-type flies clearly preferred feeding on harmless *Pe gacA* over pathogenic *Pe* WT, with significantly higher consumption of *Pe gacA* as of 5 h after the start of the experiment (Figure 23B and C, right panel).

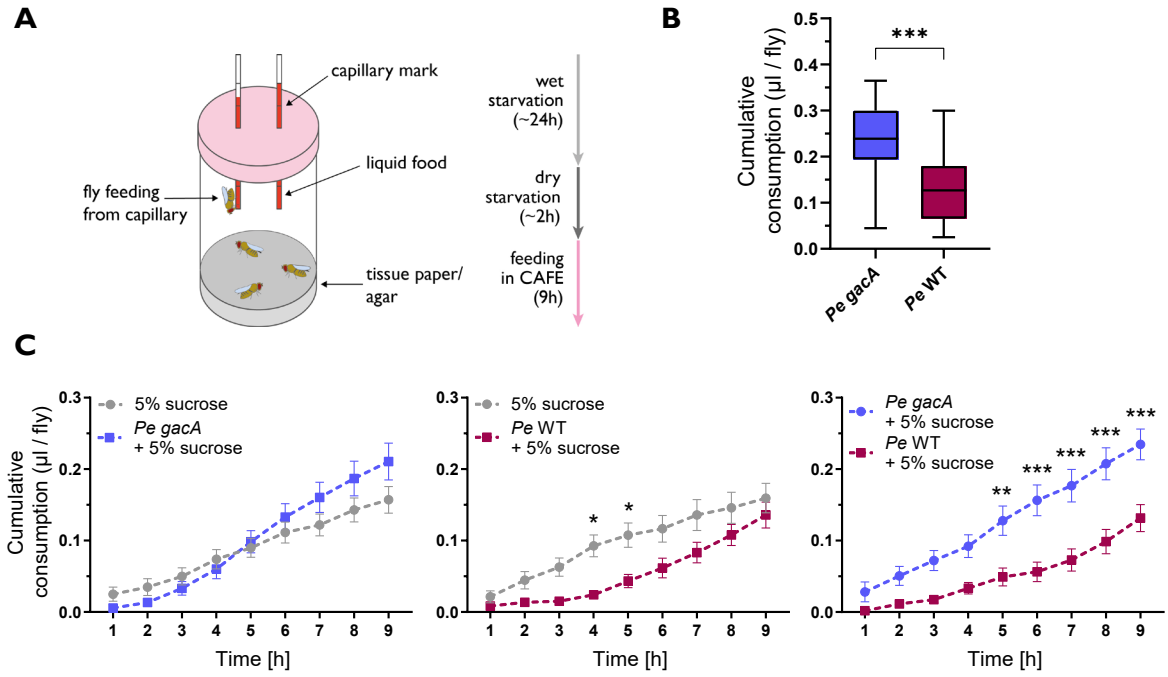

Figure 23 - Wild-type feeding preferences for *Pe* in the CAFE

(A) Illustration of a CAFE vial and general experimental protocol. **(B)** Total cumulative consumption in µl/fly of wild-type OrR flies after 9 h for the feeding choice between *Pe gacA* and *Pe* WT. **(C)** Cumulative consumption in µl/fly over time for the feeding choices harmless *Pe gacA* vs. sucrose (n = 16), pathogenic *Pe* WT vs. sucrose (n = 18) and *Pe gacA* vs. *Pe* WT (9 h-values in panel B, n = 17), mean ± SEM. p-values calculated via repeated-measures two-way ANOVA followed by Bonferroni's post hoc test for multiple comparisons.

It should be noted, however, that feeding choices involving *Pe* are challenging to test in the CAFE assay, as *Pe* are slimy bacteria and thus difficult to fill into the capillaries. Moreover, even though on average 65% of wild-type CS flies survived the oral infection with *Pe*, *Pe* is lethal for weaker and smaller fly lines such as wild-type OrR (see Figure 10) and hence potentially also for the mutant lines necessary for further behavioural testing. It is moreover questionable whether flies exhibit lasting behavioural adaptations upon experiencing the post-ingestive effects of pathogenic infection in cases where most of the population dies within less than 24 h. Accordingly, using highly virulent *Pe* as the stimulus inducing negative post-ingestive effects would prevent potential future experiments as to the investigation of more long-term effects of pathogen-modulated feeding behaviour. In addition, later flyPAD experiments (Figure 25) suggested that taste rather than post-ingestive effects might contribute to the feeding choice between pathogenic and harmless *Pe*. Consequently, all further CAFE experiments were conducted using *Ecc15* as a mildly

virulent, yet still harmful bacterial strain to infect flies and elicit negative post-ingestive effects and potentially learning.

3.5.1.2 *Ecc15* feeding preferences

Regarding preferences for food containing *Ecc15*, I not only tested wild-type OrR flies (Figure 24A), but also CS as a stronger and healthier wild-type fly line (Figure 24B) as well as w^{-}, a commonly used control strain (Figure 24C). All three preferred the more nutritive harmless *Ecc15 evf* over pure sucrose by the end of the experiment, i.e. after 9 h (Figure 24A-C, left panels). Overall consumption was considerably lower for the choice between sucrose and pathogenic *Ecc15 pOM1*, where only CS flies showed a slight preference for pathogenic bacteria after 9 h, while the OrR and w^{-} fly strains remained indifferent over the entire duration of the experiment (Figure 24A-C, middle). If given a direct choice between harmless *Ecc15 evf* and pathogenic *Ecc15 pOM1* bacteria, which are highly similar regarding their nutritional value, flies from all three wild-type strains initially fed on both strains, but shifted their preference towards the harmless strain after approximately 4-6 h (Figure 24A-C, right panels).

Flies of all three wild-type or control strains had thus consumed significantly more *Ecc15 evf* than *Ecc15 pOM1* by the end of the CAFE feeding choice experiment (Figure 24D), indicating that flies avoid pathogenic *Ecc15* if another protein source is available irrespective of their genetic background.

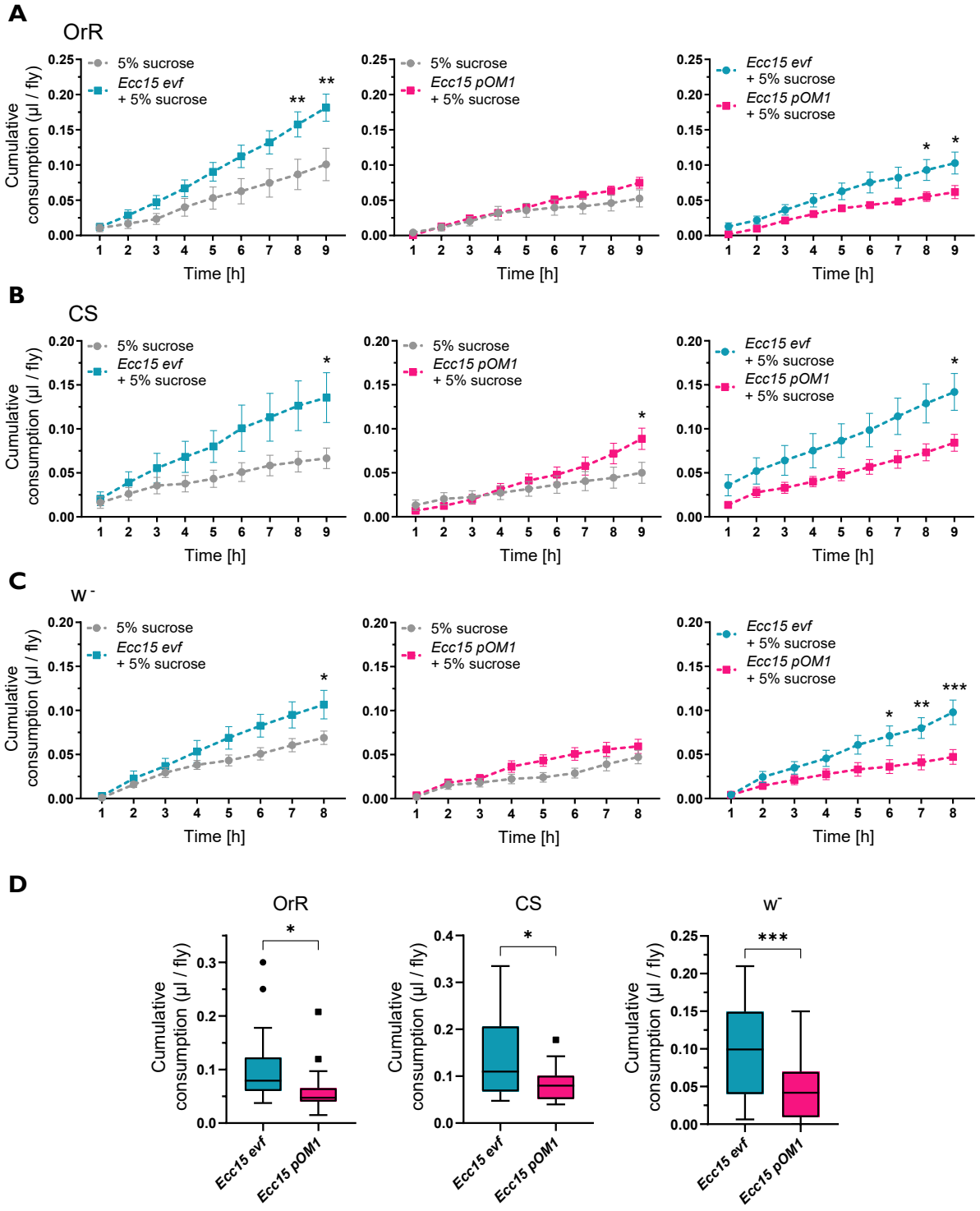

Figure 24 - Wild-type feeding preferences for *Ecc15* in the CAFE

(A) Cumulative consumption in µl/fly of wild-type OrR over time for the feeding choices sucrose vs. *Ecc15 evf* (n = 19), sucrose vs. *Ecc15 pOM1* (n = 20) and *Ecc15 evf* vs. *Ecc15 pOM1* (n = 20). **(B)** Cumulative consumption in µl/ fly of wild-type CS for the feeding choices sucrose vs. *Ecc15 evf* (n = 16), sucrose vs. *Ecc15 pOM1* (n = 16), *Ecc15 evf* vs. *pOM1* (n = 17). **(C)** Cumulative consumption in µl/fly of w⁻ flies over time at 30°C for the feeding choices sucrose vs. *Ecc15 evf* (n = 22), sucrose vs. *Ecc15 pOM1* (n = 23), *Ecc15 evf* vs. *pOM1* (n = 22). **(D)** Total cumulative consumption at the end of the assay for the *Ecc15 evf* vs. *pOM1* feeding choices from A-C. p-values calculated via repeated-measures two-way ANOVA followed by Bonferroni's post hoc test for multiple comparisons. Graphs in A-C depict mean ± SEM.

Taken together, the examined feeding choices in the CAFE assay show that *Drosophila* distinguishes harmless from pathogenic bacteria when feeding on them and clearly favours harmless *Ecc15* and *Pe* over the corresponding pathogenic strains. In the case of *Pe*, flies exhibited a strong preference for harmless *Pe gacA* soon after the first exposure to the choice between *Pe gacA* and *Pe* WT. By contrast, flies initially fed on both *Ecc15* strains and only later shifted their preference to harmless *Ecc15 evf* so that they consumed significantly more of the harmless strain towards the end of the experiment, i.e. after 8-9 h.

3.5.2 Feeding aversion to pathogens in the flyPAD

The CAFE assay allows the direct, quantitative measurement of liquid food uptake and can be used to monitor feeding behaviour over longer periods of time. However, it is not well suited to determine how fast *Drosophila* shifts its preference to feeding harmless as opposed to pathogenic bacterial strains. In order to get a higher resolution image especially during the first hours of pathogen feeding and to confirm the observations from the CAFE assay, I thus used the flyPAD (Itskov et al., 2014) to find out whether flies are immediately repelled by pathogen-containing food or start avoiding it at a later point; the former indicating a potential contribution of taste, the latter hinting at post-ingestive effects.

3.5.2.1 Immediate feeding preference for harmless *Pe*

For a more detailed investigation of feeding preferences towards highly virulent *Pe*, the master student I supervised, Irina Petcu, subjected 4-5 h dry starved wild-type CS flies to a feeding choice between harmless *Pe gacA* and pathogenic *Pe* WT in the flyPAD (Figure 25A). The cumulative number of sips as the strongest indicator of food intake shows that flies clearly preferred feeding on *Pe gacA* to *Pe* WT already a few minutes after experiment onset (Figure 25B), with the number of sips being significantly higher for the harmless strain at all time points analysed (Figure 25C).

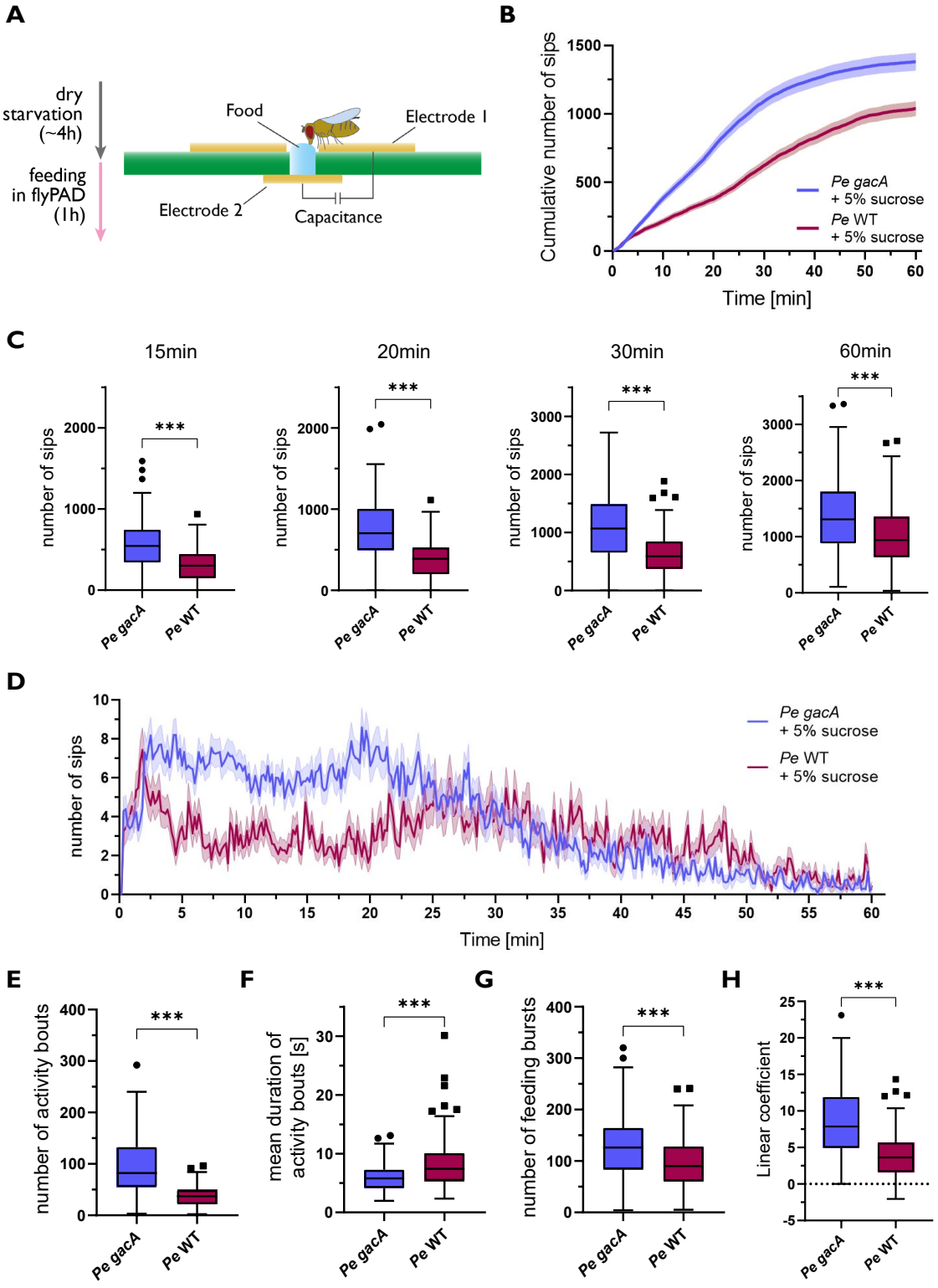

Figure 25 - Feeding preference for harmless over pathogenic *Pe* in the flyPAD

(A) Illustration of the flyPAD and general protocol for flyPAD experiments. **(B)-(H)** Feeding preferences of wild-type CS flies for the choice *Pe gacA* vs. *Pe* WT. **(B)** Cumulative number of sips, mean ± SEM. **(C)** Total number of sips after 15, 20, 30 and 60 min, p-values calculated by comparing *Pe gacA*/*Pe* WT ratios to 1 via the Wilcoxon signed rank test. **(D)** Non-cumulative number of sips, mean ± SEM. **(E)** Number of activity bouts. **(F)** Duration of activity bouts **(G)** Number of feeding bursts. **(H)** Linear coefficient. p-values in E-H calculated via the Wilcoxon matched-pairs signed rank test, n = 137. Experiments performed by Irina Petcu.

The distribution of sips over time shows that *Pe gacA* consumption was especially high during the first 25 minutes, while *Pe* WT consumption was low, yet steady for the most part of the 1h-long experiment (Figure 25D). Other parameters that correlate with food intake such as the number of activity bouts (Figure 25E) further support the observation that *Drosophila* avoids feeding on pathogenic *Pe* WT, while the duration of activity bouts as a third indicator of feeding was curiously shorter for the harmless strain (Figure 25F). Yet flies also exhibited significantly more feeding bursts, i.e. several sips in quick succession, and a higher motivation to feed – described by the linear coefficient – for harmless *Pe gacA* (Figure 25G and H), which is consistent with an overall higher preference for harmless *Pe gacA*.

Thus, almost all of the flyPAD parameters for the feeding choice between *Pe gacA* and *Pe* WT confirm the data obtained from CAFE experiments in that *Drosophila* can distinguish harmless from pathogenic *Pe* when feeding on them and prefers the harmless bacterial strain. The flyPAD data additionally show that this distinction occurs immediately after feeding onset and thus potentially due to taste.

3.5.2.2 Delayed feeding aversion to pathogenic *Ecc15*

In order to get a similar, more high-resolution analysis of feeding preferences towards the mildly virulent *Ecc15* strain, I offered starved wild-type CS flies a choice between harmless *Ecc15 evf* and pathogenic *Ecc15 pOM1* in the flyPAD. Again confirming what was seen in the CAFE, flies preferred the harmless over the pathogenic *Ecc15* strain (Figure 26A). Interestingly, flies only shifted their preference to harmless *Ecc15 evf* after feeding on both strains for approximately 15 minutes (Figure 26B and C). Other indicators of food uptake such as the number and duration of activity bouts were also significantly higher for harmless as compared to pathogenic *Ecc15* (Figure 26D and E). Finally, flies were more motivated to feed on *Ecc15 evf* as indicated by the linear coefficient and exhibited more feeding bursts for the harmless strain (Figure 26F and G), thus further underscoring the feeding preference for harmless over pathogenic *Ecc15* bacteria.

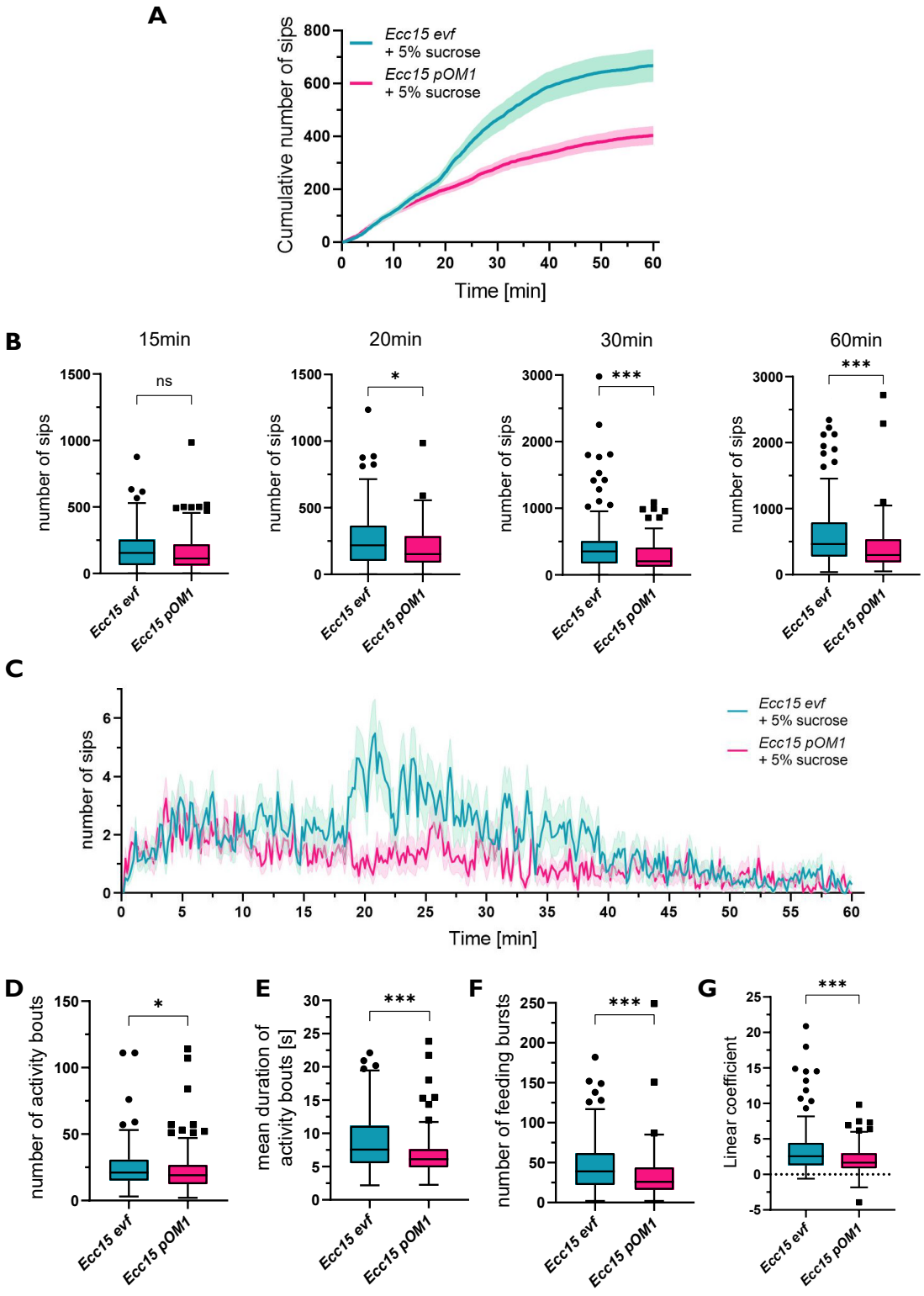

Figure 26 - Feeding preference for harmless over pathogenic *Ecc15* in the flyPAD

Feeding preferences of wild-type CS flies for the choice *Ecc15 evf* vs. *Ecc15 pOM1*. **(A)** Cumulative number of sips, mean ± SEM. **(B)** Total number of sips after 15, 20, 30 and 60 min; p-values calculated by comparing *Ecc15 evf/Ecc15 pOM1* ratios to 1 via the Wilcoxon signed rank test. **(C)** Non-cumulative number of sips, mean ± SEM. **(D)** Number of activity bouts. **(E)** Duration of activity bouts. **(F)** Number of feeding bursts. **(G)** Linear coefficient. p-values in D-G calculated via the Wilcoxon matched-pairs signed rank test, n = 132. Extreme outliers in B, F and G are removed from graphs, but included in the analysis.

To further determine whether wild-type flies would avoid pathogenic *Ecc15* under all circumstances or feed on it if it was the most nutritive food source available, I conducted a second flyPAD experiment to investigate feeding preferences for the choice between LB medium (+ sucrose) and *Ecc15 pOM1* (+ sucrose). In this case, starved wild-type flies clearly preferred feeding on pathogenic *Ecc15 pOM1* over LB (Figure 27A and B), with the higher consumption of *Ecc15 pOM1* being apparent soon after experiment onset (Figure 27C). This suggests that flies prefer the more nutritive, yet potentially harmful food source to the plain LB/sucrose mixture. Thus, similar to what was seen in CAFE experiments where CS flies preferred *Ecc15 pOM1* to sucrose (see Figure 24B), starved flies do not per se reject pathogenic *Ecc15* in the flyPAD, but feed on it depending on other available options.

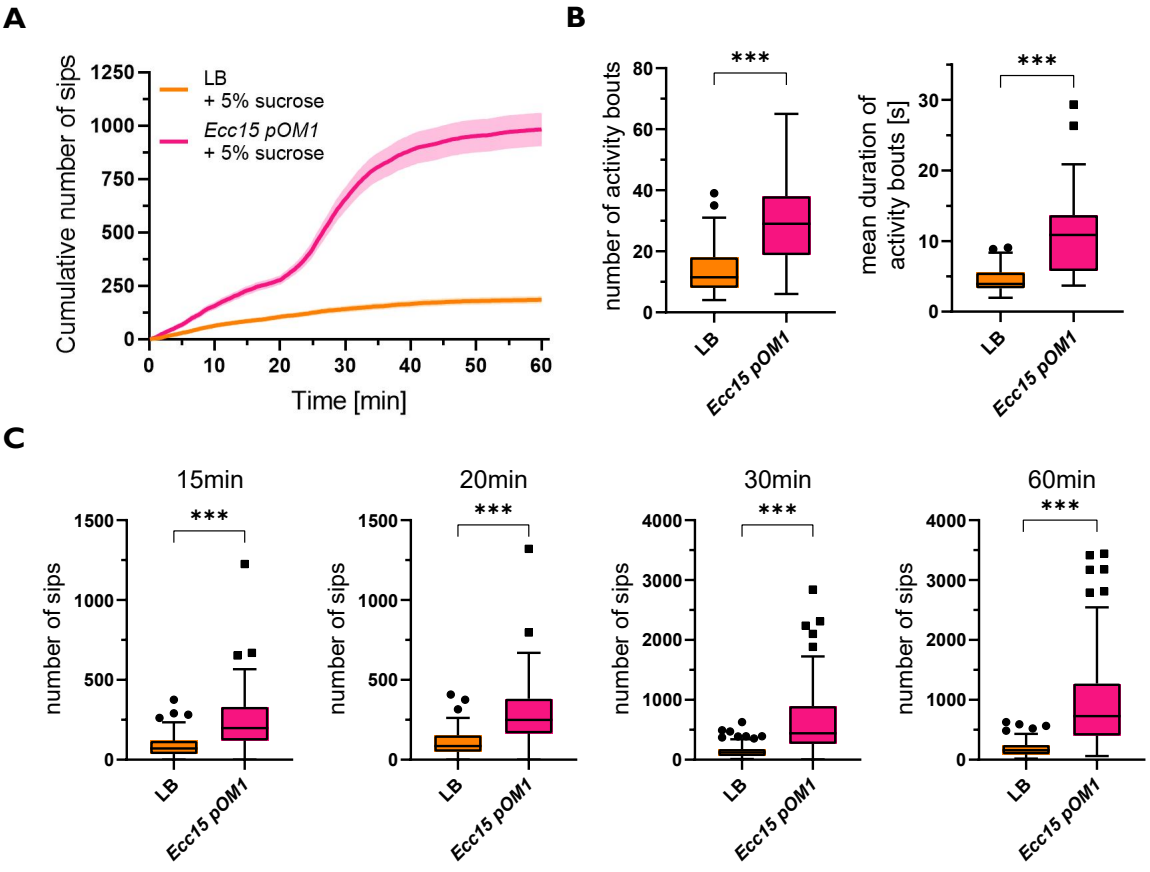

Figure 27 - Feeding preference for pathogenic *Ecc15* over LB in the flyPAD

Wild-type CS feeding preferences for the choice LB + 5% sucrose vs. *Ecc15 pOM1* + 5% sucrose. **(A)** Cumulative number of sips, mean ± SEM. **(B)** Number and duration of activity bouts. p-values calculated via the Wilcoxon matched-pairs signed rank test. **(C)** Total number of sips after 15, 20, 30 and 60 min. p-values calculated by comparing LB/*pOM1* ratios to 1 via the Wilcoxon signed rank test, n = 133. Extreme outliers in C are removed from graphs, but included in the analysis.

Another possibility to compare pathogenic and harmless food sources is to use heat-shock killed pathogenic bacteria instead of a mutant strain as the harmless control (Surendran et al., 2017). Therefore, I tested starved wild-type CS flies for their feeding preferences for the choice between dead and live *Ecc15 pOM1*. At the beginning of the 1h-feeding period, flies fed more from the food substrate containing dead *pOM1* than from live *Ecc15 pOM1*, yet the number of sips taken per substrate converged towards the end, suggesting equal feeding from both food sources (Figure 28A and C). Other indicators of food uptake also yielded ambiguous results, as flies exhibited more activity bouts for dead *pOM1*, which however lasted longer in the case of live *Ecc15 pOM1* (Figure 28B). Therefore, it cannot be stated with certainty if flies have a clear preference for dead, i.e. harmless *pOM1* as opposed to live *Ecc15 pOM1*.

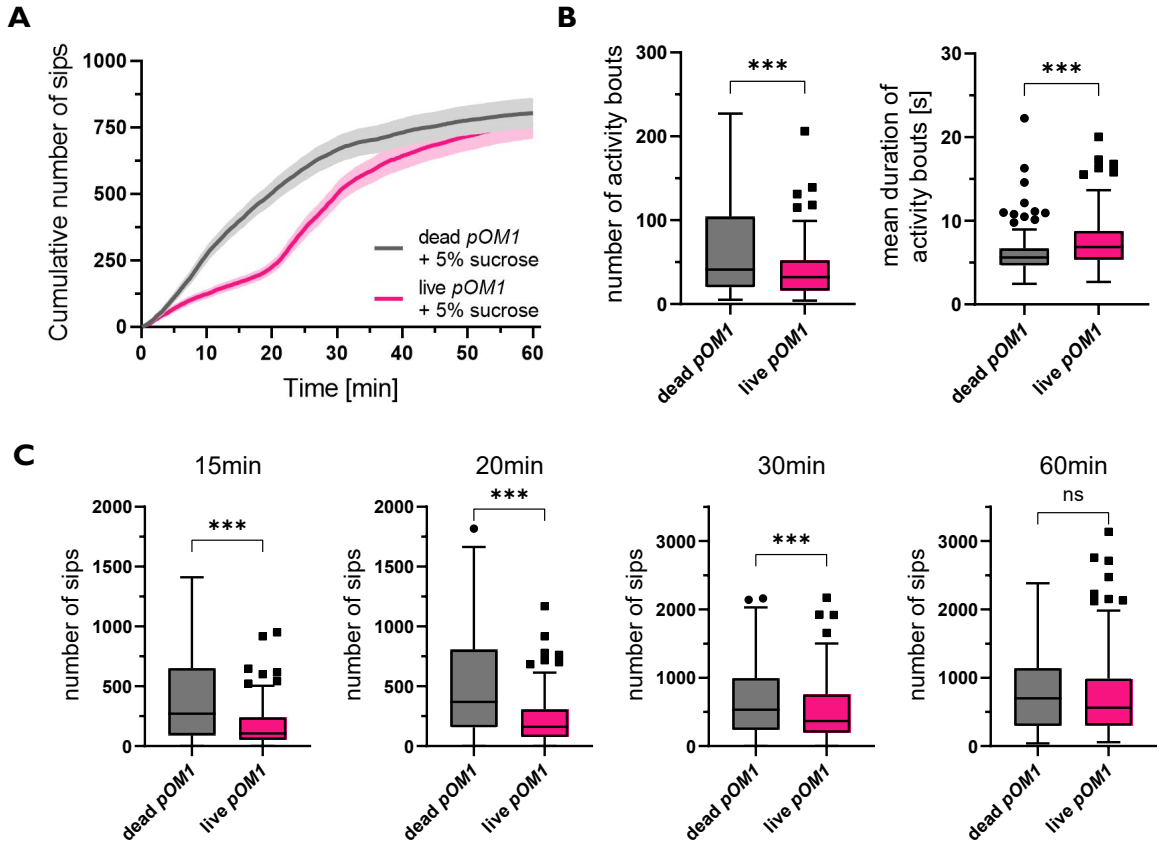

Figure 28 - Feeding preference for dead vs. live *Ecc15 pOM1* in the flyPAD

Wild-type CS feeding preferences for the choice dead vs. live *Ecc15 pOM1*. **(A)** Cumulative number of sips, mean ± SEM. **(B)** Number and duration of activity bouts. p-values calculated via the Wilcoxon matched-pairs signed rank test. **(C)** Total number of sips after 15, 20, 30 and 60 min. p-values calculated by comparing LB/*pOM1* ratios to 1 via the Wilcoxon signed rank test, n = 131.

3.5.2.3 Sucrose feeding in the flyPAD and the contribution of taste

One could object that the main reason why flies prefer feeding on harmless over pathogenic bacteria is in fact a difference in taste between the two strains. However, a first indicator that taste does not play a dominant role for the distinction between good and bad at least in the case of *Ecc15* is that flies initially fed on both strains in the flyPAD and only shifted their preference to harmless *Ecc15 evf* after approximately 15-20 min. Yet differences in taste should be reflected in a much faster divergence of sips taken from the two substrates. To confirm this hypothesis and to get a more detailed picture of the time course of feeding in the flyPAD, I conducted another flyPAD experiment with a feeding choice between two easily discernible food sources, i.e. 1% sucrose and 10% sucrose. As expected and previously reported (Itskov et al., 2014), starved flies immediately favoured the higher over the lower sucrose concentration (Figure 29A).

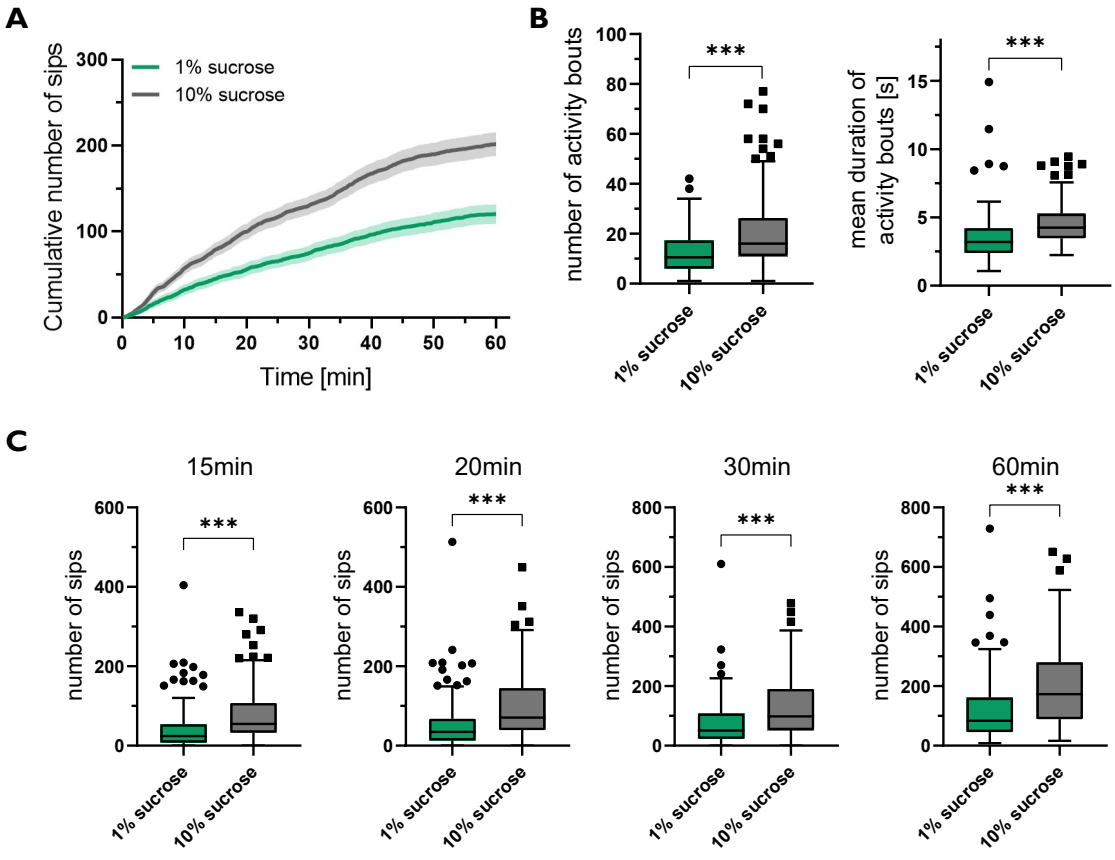

Figure 29 - Sucrose feeding preferences in the flyPAD

Wild-type CS feeding preferences for the choice 1% vs. 10% sucrose. **(A)** Cumulative number of sips, mean ± SEM. **(B)** Number and duration of activity bouts. p-values calculated via the Wilcoxon matched-pairs signed rank test. **(C)** Total number of sips after 15, 20, 30 and 60 min. p-values calculated by comparing 1% sucrose/10%sucrose ratios to 1 via the Wilcoxon signed rank test, n = 126.

Other indicators of food intake such as the number and duration of activity bouts also show that flies preferred the 10% sucrose food substrate to food containing only 1% sucrose (Figure 29B). It should also be noted that the difference in sips taken was already highly significant 15 minutes after experiment onset (Figure 29C), which was also observed for *Pe*, but not in the case of *Ecc15* feeding. Thus, while a contribution of taste cannot be excluded for the feeding aversion to pathogenic *Pe*, the preference for harmless over pathogenic *Ecc15* does most likely not result from an innate taste preference but rather seems to constitute an acquired aversion, potentially due to post-ingestive signals. Consequently, all further experiments focused on *Ecc15* to investigate how the post-ingestive consequences of pathogen ingestion and infection induce lasting behavioural changes.

3.6 The role of associative memory formation for feeding aversion

3.6.1 Feeding preferences upon inactivation of the mushroom body

As explored in the preceding sections, *Drosophila* is innately attracted to the odour of pathogenic bacteria, but avoids feeding on them and instead prefers the harmless versions of the same strain. Moreover, in the case of *Ecc15*, taste does not seem to be the major determinant for the feeding preference for harmless as opposed to pathogenic bacteria. The time lag between feeding onset and the occurrence of a shift in preference towards the harmless strain instead suggests this behaviour to be an acquired aversion. To test the hypothesis whether the observed feeding aversion to pathogenic *Ecc15* indeed constitutes an adaptive behaviour requiring higher brain centres, I investigated the role of the MB as the learning and memory centre of the fly brain in the observed feeding aversion. To that aim, I reversibly blocked synaptic output from all KCs of the MB during the *Ecc15* feeding choice via the expression of the temperature-sensitive dynamin mutant allele *shibire*^{ts1} in the MB. In the CAFE, MB10B-GAL4 > UAS-shi^{ts1} flies fed equally from both *Ecc15 evf* and *Ecc15 pOM1*, while pBDP-GAL4U > UAS-shi^{ts1} control flies (so-called 'empty' control, Pfeiffer et al., 2010) exhibited the known feeding preference for harmless *Ecc15 evf* (Figure 30).

Thus, flies need their MB to distinguish between pathogenic and harmless food sources, suggesting this to be an adaptive behaviour that might require associative memory formation.

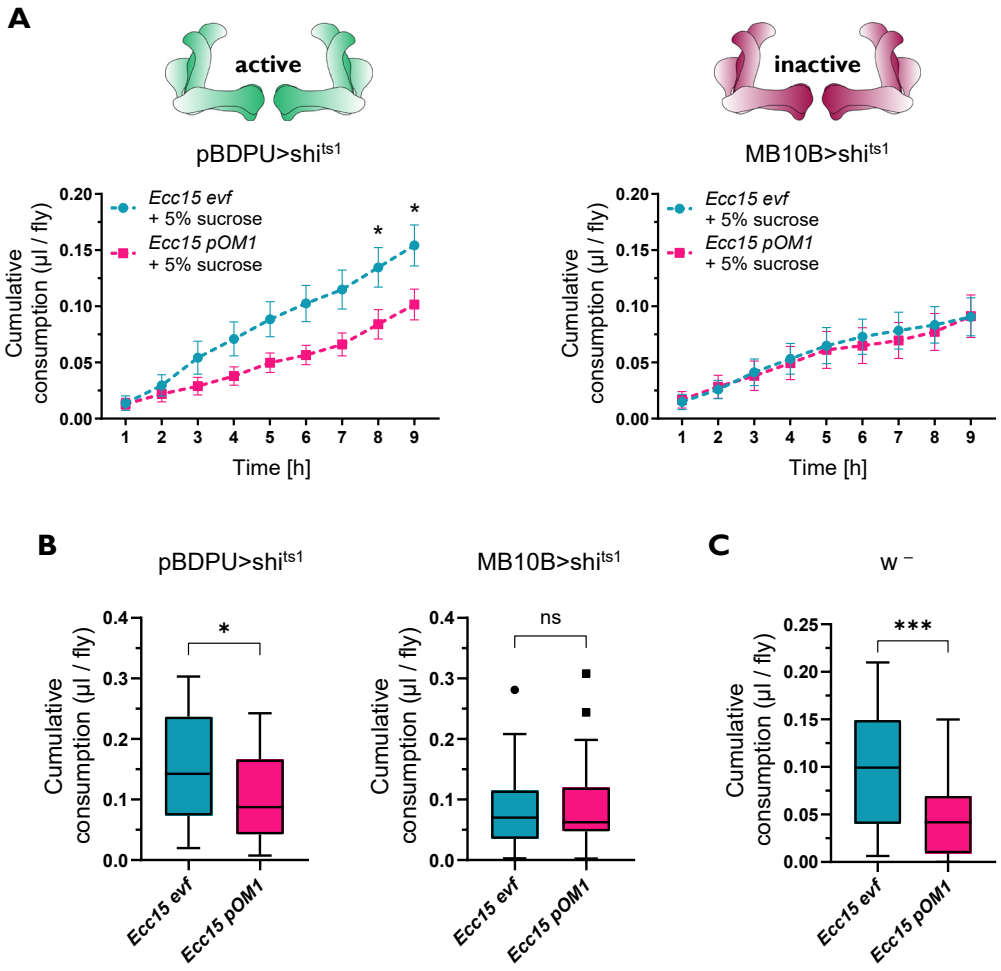

Figure 30 - *Ecc15* feeding preferences in the CAFE upon silencing of all MB KCs

(A) Cumulative consumption in µl/fly of control (pBDP-GAL4U > UAS-shi^{ts1}, n = 24) and MB-inactivated flies (MB10B-GAL4 > UAS-shi^{ts1}, n = 19) over time for the feeding choice *Ecc15 evf* vs. *Ecc15 pOM1* at 30°C; mean ± SEM **(B)** Total cumulative consumption from (A) after 9 h. **(C)** Total cumulative consumption of w$^-$ control flies also shown in Figure 24 at 30°C after 8 h for additional comparison (n = 22). p-values calculated via repeated-measures two-way ANOVA followed by Bonferroni's post hoc test for multiple comparisons.

3.6.2 Feeding preferences of *rutabaga* learning mutant flies

Adaptive behaviour and memory formation require synaptic plasticity. One enzyme that is known to mediate associative synaptic plasticity is the Ca^{2+}/calmodulin-dependent adenylyl cyclase rutabaga. *Rutabaga* mutant flies exhibit learning defects and are unable to form associative memories (Livingstone et al., 1984). To further investigate a putative contribution of learning and memory formation to the observed feeding aversion to pathogenic bacteria, I hence conducted bacterial feeding choice experiments in the CAFE using *rutabaga* mutant flies. These flies preferred harmless, but not pathogenic bacteria to

mere sucrose (Figure 31A), indicating that they were still able to distinguish food sources of different nutritive value. Interestingly, flies lacking rutabaga did not distinguish between good and bad bacterial food sources, i.e. they fed equally from both *Ecc15 evf*- and *Ecc15 pOM1*-containing capillaries (Figure 31A and C).

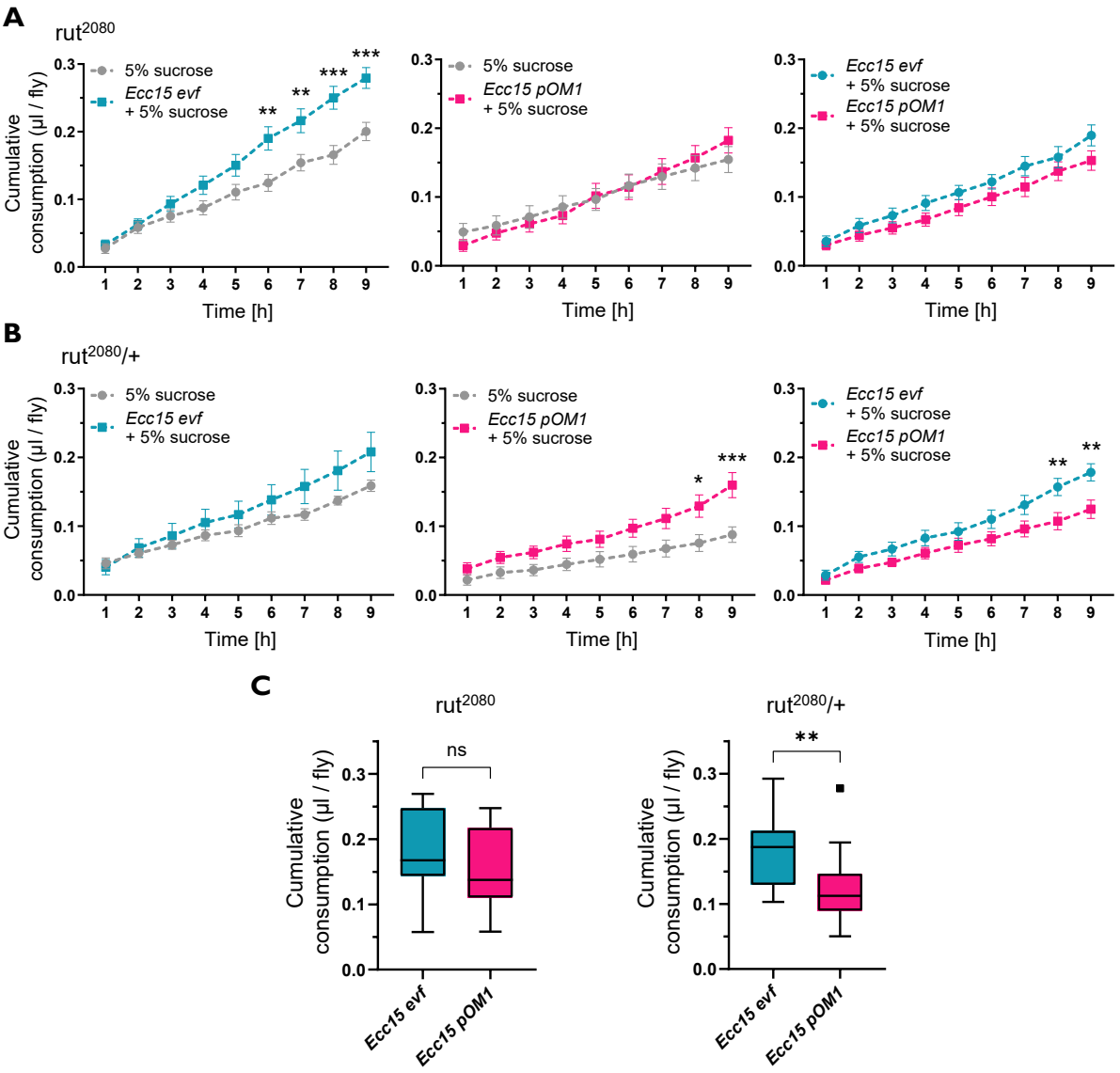

Figure 31 - *Ecc15* feeding preferences of *rutabaga* mutant flies in the CAFE

(A) Cumulative consumption in µl/fly of rutabaga^{2080} flies over time for the feeding choices sucrose vs. *Ecc15 evf*, sucrose vs. *Ecc15 pOM1* and *Ecc15 evf* vs. *Ecc15 pOM1* (all n = 17). **(B)** Cumulative consumption in µl/fly of rutabaga^{2080}/+ heterozygous control flies over time for the feeding choices sucrose vs. *Ecc15 evf* (n = 16), sucrose vs. *Ecc15 pOM1* (n = 17) and *Ecc15 evf* vs. *Ecc15 pOM1* (n = 17). **(C)** Total cumulative consumption from (A) and (B) for the feeding choices *Ecc15 evf* vs. *Ecc15 pOM1* after 9 h. All p-values calculated via repeated-measures two-way ANOVA followed by Bonferroni's post hoc test for multiple comparisons. Graphs in A and B depict mean ± SEM.

Contrary to *rutabaga*-deficient flies, heterozygous control flies with one functional copy of *rutabaga* were able to make this distinction and shifted their preference towards *Ecc15 evf* after a few hours, thus consuming significantly more harmless than pathogenic *Ecc15* by the end of the assay (Figure 31B and C). In addition, heterozygous flies did not clearly differentiate between sucrose and *Ecc15 evf* and consumed more *Ecc15 pOM1* than sucrose towards the end of the assay (Figure 31B), suggesting once again that flies avoid detrimental bacteria only if another protein source is available.

The fact that both MB-inactivated flies as well as flies deficient in short-term memory formation were unable to distinguish a harmless from a detrimental food source suggests that the observed aversion to a food source containing pathogenic bacteria could rely on an associative learning mechanism.

3.7 Contribution of Imd pathway components to feeding aversion

Having shown that the fly's memory brain centre is necessary to distinguish good from bad bacteria, I next investigated how the detection of pathogens in the periphery contributes to the observed feeding behaviour and how the nervous system could sense that pathogens have been ingested. One of the first lines of defence against pathogens that enter the body is the immune response. The *Drosophila* innate immune system recognizes and fights Gram-negative bacteria such as *Ecc15* via the Imd pathway, which comprises the recognition of bacterial cell wall PGN by PGRPs and the consequential activation of an NF-κB signalling cascade that leads to the transcription of AMPs and other effectors (Figure 3). Apart from the direct antimicrobial response it initiates, the Imd pathway has also been implicated in steering adaptive behavioural strategies to lower the negative impact of an infection (Kurz et al., 2017). The observed acquired feeding aversion to pathogenic *Ecc15* also seems to represent such a behavioural adaptation induced by pathogenic bacteria and the associated post-ingestive effects and might hence similarly rely on Imd pathway activation. To test this hypothesis, I subjected mutants for different components of the Imd pathway to the *Ecc15* feeding choice in the CAFE.

3.7.1 Peptidoglycan recognition proteins

PGRPs as upstream components of the Imd pathway activate the signalling cascade upon recognizing bacterial PGN and thus constitute the first step of the innate immune response. PGRP-LC is a transmembrane PGRP that can bind both polymeric and monomeric PGN. Flies lacking this receptor fed more *Ecc15 evf* and *Ecc15 pOM1*, respectively, if the alternative was only sucrose, and were hence still able to make the distinction between the protein-rich bacteria-sucrose mixture and mere sucrose. Interestingly, however, *PGRP-LC* mutant flies completely lost the ability to discriminate between harmless and pathogenic *Ecc15* (Figure 32A and C). Heterozygous control flies that still retained one functional copy of PGRP-LC preferred *Ecc15 evf*, but not *Ecc15 pOM1*, to sucrose and clearly chose avirulent *Ecc15 evf* over detrimental *Ecc15 pOM1* (Figure 32B and C).

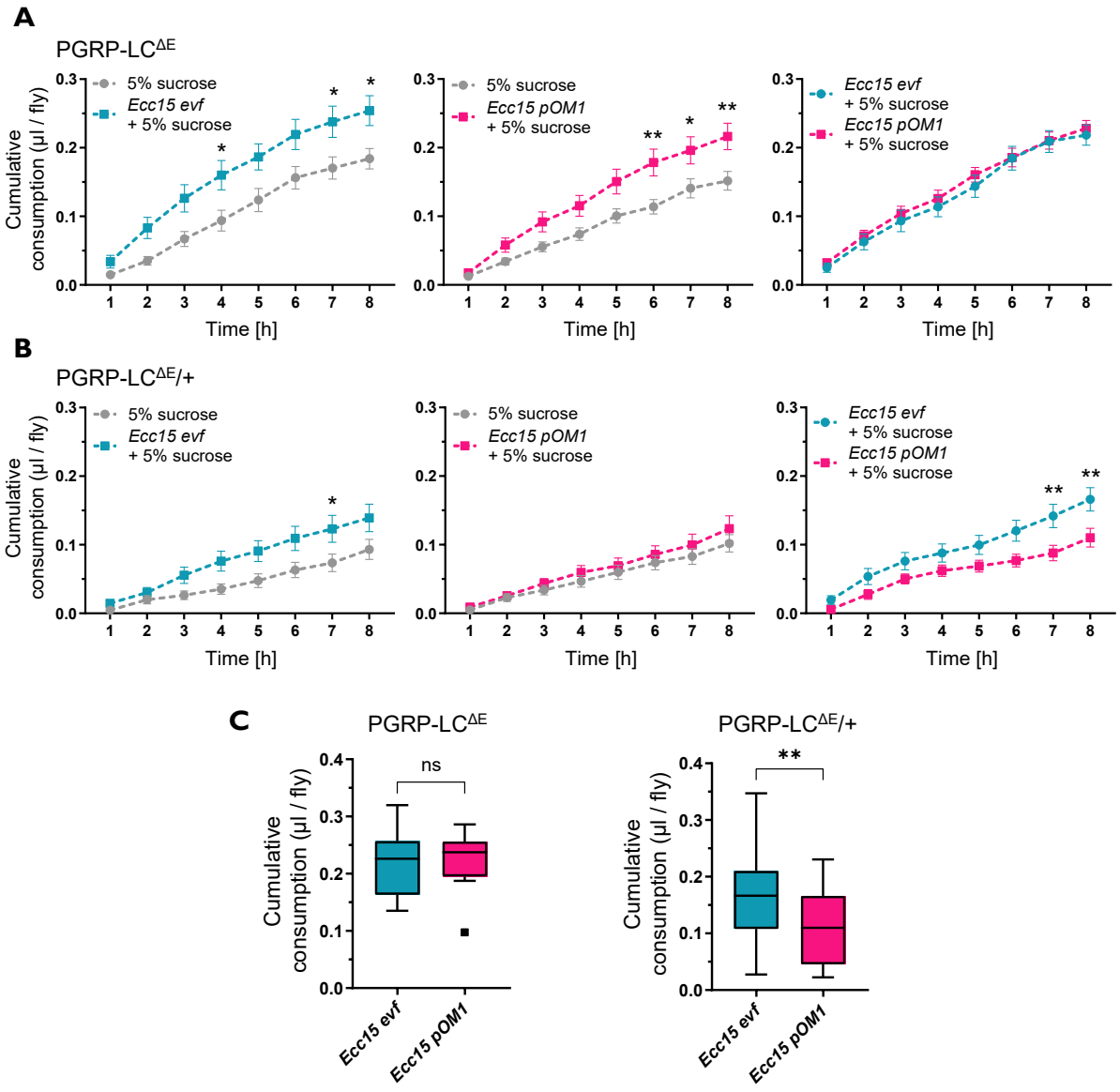

Figure 32 - *Ecc15* feeding preferences of flies lacking PGRP-LC in the CAFE

(A) Cumulative consumption in µl/fly of PGRP-LC$^{\Delta E}$ flies over time for the feeding choices sucrose vs. *Ecc15 evf,* sucrose vs. *Ecc15 pOM1* and *Ecc15 evf* vs. *Ecc15 pOM1* (all n = 16). **(B)** Cumulative consumption in µl/fly of PGRP-LC$^{\Delta E}$/+ heterozygous control flies over time for the feeding choices sucrose vs. *Ecc15 evf,* sucrose vs. *Ecc15 pOM1* and *Ecc15 evf* vs. *Ecc15 pOM1* (all n = 24). **(C)** Total cumulative consumption from (A) and (B) for the feeding choices *Ecc15 evf* vs. *Ecc15 pOM1* after 8 h. All p-values calculated via repeated-measures two-way ANOVA followed by Bonferroni's post hoc test for multiple comparisons. Graphs in A and B depict mean ± SEM.

A second PGRP, PGRP-LE, recognizes and binds monomeric PGN in the cytosol. The absence of this receptor during *Ecc15* feeding choices similarly abolished the previously observed preference for harmless over pathogenic *Ecc15* (Figure 33A and C).

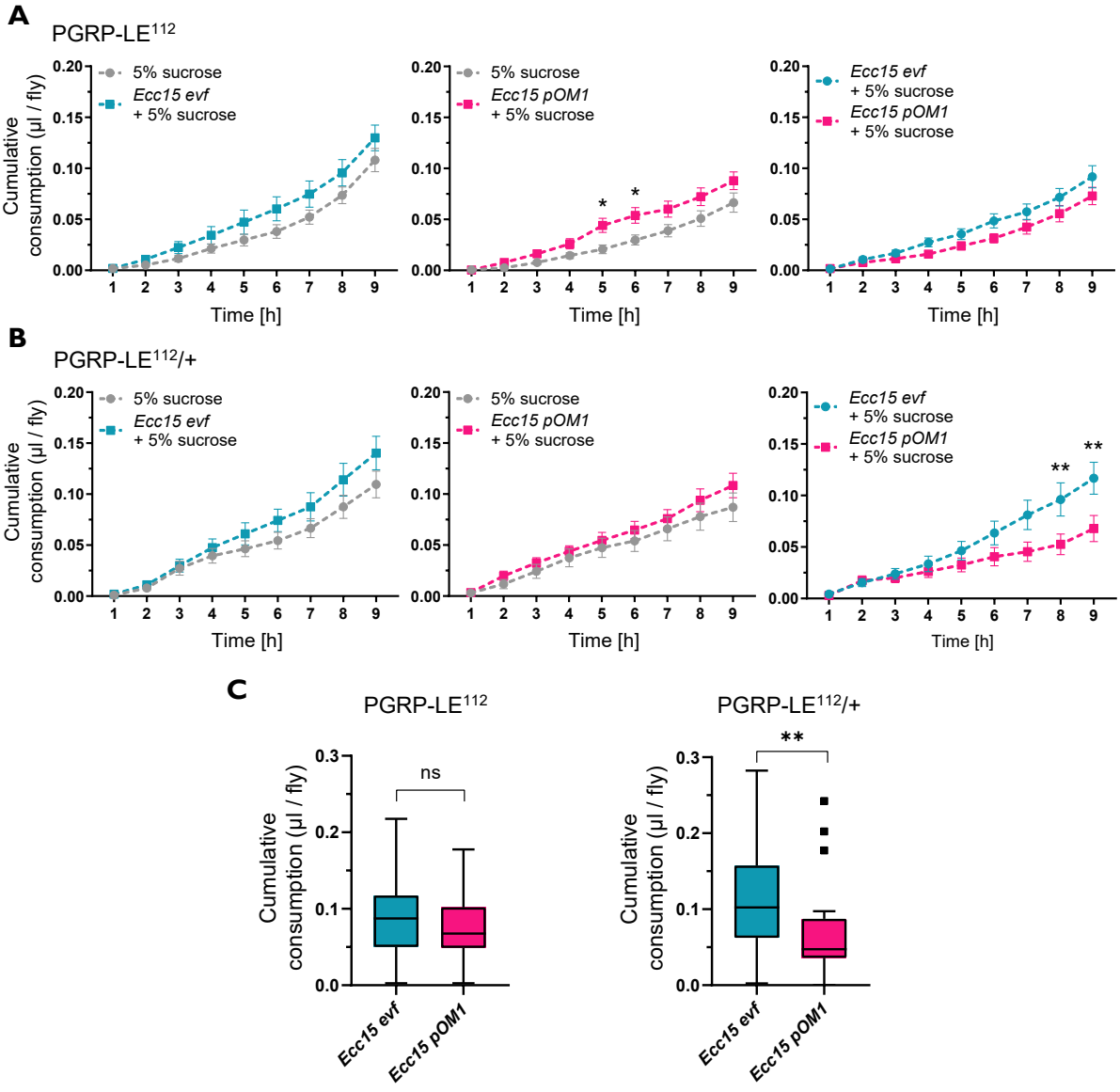

Figure 33 - *Ecc15* feeding preferences of flies lacking PGRP-LE in the CAFE

(A) Cumulative consumption in µl/fly of PGRP-LE^{112} flies over time for the feeding choices sucrose vs. *Ecc15 evf*, sucrose vs. *Ecc15 pOM1* and *Ecc15 evf* vs. *Ecc15 pOM1* (all n = 24). **(B)** Cumulative consumption in µl/fly of PGRP-LE^{112}/+ heterozygous control flies over time for the feeding choices sucrose vs. *Ecc15 evf* (n = 24), sucrose vs. *Ecc15 pOM1* (n = 22) and *Ecc15 evf* vs. *Ecc15 pOM1* (n = 23). **(C)** Total cumulative consumption from (A) and (B) for the feeding choices *Ecc15 evf* vs. *Ecc15 pOM1* after 9 h. All p-values calculated via repeated-measures two-way ANOVA followed by Bonferroni's post hoc test for multiple comparisons. Graphs in A and B depict mean ± SEM.

PGRP-LE-deficient flies did not favour *Ecc15 evf* over sucrose, while slightly preferring pathogenic *Ecc15 pOM1* to mere sucrose (Figure 33A). Heterozygous control flies did not

differentiate in case of the *Ecc15* vs. sucrose feeding choices, but otherwise started preferring *Ecc15 evf* to *Ecc15 pOM1* towards the end of the assay (Figure 33B and C). Thus, the preceding data indicate that *Drosophila* relies on both the transmembrane PGRP-LC and the cytosolic PGRP-LE immune receptors to differentiate between harmless and pathogenic *Ecc15* and adapt its behaviour to avoid a detrimental food source.

3.7.2 Downstream components of the Imd pathway

PGRPs activate a downstream NF-κB signalling cascade, which involves several steps and culminates in the nuclear translocation of the NF-κB transcription factor Relish. Relish in turn induces the transcription of immune effectors such as AMPs (Figure 3).

Flies that lack a specific component of the Imd pathway, namely the caspase Dredd that normally cleaves Relish, still prefer harmless *Ecc15 evf* over pathogenic *Ecc15 pOM1* (Figure 34A and C). *Dredd* mutant flies hence did not differ from wild-type behaviour (see Figure 24), suggesting that Dredd does not play a major role for the acquired aversion to pathogenic bacteria. In addition, however, it should be noted that heterozygous control flies did not exhibit the expected feeding preference for harmless *Ecc15 evf* over pathogenic *Ecc15 pOM1* (Figure 34B and C), potentially due to the high variation between different groups of flies.

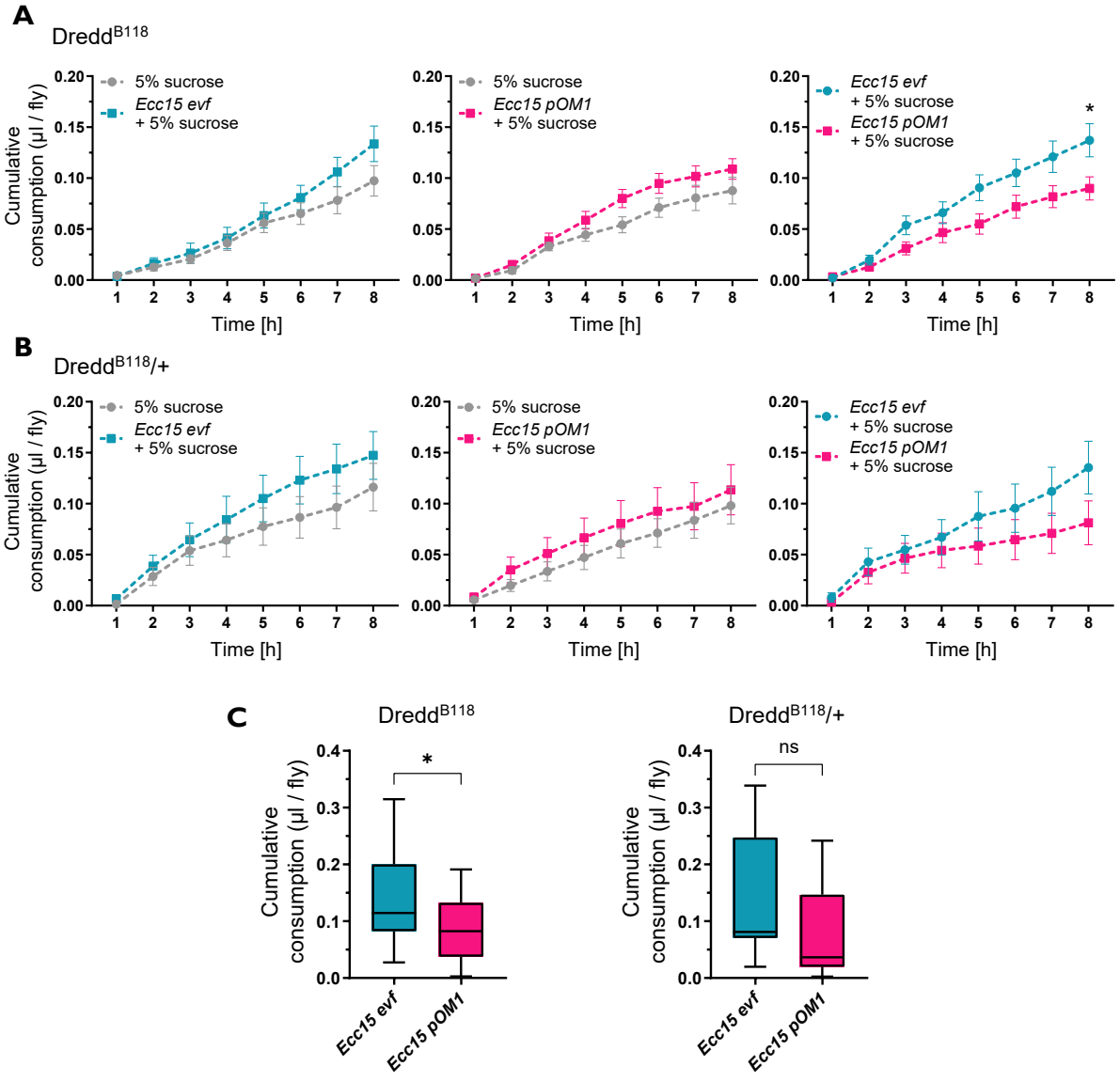

Figure 34 - *Ecc15* feeding preferences of *dredd*-deficient flies in the CAFE

(A) Cumulative consumption in µl/fly of Dredd^{B118} flies over time for the feeding choices sucrose vs. *Ecc15 evf* (n = 22), sucrose vs. *Ecc15 pOM1* (n = 23) and *Ecc15 evf* vs. *Ecc15 pOM1* (n = 23). **(B)** Cumulative consumption in µl/fly of Dredd^{B118}/+ heterozygous control flies over time for the feeding choices sucrose vs. *Ecc15 evf* (n = 19), sucrose vs. *Ecc15 pOM1* (n = 17) and *Ecc15 evf* vs. *Ecc15 pOM1* (n = 16). **(C)** Total cumulative consumption from (A) and (B) for the feeding choices *Ecc15 evf* vs. *Ecc15 pOM1* after 8 h. All p-values calculated via repeated-measures two-way ANOVA followed by Bonferroni's post hoc test for multiple comparisons. Graphs in A and B depict mean ± SEM.

Another central component of the Imd pathway is the NF-κB transcription factor Relish, which induces the transcription of a variety of immune effector genes. *Relish* mutant flies did not distinguish in any of the feeding choices offered, including the choice between *Ecc15 evf* and *Ecc15 pOM1* (Figure 35A and C). By contrast, w$^-$ control flies showed the frequently

observed preference for *Ecc15 evf* over sucrose as well as the expected increased feeding of *Ecc15 evf* as compared to *Ecc15 pOM1* (Figure 35B and C).

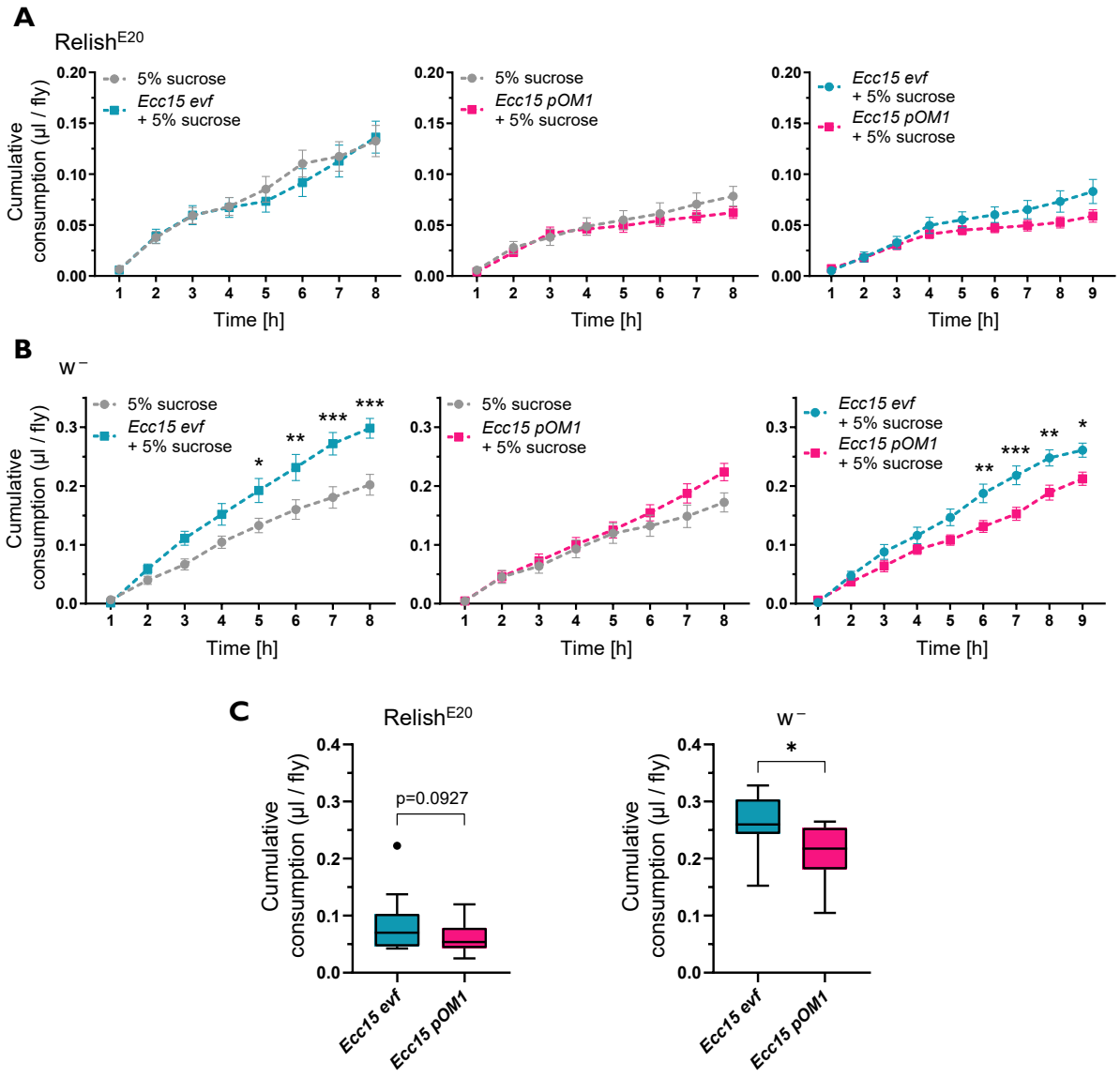

Figure 35 - *Ecc15* feeding preferences of *relish* mutant flies in the CAFE

(A) Cumulative consumption in µl/fly of isogenized Relish^{E20} flies over time for the feeding choices sucrose vs. *Ecc15 evf*, sucrose vs. *Ecc15 pOM1* and *Ecc15 evf* vs. *Ecc15 pOM1* (all n = 16). **(B)** Cumulative consumption in µl/fly of isogenized w$^-$ control flies over time for the feeding choices sucrose vs. *Ecc15 evf* (n = 15), sucrose vs. *Ecc15 pOM1* (n = 14) and *Ecc15 evf* vs. *Ecc15 pOM1* (n = 16). **(C)** Total cumulative consumption from (A) and (B) for the feeding choices *Ecc15 evf* vs. *Ecc15 pOM1* after 9 h. All p-values calculated via repeated-measures two-way ANOVA followed by Bonferroni's post hoc test for multiple comparisons. Graphs in A and B depict mean ± SEM.

The Imd pathway culminates in the expression of AMPs as the central effectors of the innate immune response in *Drosophila*. To investigate whether AMPs contribute to the acquired avoidance to pathogenic bacteria, I used flies where all immune-inducible AMPs

except for the four cecropins were deleted and which are hence highly susceptible to an infection with *Ecc15* (Hanson et al., 2019). AMP mutant flies preferred *Ecc15 evf* to sucrose and were indifferent to the sucrose-*Ecc15 pOM1* choice. Interestingly, these AMP mutant flies fed equal amounts of *Ecc15 evf* and *pOM1* (Figure 36, for control see also Figure 35B), apparently not being able to distinguish between harmless and pathogenic food sources anymore.

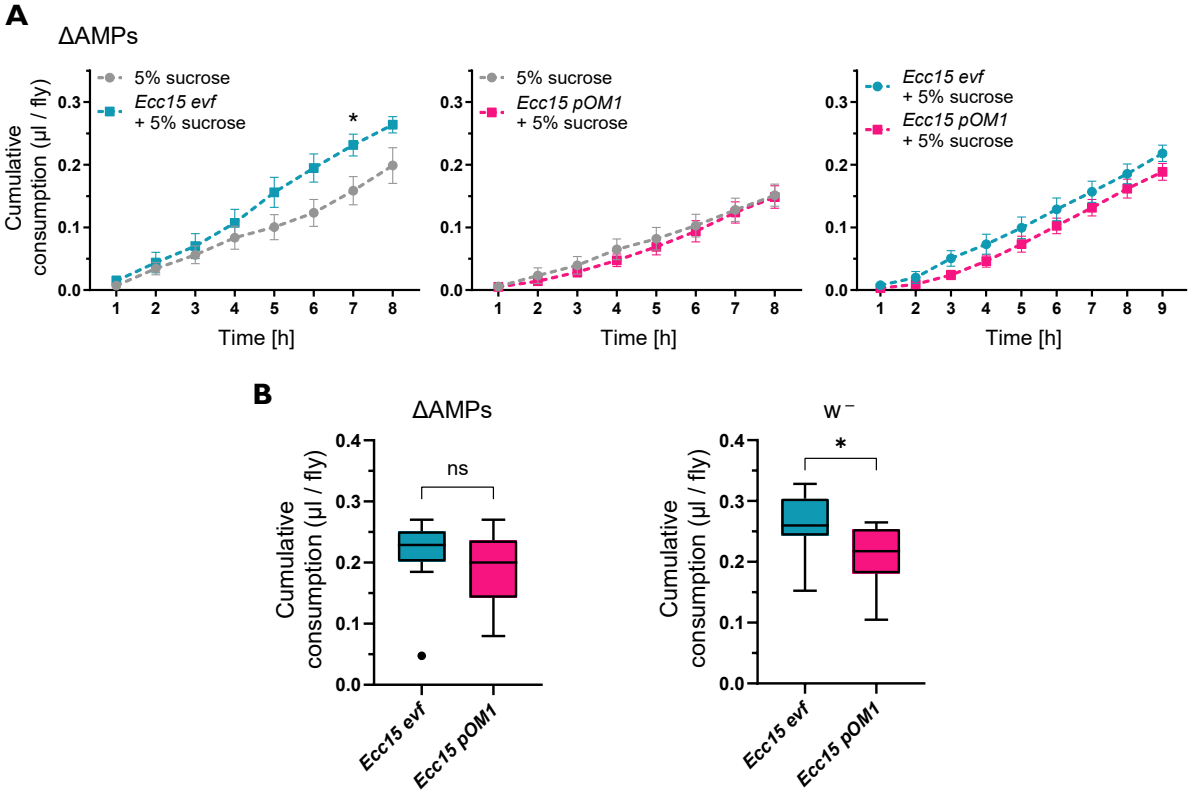

Figure 36 - *Ecc15* feeding preferences of flies lacking most AMPs in the CAFE

(A) Cumulative consumption in µl/fly of ΔAMP flies over time for the feeding choices sucrose vs. *Ecc15 evf*, sucrose vs. *Ecc15 pOM1* and *Ecc15 evf* vs. *Ecc15 pOM1* (all n = 16); mean ± SEM. **(B)** Total cumulative consumption from (A) and of isogenized w^- control from Figure 35B for the feeding choice *Ecc15 evf* vs. *Ecc15 pOM1* after 9 h (n = 16). All p-values calculated via repeated-measures two-way ANOVA followed by Bonferroni's post hoc test for multiple comparisons. Isogenized w^{1118} control tested on the same day.

The data presented in this section implicate several components of the Imd pathway in the behavioural adaptation following contact with contaminated food. The two immune receptors PGRP-LC and PGRP-LE as well as AMPs and the NF-κB transcription factor Relish were shown to be necessary for the acquired feeding aversion to pathogenic *Ecc15*. By contrast, the caspase Dredd was apparently dispensable for the distinction between harmless and pathogenic *Ecc15*.

3.8 Downregulation of PGRP-LC during pathogen feeding

3.8.1 Whole body-knockdown of PGRP-LC mimics mutant phenotype

Among the Imd pathway mutants tested, the strongest phenotype for *Ecc15* feeding choices in the CAFE was observed in flies lacking the transmembrane immune receptor PGRP-LC. PGRP-LC-deficient flies did not acquire the feeding aversion to pathogenic *Ecc15* observed in wild-type flies. PGRP-LC activates Imd pathway signalling primarily in the fat body and the anterior parts of the gut, but has also been implicated in presynaptic homeostasis in the nervous system (Harris et al., 2015). In order to understand how PGRP-LC is involved in the adaptation of behaviour following contact with pathogenic bacteria, it was thus essential to narrow down the parts of the body in which PGRP-LC might be required to suppress feeding on pathogenic *Ecc15*. To that aim, I employed RNAi to downregulate PGRP-LC in midgut enterocytes, the fat body or the nervous system. However, before investigating specific bodily regions, I tested *Ecc15* feeding preferences of flies where PGRP-LC had been downregulated globally via the ubiquitous Actin-GAL4 driver in order to confirm the PGRP-LC mutant phenotype and the function of the RNAi construct. Just as PGRP-LC mutant flies, Act > PGRP-LC^{RNAi} flies consumed equal amounts of harmless *Ecc15 evf* and pathogenic *Ecc15 pOM1* (Figure 37A and C). Additionally, they did not differentiate between sucrose and *Ecc15 pOM1*, but consumed more harmless *Ecc15 evf* than sucrose, suggesting that global knockdown of PGRP-LC did not interfere with the flies' ability to distinguish food sources of different nutritive value (Figure 37A and C). By contrast, control flies (Act-GAL4 > +) exhibited a wild-type-like phenotype in that they clearly preferred harmless over pathogenic *Ecc15* (Figure 37B and C). These results further corroborate the finding that *Drosophila* relies on PGRP-LC to distinguish between good and potentially detrimental food sources.

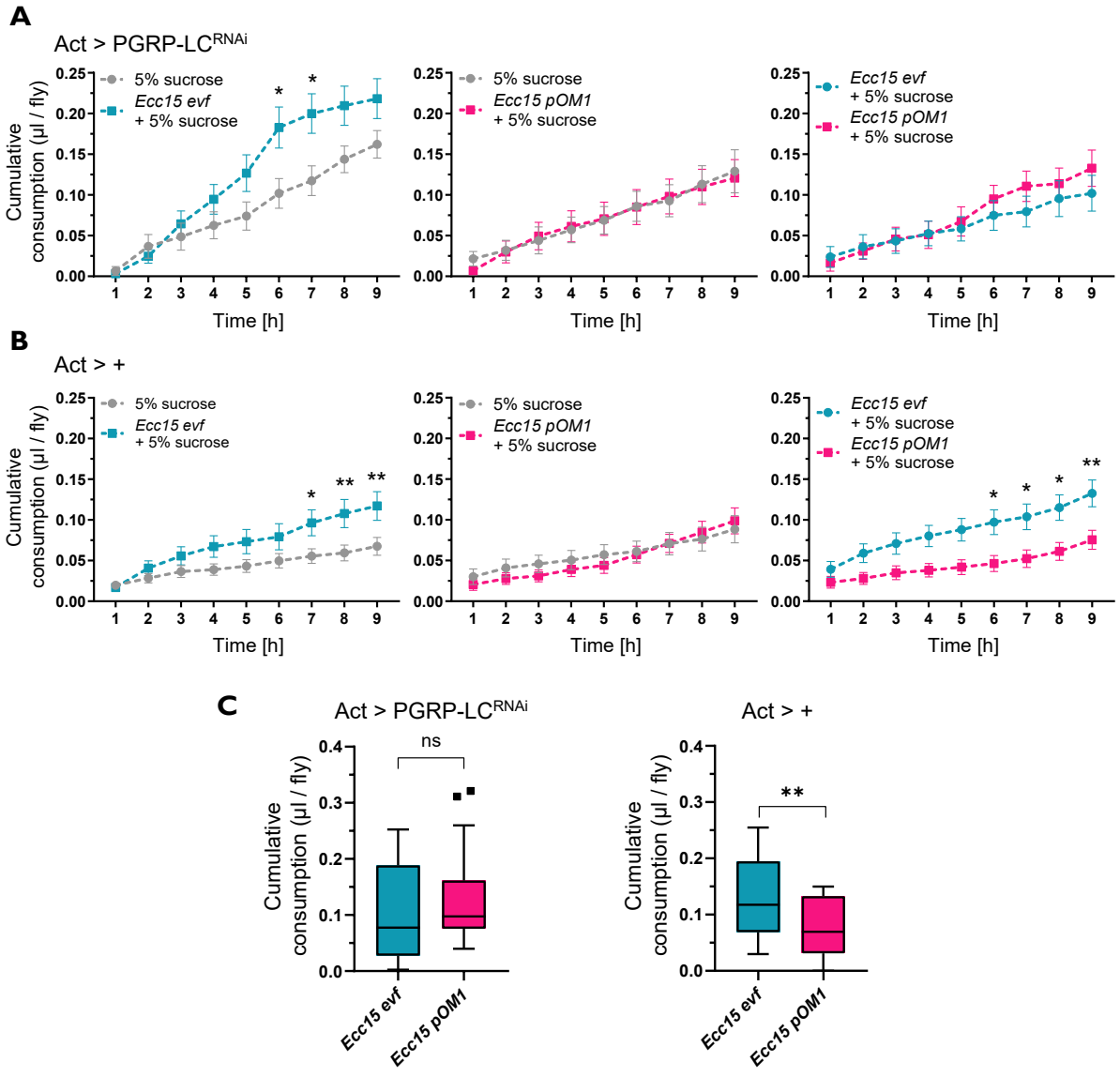

Figure 37 - *Ecc15* feeding preferences in the CAFE upon global knockdown of PGRP-LC

(A) Cumulative consumption in µl/fly of Act > PGRP-LC^{RNAi} flies over time for the feeding choices sucrose vs. *Ecc15 evf*, sucrose vs. *Ecc15 pOM1* and *Ecc15 evf* vs. *Ecc15 pOM1* (all n = 16). **(B)** Cumulative consumption in µl/fly of Act > + control flies over time for the feeding choices sucrose vs. *Ecc15 evf* (n = 19), sucrose vs. *Ecc15 pOM1* (n = 20) and *Ecc15 evf* vs. *Ecc15 pOM1* (n = 20). **(C)** Total cumulative consumption from (A) and (B) for the feeding choices *Ecc15 evf* vs. *Ecc15 pOM1* after 9 h. All p-values calculated via repeated-measures two-way ANOVA followed by Bonferroni's post hoc test for multiple comparisons. Graphs in A and B depict mean ± SEM.

To exclude that any potential phenotypes observed in the RNAi knockdown experiments are due to the genetic background, I additionally tested the UAS-control, i.e. + > PGRP-LC^{RNAi} flies, for their *Ecc15* feeding preferences in the CAFE. As expected, + > PGRP-LC^{RNAi} flies clearly preferred feeding on harmless *Ecc15 evf* to pathogenic *Ecc15 pOM1* (Figure 38).

Moreover, they consumed both more *Ecc15 evf* and more *Ecc15 pOM1* if the alternative was only sugar (Figure 38A).

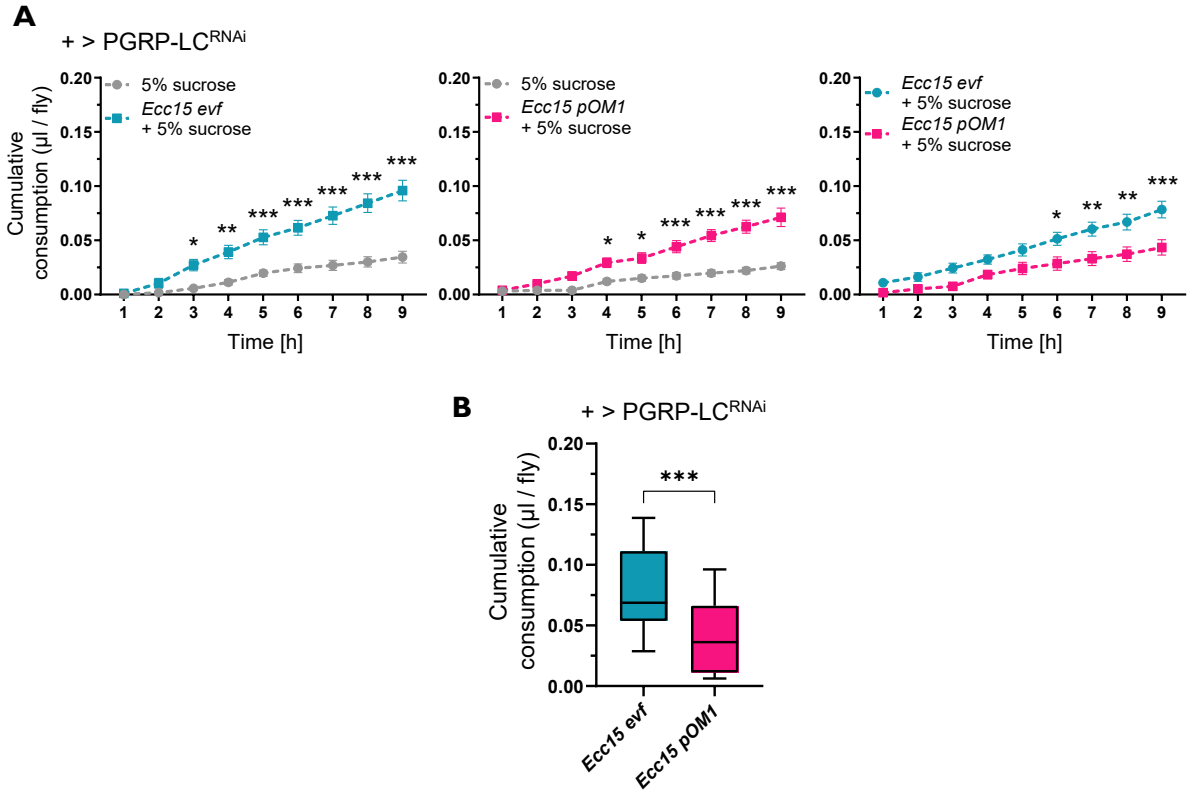

Figure 38 - UAS control for PGRP-LC^{RNAi} feeding choice experiments in the CAFE

(A) Cumulative consumption in µl/fly of + > PGRP-LC^{RNAi} flies over time for the feeding choices sucrose vs. *Ecc15 evf* (n = 17), sucrose vs. *Ecc15 pOM1* (n = 19) and *Ecc15 evf* vs. *Ecc15 pOM1* (n = 17); mean ± SEM. **(B)** Total cumulative consumption from (A) for the feeding choice *Ecc15 evf* vs. *Ecc15 pOM1* after 9 h. All p-values calculated via repeated-measures two-way ANOVA followed by Bonferroni's post hoc test for multiple comparisons.

3.8.2 Knockdown of PGRP-LC in midgut enterocytes

Following ingestion of pathogenic bacteria, the local immune response is initiated in the gut. To determine whether PGRP-LC is required in the gut for the distinction between harmless and pathogenic *Ecc15*, I downregulated PGRP-LC in midgut enterocytes using the mex-GAL4 driver line. However, knockdown of PGRP-LC in midgut enterocytes via RNAi did not abolish the shift in preference towards *Ecc15 evf* for the feeding choice between *Ecc15 evf* and *Ecc15 pOM1* (Figure 39). By contrast, Act > PGRP-LC^{RNAi} control flies retained the phenotype observed in PGRP-LC mutant flies and did not distinguish between harmless and pathogenic *Ecc15* (Figure 39B).

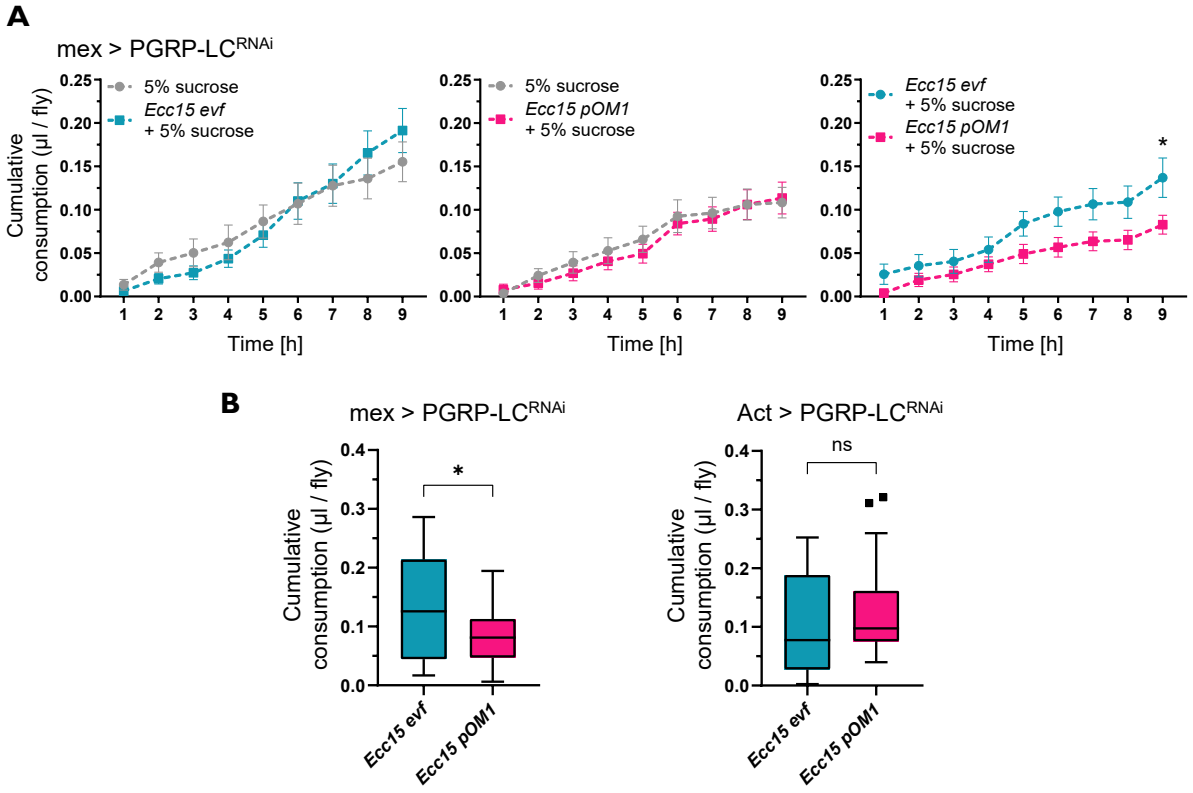

Figure 39 - *Ecc15* feeding preferences in the CAFE upon knockdown of PGRP-LC in midgut enterocytes

(A) Cumulative consumption in µl/fly of mex > PGRP-LC^{RNAi} flies over time for the feeding choices sucrose vs. *Ecc15 evf*, sucrose vs. *Ecc15 pOM1* and *Ecc15 evf* vs. *Ecc15 pOM1* (all n = 16); mean ± SEM. **(B)** Total cumulative consumption for the feeding choice *Ecc15 evf* vs. *Ecc15 pOM1* after 9 h from (A) as well as additional Act > PGRP-LC^{RNAi} control (n = 16, also shown in Figure 37). All p-values calculated via repeated-measures two-way ANOVA followed by Bonferroni's post hoc test for multiple comparisons.

3.8.3 Knockdown of PGRP-LC in the fat body

The fat body plays an important role for the initiation of a systemic immune response upon infection with pathogenic bacteria. The avoidance response to *Ecc15 pOM1* might hence start out with PGRP-LC recognizing bacterial PGN in the fat body. To test this hypothesis, I downregulated PGRP-LC in the fat body using takeout (to) - and Lipophorin (Lpp)- GAL4 drivers. Takeout is mainly expressed in the fat body surrounding the brain as well as in the cardia, crop and antennae (Sarov-Blat et al., 2000; Dauwalder et al., 2002). Downregulation of PGRP-LC in the fat body using to-GAL4 as a driver did not have any effects on the feeding preferences towards *Ecc15*, as flies preferred feeding on *Ecc15 evf* by the end of the assay (Figure 40A and C). However, even though *to* is expressed in both males and females, expression in the head fat body has been suggested to be male-specific

(Sarov-Blat et al., 2000; Dauwalder et al., 2002), which may explain the absence of a phenotype for this GAL4-driver.

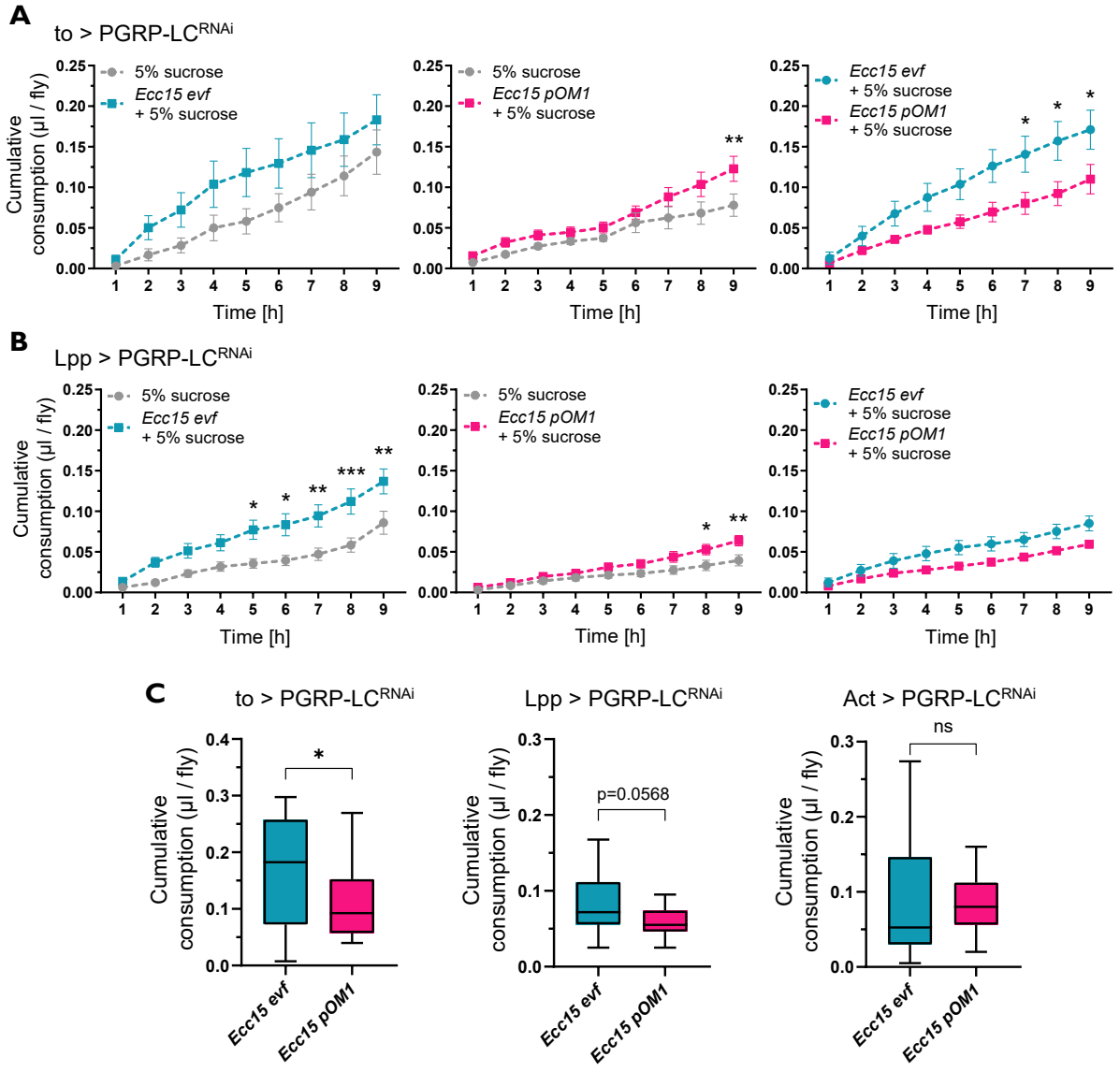

Figure 40 - *Ecc15* feeding preferences in the CAFE upon knockdown of PGRP-LC in the fat body

(A) Cumulative consumption in µl/fly of to > PGRP-LC^{RNAi} flies over time for the feeding choices sucrose vs. *Ecc15 evf* (n = 16), sucrose vs. *Ecc15 pOM1* (n = 18) and *Ecc15 evf* vs. *Ecc15 pOM1* (n = 15). **(B)** Cumulative consumption in µl/fly of Lpp > PGRP-LC^{RNAi} flies over time for the feeding choices sucrose vs. *Ecc15 evf* (n = 20), sucrose vs. *Ecc15 pOM1* (n = 19) and *Ecc15 evf* vs. *Ecc15 pOM1* (n = 20). **(C)** Total cumulative consumption for the feeding choices *Ecc15 evf* vs. *Ecc15 pOM1* after 9 h from (A) and (B) as well as additional Act > PGRP-LC^{RNAi} control (n = 17) conducted on the same days as Lpp > PGRP-LC^{RNAi} experiments. All p-values calculated via repeated-measures two-way ANOVA followed by Bonferroni's post hoc test for multiple comparisons. Graphs in A and B depict mean ± SEM.

As a next step, I hence downregulated PGRP-LC using Lpp-GAL4, Lpp being a lipoprotein specifically produced in the fat body (Palm et al., 2012). In this case, PGRP-LC knockdown in the fat body abolished the preference for harmless *Ecc15 evf* (Figure 40B and C). However, it should be noted that Lpp > PGRP-LC^{RNAi} flies overall consumed little for the choice between *Ecc15 evf* and *Ecc15 pOM1*, meaning that a potential difference in consumption was potentially more difficult to identify ($p = 0.0568$, Figure 40C). By contrast, the Act > PGRP-LC^{RNAi} flies tested on the same experimental days clearly showed no difference in consumption between harmless *Ecc15 evf* and pathogenic *Ecc15 pOM1*, thus mimicking the behaviour of PGRP-LC mutant flies (Figure 40C).

3.8.4 PGRP-LC-knockdown in the nervous system

3.8.4.1 Neuronal PGRP-LC is necessary for pathogen-harmless distinction

The gut and the fat body as primary nodes of the innate immune response are obvious candidates for a potential contribution of PGRP-LC to distinguish harmful from pathogenic *Ecc15* food sources. The data presented so far do not implicate PGRP-LC in the gut for the observed feeding aversion to *Ecc15 pOM1*, while PGRP-LC might be required in the fat body. It has been shown that PGRP-LC is expressed in the nervous system, where it is required for homeostatic plasticity (Harris et al., 2015). Therefore, another option is that PGRP-LC acts directly in the nervous system and thus constitutes a link between the immune system and the adaptation of behaviour following exposure to detrimental food sources. Accordingly, I downregulated PGRP-LC in the nervous system using the pan-neuronal driver neuronal Synaptobrevin (nSyb)-GAL4. Flies lacking PGRP-LC in the nervous system did not prefer any of the *Ecc15* strains over sucrose and most importantly did not differentiate in case of the direct choice between *Ecc15 evf* and *Ecc15 pOM1* (Figure 41A and C). By contrast, nSyb > + control flies were still able to make the distinction between harmless and pathogenic *Ecc15* (Figure 41B and C). Absence of PGRP-LC in the nervous system hence mimics the behaviour seen in flies lacking PGRP-LC globally (Figure 41C), indicating that neuronal PGRP-LC is sufficient to suppress feeding of pathogenic *Ecc15*.

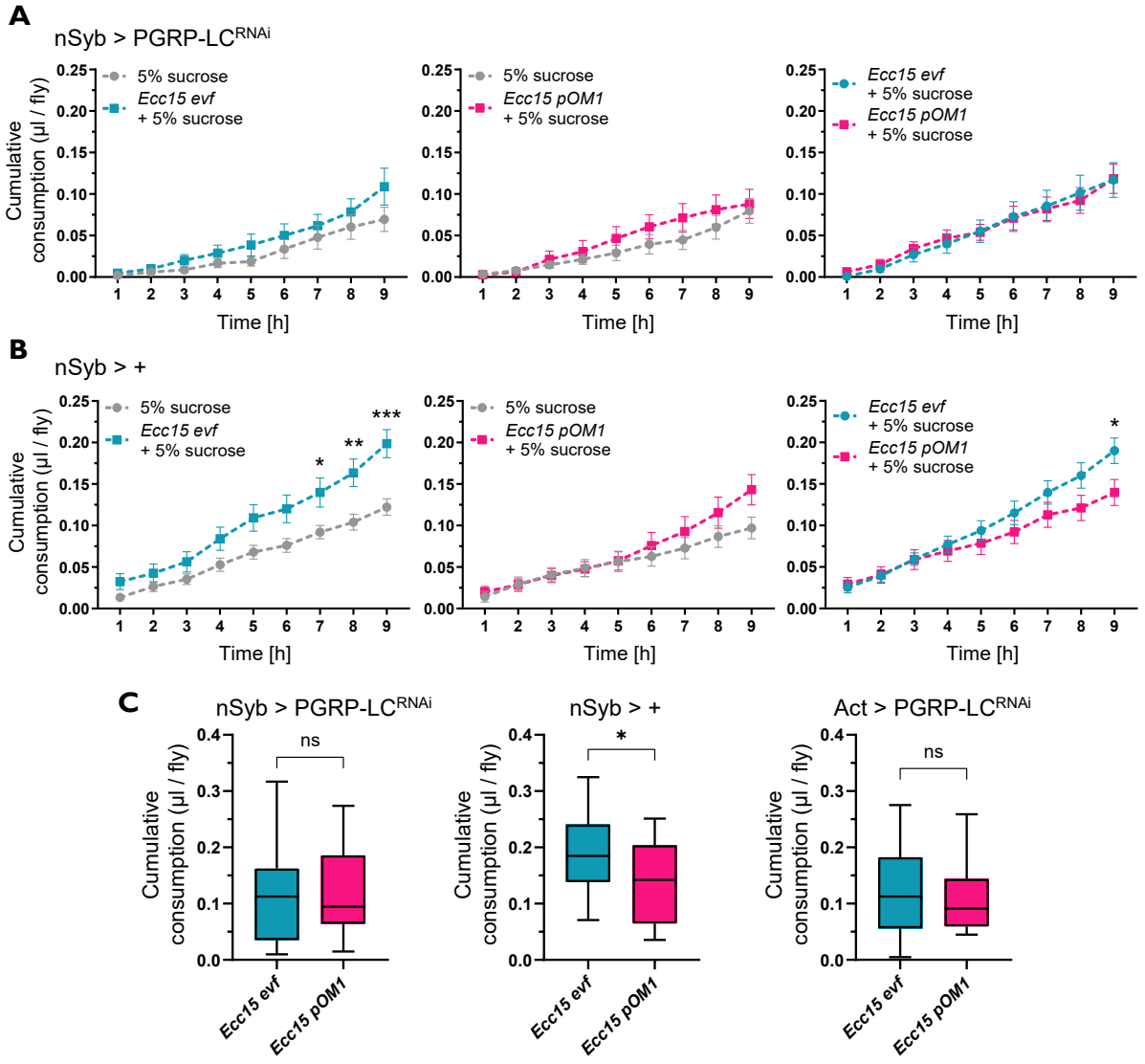

Figure 41 - *Ecc15* feeding preferences in the CAFE upon knockdown of PGRP-LC in the nervous system

(A) Cumulative consumption in µl/fly of nSyb > PGRP-LC^{RNAi} flies over time for the feeding choices sucrose vs. *Ecc15 evf* (n = 19), sucrose vs. *Ecc15 pOM1* (n = 19) and *Ecc15 evf* vs. *Ecc15 pOM1* (n = 20). **(B)** Cumulative consumption in µl/fly of nSyb > + control flies over time for the feeding choices sucrose vs. *Ecc15 evf* (n = 19), sucrose vs. *Ecc15 pOM1* (n = 19) and *Ecc15 evf* vs. *Ecc15 pOM1* (n = 21). **(C)** Total cumulative consumption for the feeding choices *Ecc15 evf* vs. *Ecc15 pOM1* after 9 h from (A) and (B) as well as additional Act > PGRP-LC^{RNAi} control (n = 20) conducted on the same days. All p-values calculated via repeated-measures two-way ANOVA followed by Bonferroni's post hoc test for multiple comparisons. Graphs in A and B depict mean ± SEM.

3.8.4.2 PGRP-LC may be required in the mushroom body

I previously showed that flies rely on their MB to distinguish between harmful and pathogenic *Ecc15* (see 3.6.1 and Figure 30). In addition, flies lacking PGRP-LC only in the nervous system were not able to make this distinction either. PGRP-LC might thus be directly required in the MB to regulate the feeding aversion to pathogenic *Ecc15*. To test this

hypothesis, I downregulated PGRP-LC in all KCs of the MB. MB10B > PGRP-LC^{RNAi} flies did indeed not differentiate between *Ecc15 evf* and *Ecc15 pOM1*, while MB10B > + control flies showed the expected preference for the harmless *Ecc15* strain (Figure 42).

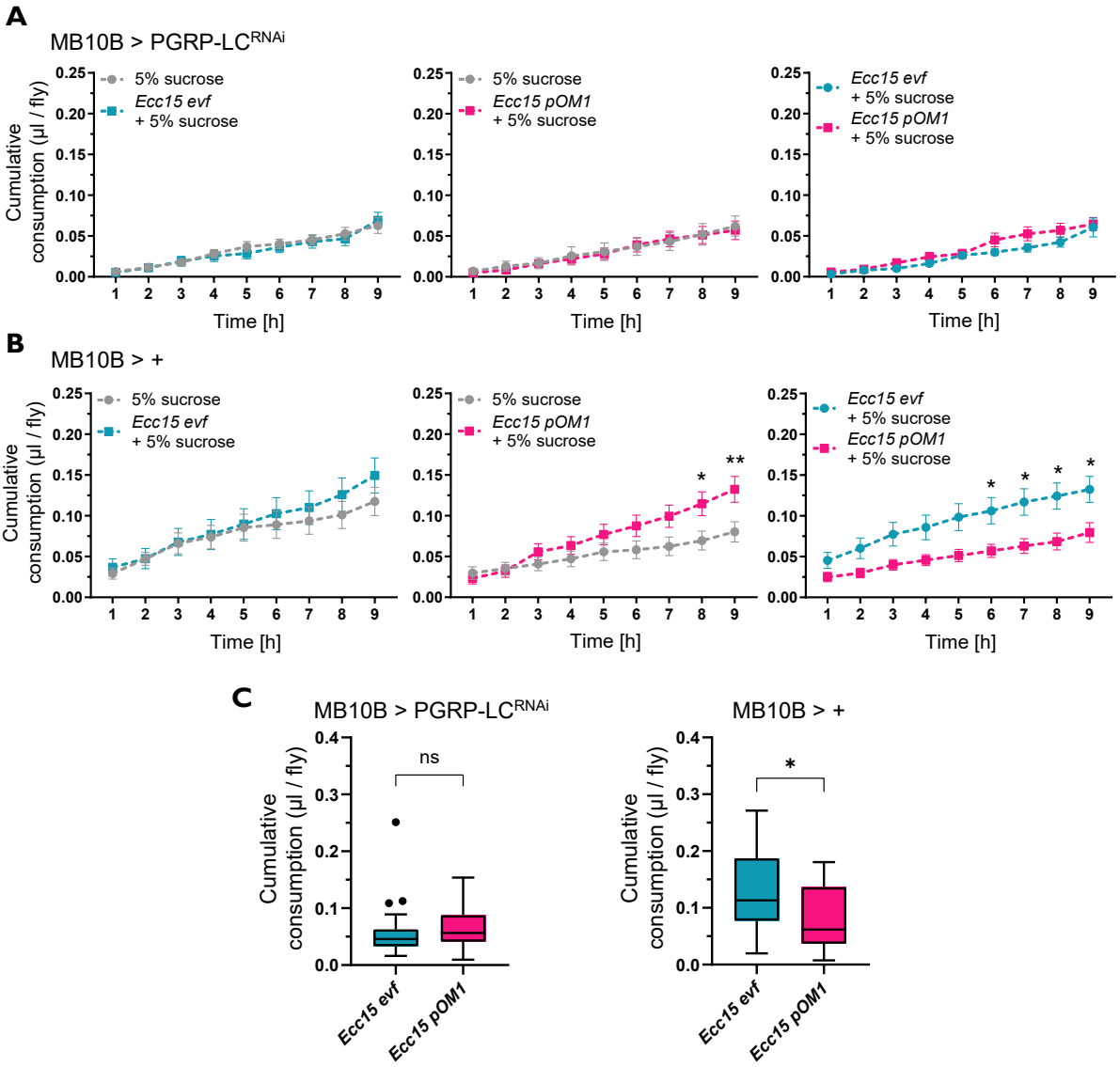

Figure 42 - Ecc15 feeding preferences in the CAFE upon knockdown of PGRP-LC in the MB

(A) Cumulative consumption in µl/fly of MB10B > PGRP-LC^{RNAi} flies over time for the feeding choices sucrose vs. *Ecc15 evf* (n = 18), sucrose vs. *Ecc15 pOM1* (n = 16) and *Ecc15 evf* vs. *Ecc15 pOM1* (n = 20). **(B)** Cumulative consumption in µl/fly of MB10B > + control flies over time for the feeding choices sucrose vs. *Ecc15 evf* (n = 18), sucrose vs. *Ecc15 pOM1* (n = 18) and *Ecc15 evf* vs. *Ecc15 pOM1* (n = 20). **(C)** Total cumulative consumption from (A) and (B) for the feeding choices *Ecc15 evf* vs. *Ecc15 pOM1* after. All p-values calculated via repeated-measures two-way ANOVA followed by Bonferroni's post hoc test for multiple comparisons. Graphs in A and B depict mean ± SEM.

However, MB10B > PGRP-LC^{RNAi} flies were weak and consumed very little overall for all three feeding choices tested, but in particular for the choice between *Ecc15 evf* and *Ecc15 pOM1*. Given the poor health of these flies as well as the low consumption of bacteria and the accompanying difficulties in precisely measuring liquid decrease in the CAFE, it cannot be stated with certainty whether PGRP-LC is required in the MB for the observed acquired feeding aversion to pathogens.

3.8.4.3 PGRP-LC is required in octopaminergic neurons for feeding aversion

It has been reported previously that a particular adaptation of behaviour following exposure to pathogens, i.e. a decrease in egg-laying upon infection, relies on PGN-mediated NF-κB pathway activation in octopaminergic neurons (Kurz et al., 2017). Moreover, octopamine signalling has also been implicated in microbe-mediated control of locomotor activity (Schretter et al., 2018). Accordingly, PGRP-LC might play a role in octopaminergic neurons for the adaptation of behaviour after feeding on pathogenic bacteria. To determine whether the absence of PGRP-LC in octopaminergic neurons is sufficient to abolish the preference for harmless over pathogenic *Ecc15*, I drove expression of the PGRP-LC^{RNAi} construct specifically in octopaminergic neurons using Tdc2-GAL4 as a driver. The *tyrosine decarboxylase 2* (*Tdc2*) gene encodes an enzyme catalysing the synthesis of the precursor of octopamine, i.e. tyramine, out of tyrosine in the nervous system (Cole et al., 2005; Busch et al., 2009).

Flies lacking PGRP-LC only in Tdc2+ octopaminergic neurons preferred *Ecc15 evf* and *Ecc15 pOM1* over sucrose, respectively, and were thus still able to distinguish food sources of different nutritive value, but did not show a preference for a particular bacterial strain in case of a choice between *Ecc15 evf* and *Ecc15 pOM1* (Figure 43A and C). Tdc2 > + control flies similarly preferred *Ecc15* over mere sucrose, but were able to differentiate between pathogenic and harmless *Ecc15* in that they shifted their preference to harmless *Ecc15 evf* after a few hours of feeding (Figure 43B and C). Thus, PGRP-LC is required in octopaminergic neurons to induce the avoidance of a food source containing pathogenic bacteria.

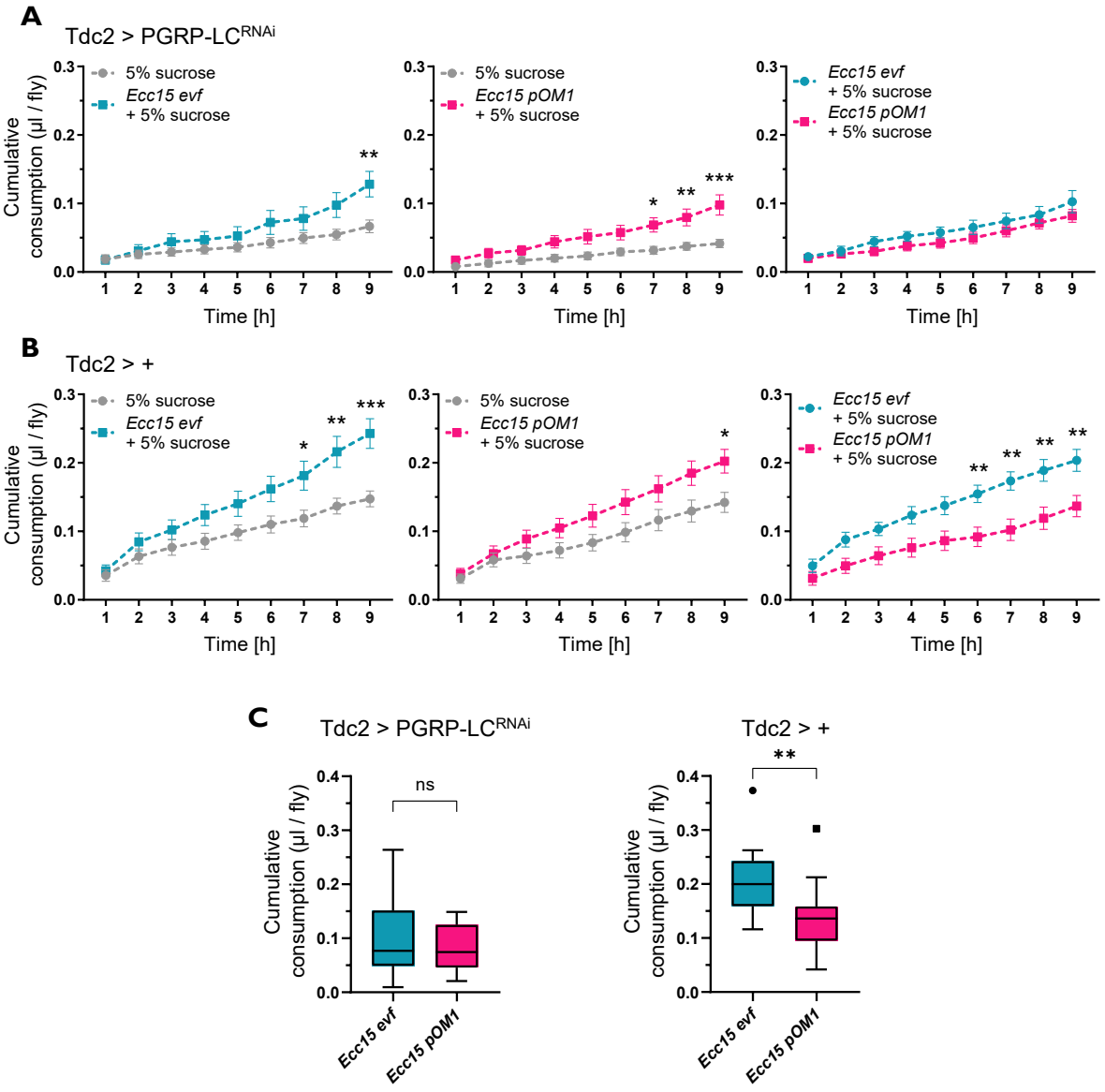

Figure 43 - *Ecc15* feeding preferences in the CAFE upon knockdown of PGRP-LC in octopaminergic neurons

(A) Cumulative consumption in µl/fly of Tdc2 > PGRP-LC^{RNAi} flies over time for the feeding choices sucrose vs. *Ecc15 evf* (n = 19), sucrose vs. *Ecc15 pOM1* (n = 17) and *Ecc15 evf* vs. *Ecc15 pOM1* (n = 20). **(B)** Cumulative consumption in µl/fly of Tdc2 > + control flies over time for the feeding choices sucrose vs. *Ecc15 evf* (n = 17), sucrose vs. *Ecc15 pOM1* (n = 17) and *Ecc15 evf* vs. *Ecc15 pOM1* (n = 16). **(C)** Total cumulative consumption from (A) and (B) for the feeding choices *Ecc15 evf* vs. *Ecc15 pOM1* after 9 h. All p-values calculated via repeated-measures two-way ANOVA followed by Bonferroni's post hoc test for multiple comparisons. Graphs in A and B depict mean ± SEM.

4. Discussion

4.1 Summary of results

The ability to avoid detrimental food and remember food sources that have caused sickness in the past is essential for an animal's survival. While the initial evaluation prior to ingestion relies on the external sensory systems, in particular smell and taste, pathogenic bacteria that are taken up with food trigger the activation of the immune response and elicit post-ingestive effects such as intestinal malaise. The immune system as well as immediate behavioural adaptations and remembering the detrimental food source allow the animal to fight off the pathogens, alleviate the consequences of the infection and avoid repeated infections in the future. Although these conditioned food aversions are present in many animal species, it is unknown how exactly the post-ingestive effects caused by an infection translate into a lasting avoidance of detrimental food.

The aim of this thesis was to shed light on the mechanisms underlying these behavioural adaptations to spoiled food. In the present study, I have now shown that the fruit fly *Drosophila melanogaster* develops an aversion to pathogenic bacteria after feeding on them and that this aversion is mediated by an immune receptor in octopaminergic neurons and requires the learning and memory centre of the fly brain, the MB (Figure 44).

Even though the oral infection with the two Gram-negative bacterial strains used in this study, *Ecc15* and *Pe*, is lethal to some or even to all flies (Figure 10), only *Ecc15*-infected flies transiently exhibited reduced locomotor activity, while the infection with both *Ecc15* and *Pe* did not affect circadian rhythm (Figure 12 - Figure 14). Surprisingly, flies were innately attracted to the odour of pathogenic bacteria, even if the other option was not mere humidified air, but the odour of the corresponding harmless strain (Figure 16 & Figure 19). Prior bacterial feeding abolished this preference; however, both flies infected with harmless and those infected with pathogenic strains were indifferent to the choice between harmless and pathogenic bacterial odours following bacteria feeding (Figure 22), indicating that oral infection with pathogenic bacteria was not sufficient to induce specific olfactory aversion. By contrast, feeding elicited a lasting aversion of food containing pathogenic bacteria (Figure 23 - Figure 26). While flies immediately preferred harmless over pathogenic *Pe* strains in two

different feeding choice assays (Figure 23 & Figure 25), the delayed preference for harmless over pathogenic *Ecc15* (Figure 24 & Figure 26) provided a first indication that this behaviour constitutes an acquired aversion due to post-ingestive effects rather than a preference based on taste alone. Thermogenetic silencing of all synaptic output from the MB (Figure 30) as well as testing the learning mutant *rutabaga* (Figure 31) for the *Ecc15* feeding choice subsequently supported the hypothesis that the avoidance of a contaminated food source requires an associative learning mechanism.

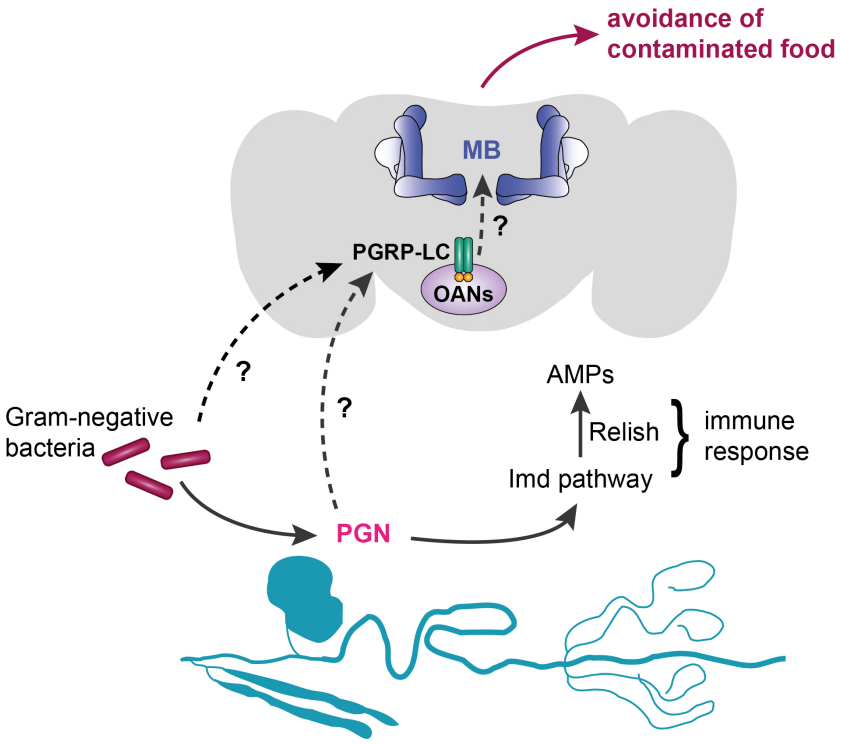

Figure 44 - Model for PGRP-LC-mediated acquired feeding aversion to pathogens

Ingestion of virulent Gram-negative bacteria triggers the activation of the immune response and induces PGRP-LC signalling in octopaminergic neurons, for example via sensing of PGN or another bacterial factor. Octopaminergic neurons could relay the information about the harmful food source to the MB, which is necessary for the acquired feeding aversion to pathogenic bacteria and where the appropriate behavioural output is generated.

Further *Ecc15* feeding choice experiments with different immune mutant lines revealed that several components of the Imd immune signalling pathway were necessary to distinguish harmless from pathogenic bacteria, namely the cytosolic receptor PGRP-LE, the NF-κB transcription factor Relish, AMPs and in particular the transmembrane receptor PGRP-LC (Figure 32 - Figure 36). By downregulating PGRP-LC in different parts of the body via RNAi, I found that flies lacking this receptor in the nervous system, and specifically in

octopaminergic neurons, were unable to differentiate between harmless food and food containing pathogenic bacteria (Figure 41 & Figure 43). Thus, being able to avoid detrimental food presupposes immune signalling directly in the nervous system. Following ingestion of pathogenic Gram-negative bacteria, PGRP-LC signalling in octopaminergic neurons induces avoidance of pathogen-infested food, potentially by propagating the information about the infection to the MB, where the appropriate behavioural output is generated (Figure 44).

4.2 Survival after infection and 'sickness behaviour'

To study the adaptive behaviour of *Drosophila* following infection with pathogenic bacteria, I employed an oral infection paradigm using Gram-negative bacteria. When investigating the immune defence against pathogens and to disentangle immune signalling pathways, it is common to infect flies not *per os*, but by pricking them, i.e. by introducing bacteria directly into the body cavity with a thin needle, which elicits a systemic immune response (Neyen et al., 2014). However, since I was interested in the behavioural adaptations and the potential memory formation following the post-ingestive effects caused by pathogen ingestion, it was crucial to select an oral route of infection that was as natural as possible in a laboratory setting. To increase the motivation to feed and ensure that all flies ingested bacteria, flies were dry starved prior to bacteria feeding, and the bacteria preparations were supplemented with sucrose and offered as the only food source available. Even though I cannot exclude that individual flies consumed little or no bacteria, these measures make it highly unlikely that a relevant number of flies refrained from ingesting bacteria.

Survival analysis following bacteria feeding confirmed the previously known virulence of the two strains used (Figure 10). *Pe* is a highly virulent entomopathogenic bacterial strain that was shown to kill a large fraction of flies in the 10 h following pricking and within less than two days after natural oral infection (Liehl et al., 2006). Not surprisingly, wild-type CS flies were more likely to survive *Pe* infection than flies of the smaller and weaker OrR wild-type strain. By contrast, *Ecc15 pOM1* was mildly pathogenic, with 80 - 90% of bacteria-fed flies surviving the infection. In fact, ingestion of wild-type *Ecc15* is typically non-lethal for adult flies and only induces a local immune response (e.g. Zaidman-Rémy et al., 2006; Chakrabarti et al., 2012). However, *Ecc15 pOM1-evf* (abbreviated as *Ecc15 pOM1* in this study) is a more pathogenic *Ecc15* strain due to an overexpression of virulence factors (Basset et al.,

2003) that leads to an increased persistence of bacteria in the fly gut and thus killed 10-20% of flies within less than 24 h after feeding.

One of the immediate consequences of an infection with pathogens are so-called 'sickness behaviours' accompanying the infection, which in the narrower sense comprise behavioural adjustments to accelerate recovery (Hart, 1988; Pacheco-López et al., 2011). A common adaptation following pathogenic infection is a reduction in overall activity and an increase in rest phases, presumably to preserve energy resources for fighting the infection. The reciprocal relationship between sleep and the immune system not only implies that the susceptibility to an infection is higher following sleep loss, but also that an infection conversely causes increased sleep and changes sleep patterns (Besedovsky et al., 2019). In *Drosophila*, too, the immune system and sleep are tightly linked, as bacterial infection for instance seems to promote sleep via the NF-κB transcription factor Relish (Kuo et al., 2010).

In the present study, oral infection of flies with *Ecc15* or *Pe* did not change the regularity of their activity patterns or circadian rhythm (Figure 14). In addition, *Pe*-fed flies were not impaired in their general locomotor activity throughout the three days after infection. By contrast, flies fed with pathogenic *Ecc15* exhibited reduced locomotor activity during the first hours after bacterial exposure, but quickly recovered and had returned to the activity levels of harmless *Ecc15*-fed flies 17 h post-infection (Figure 12, appendix Figure 46). Interestingly, this time course parallels what has been reported for pheromone production following *Ecc15* infection via pricking, which was shown to peak 8 h post-infection and then returned to control levels (Keesey et al., 2017). Moreover, this corresponds to the CAFE experiments in the present study demonstrating that flies develop an aversion to *Ecc15*-contaminated food during a 9 h-feeding period (see Figure 26 and discussion in 4.4.1), suggesting that *Ecc15* infection might have the strongest impact on the behaviour of infected flies during the first hours post-ingestion.

While I did not quantify sleep in particular, the transient reduction of activity and corresponding increase in rest phases after *Ecc15* infection is in accordance with previous work on sleep-immune interactions. Bacterial infections seem to increase sleep in flies, and flies that had slept more prior to the infection displayed a higher resistance to bacterial infections (Kuo et al., 2010; Kuo et al., 2014). Similarly, oral viral infection with *Drosophila* C virus has been shown to reduce overall activity in female flies while increasing sleep (Vale

et al., 2015). The observation that bacteria can modulate sleep, but do not affect circadian rhythm was also made in flies colonized by the endosymbiotic, i.e. non-pathogenic bacterial strain *Wolbachia*, with *Wolbachia*-infected flies exhibiting decreased activity and increased sleep, but the same circadian rhythmicity as *Wolbachia*-free flies (Vale et al., 2015; Bi et al., 2018). Since the NF-κB transcription factor Relish was required to promote sleep following infection (Kuo et al., 2010), it is possible that the decreased activity of *Ecc15*-fed flies observed in the present study could likewise be mediated by Relish.

However, it is unclear at this point why an infection with the much more virulent bacterial strain *Pe* did not have an effect on locomotor activity. *Pe* feeding triggers a systemic immune response and heavily damages the gut epithelium (Vodovar et al., 2005; Liehl et al., 2006; Chakrabarti et al., 2012). Seemingly reduced activity more than 24 h after infection ($p = 0.0530$ for the 29-34 h post-infection time period) implies that a potential effect was nevertheless not big enough to be detected. Similarly, pheromone production following infection with *Pe* via pricking peaked only after 24 h (Keesey et al., 2017). Thus, what is certainly true is that the time course of the *Pe* infection is considerably slower and more variable than for an infection with *Ecc15*, suggesting that brief periods of reduced activity in individual *Pe*-infected flies could be distributed across a wider time span and thus fail to be detected. Nonetheless, this demonstrates that flies can suffer from a severe pathogenic infection without showing apparent sickness behaviours such as reduced activity. Lack of obvious sickness behaviours is not uncommon even in vertebrates: while rats display clear signs of nausea in CTA paradigms after lithium chloride injections, e.g. lying-on-belly, these signs are absent in mice, even though mice, too, form aversive memories of saccharin following lithium chloride administration (Welzl et al., 2001).

Hence, flies infected with pathogenic bacteria do not exhibit prolonged sickness behaviours visible as a decrease in general motor behaviour. This however does of course not interfere with the fact that *Ecc15* and *Pe* trigger the immune response (Basset et al., 2000; Vodovar et al., 2005) (*Ecc15*: Figure 11) and can cause negative post-ingestive effects and other behavioural adaptations beyond locomotor activity such as feeding aversions. Importantly, the merely brief and transient reduction in activity in *Ecc15*-fed flies as well as the observation that *Pe* infection did not impair activity indicates that infected flies were fit enough for subsequent behavioural tests.

4.3 Smells like danger? Perception of bacterial odours

4.3.1 The odour of pathogenic bacteria is innately attractive

Odours are important cues for any animal when navigating its environment. They are essential to find food sources or a mating partner, but are also important danger signals that indicate the presence of a threat such as pathogens in a food source or the approach of a predator and can thus elicit innate avoidance reactions. Mice for example rely on a specific olfactory receptor type to identify predator urine (Dewan et al., 2013), and the nematode *C. elegans* uses chemosensation to perceive and avoid a compound emitted by pathogenic bacteria (Pradel et al., 2007). The olfactory system is moreover crucial to detect detrimental microbes in a food source: *Drosophila* recognizes and avoids the volatile compound geosmin, which is produced by some toxic moulds and bacteria, via a dedicated olfactory circuit (Stensmyr et al., 2012). By contrast, flies were innately attracted to the odour of both pathogenic *Pe* and *Ecc15* as compared to mere humidified air, suggesting that the odour of pathogenic bacteria is not repulsive per se (Figure 16). In the case of *Ecc15*, flies additionally preferred bacterial odours to pure LB medium.

Prior to the olfactory choice assay, flies were starved for a minimum of 15 h and one could thus object that the attraction to pathogenic bacterial odours is due to the flies' hunger state and the bacteria representing the only available potential food source. Hungry flies, like other animals, will direct all their efforts towards finding food (e.g. Sayin et al., 2019) and this naturally entails changes in their olfactory system and behaviour as well. Starvation promotes food-search behaviour and increases olfactory sensitivity towards attractive odours, while decreasing avoidance of innately aversive compounds (Root et al., 2011; Bräcker et al., 2013). Indeed, when given a choice between the odour of pathogenic *Pe* and that of a highly attractive protein source, namely yeast, hungry flies clearly preferred pure yeast not only to *Pe* odour alone but also to a mixture of *Pe* and yeast (Figure 17). The observation that this behaviour was dependent on the obligate olfactory co-receptor ORCO is supported by another study reporting that the olfactory attraction to yeast alone likewise required ORCO (Qiao et al., 2019). *Drosophila* is highly attracted to volatiles from yeast (Becher et al., 2012). Accordingly, the data showing that hungry flies preferred pure yeast to yeast containing pathogenic *Pe* suggests that they either detect an aversive olfactory

compound of the *Pe* strain or that the attraction to yeast overrides any other potentially existing olfactory preferences or aversions to pathogenic *Pe*. Interestingly however, flies slightly preferred the bacteria/yeast mixture to pure yeast in the case of pathogenic *Ecc15*. Here, it cannot be excluded that flies were unable to properly distinguish between this mixture and pure yeast. Nevertheless, the different degrees of attraction to yeast versus the yeast-bacteria mixture for *Ecc15* and *Pe* could for example be due to a difference in virulence between the two strains, with *Pe* being much more harmful than *Ecc15*. It could also indicate that pathogenic *Ecc15* emit an attractive airborne compound.

However, it is not the case that hungry flies are merely attracted to pathogenic bacterial odours if they can detect no other potential food source. The clear preference for the odour of pathogenic bacteria to that of the corresponding harmless strains (Figure 19) shows that flies not only distinguish between the odours of the respective strains, but that they are, surprisingly, innately attracted to detrimental bacteria that pose a threat to survival if ingested. This attraction, too, required ORCO-mediated olfaction, and it could for instance constitute a strategy of the bacteria to increase their spread. Hence, while starvation cannot be excluded as the driving force for the preference for pathogenic bacterial odours to air, the preference even over the corresponding harmless strains, which have the same nutritive value and only differ as to their virulence, supports the notion of an innate olfactory preference towards pathogenic bacteria. On a side note, it has been previously reported that *Drosophila* does not respond to *Pe* odour, while being attracted to the odour of infected flies due to an increased pheromone production (Keesey et al., 2017). However, this described indifference to *Pe* odour applied to an olfactory choice between *Pe* and culture media, which conforms to my own data (Figure 16). Yet flies were clearly attracted to the odour of pathogenic *Pe* if no other option was available or if the choice was between pathogenic and harmless strains, a behaviour that depended on ORCO-mediated olfaction, thus showing that flies can in fact detect *Pe* via their olfactory system.

It should be added that an olfactory preference for pathogenic bacteria does not preclude the existence of other avoidance behaviours. For example, adult flies are indifferent to the odour of parasitoid wasps, which are highly lethal for larvae, but can detect it via their olfactory system and strongly avoid it during oviposition (Ebrahim et al., 2015).

Nevertheless, the data presented here show that *Drosophila* would fail to detect the threat of food source contaminated with pathogenic *Ecc15* or *Pe* if it relied merely on olfaction.

4.3.2 Odours are not enough: prior infection does not induce olfactory avoidance

Knowing that flies are innately attracted to the odour of pathogenic bacteria and that infection with those bacteria severely harms the flies, the expectation was that prior ingestion of pathogenic bacteria might decrease this innate attraction or turn it into aversion. Contrary to the innate olfactory attraction to pathogenic bacteria seen in naïve flies, both flies infected with harmless and with pathogenic strains reduced their preference to bacterial odours following feeding on the respective strains (Figure 20 - Figure 22). Interestingly, while flies were indifferent to the various olfactory choices in most conditioning paradigms tested, weaker fly strains or flies that were starved longer still preferred bacteria to air – again regardless of the pathogenicity of the strain they had been infected with (Figure 21). This suggests that the observed difference between naïve and bacteria-fed flies could be due to different degrees of starvation, as naïve flies only had access to water prior to testing, while the bacteria-sucrose solutions dry out after a few hours, but still provide more nutritive value than pure water. Nonetheless, following bacteria feeding, it was not only flies tested for the olfactory choice between bacteria and air, but also those choosing between harmless and pathogenic bacterial odours that did not differ regarding the pathogenicity of the strain they had been feeding on prior to testing (Figure 20 - Figure 22). This indicates that potentially formed conditioned food aversions either evaded detection in an olfactory choice assay, i.e. that a mere olfactory stimulus was not sufficient to recall memory of spoiled food, or that *Drosophila* does not form a purely olfactory memory of food that has caused sickness in the past.

One of the difficulties in these olfactory conditioning paradigms was to accurately determine the timing of conditioning paradigms that aim at establishing conditioned food aversions. Classical aversive learning in *Drosophila* involves an association between an odour and an electric shock. Depending on the conditioning paradigms and the frequency and timing of US-CS pairings, flies form strong short-term or lasting memories (Tully et al., 1985; Tully et al., 1994). However, this requires concurrent presentation of the CS and the US, and trace conditioning paradigms have shown that the delay between the odour and onset of the

aversive stimulus must not be longer than 25 s for flies to form aversive olfactory memories (Galili et al., 2011). In conditioned food aversion learning, the onset of malaise typically occurs minutes if not hours after feeding and the chemosensory perceptions accompanying it. Remembering food that has caused infections thus requires forming an association between the chemosensory perceptions of the food source and the delayed onset of negative post-ingestive consequences. In *Drosophila*, it is not known whether the ingestion of pathogenic bacteria induces merely short-term, immediate behavioural adaptations such as decreased feeding and oviposition or in addition long-lasting avoidance behaviours and memory formation. In the present study, flies were commonly tested for their olfactory preference behaviour after approximately 16-20 h of bacteria feeding. However, it is difficult to assess whether this testing protocol captured potential memory formation. Previous studies as well as activity monitoring following infection (Figure 12 & Figure 13) suggest that although natural infection with *Pe* as well as *Ecc15* induces rapid expression of AMPs that is sustained more than 24 h after feeding (Basset et al., 2000; Liehl et al., 2006), the time course of *Ecc15* infection differs considerably from infection with *Pe*. An infection with *Ecc15* is characterized by fast lethality as well as quick recovery, while the time course of an infection with *Pe* is much more variable and longer (as was reported for pheromone production in Keesey et al., 2017). Thus, it is conceivable that the conditioning and testing protocols used in the present study might not have accurately captured potential olfactory aversion due to a different time course of memory formation.

Furthermore, starvation might account for the disparity between naïve and bacteria-fed flies, but it does not explain the absence of a difference between flies fed with either harmless or pathogenic bacteria. Accordingly, the observation that pre-exposed flies were indifferent even for the choice between harmless and pathogenic bacterial odours (Figure 22) points to an explanation other than hunger state for the absence of olfactory aversion after bacterial infection. Flies fed with harmless strains might either retain their innate preference for pathogenic bacterial odours or even form a positive association and exhibit an increased attraction to the odour of the harmless strain. A recent study showed that pre-exposure to certain beneficial microbes that are part of the fly's microbiome changed olfactory preference behaviour, as yeast-fed flies subsequently increased their preference to yeast odour as well

as their aversion of the odour of an *Acetobacter* strain as compared to naïve flies (Qiao et al., 2019).

Regarding pathogenic bacteria, it has been reported that flies exposed to a *Pe* WT-contaminated food source scented with an arbitrary odour subsequently reduced their attraction to this odour (Babin et al., 2014). In the conditioning paradigms tested in this study, flies did not show similar avoidance learning. Were an olfactory stimulus to be enough to form an aversive memory of a contaminated food source, flies infected with pathogenic bacteria should have decreased their preference for the odour of pathogenic bacteria in comparison to flies infected with harmless control strains. However, the study by Babin and colleagues relied on an assay where, during testing, flies were able to feed and select food sources which were scented with the odours previously paired with food laced with bacteria, while the olfactory choice arena used in the present study required flies to choose between odours only. This hence suggests that olfactory cues might not be sufficient for flies to recall prior ingestion of pathogenic bacteria, and that remembering the ingestion of a detrimental food source instead requires multisensory features in order to adapt behaviours and avoid food that has caused harm.

4.4 Flies avoid pathogen-infested food

4.4.1 Pathogens modulate the feeding behaviour of *Drosophila*

The data presented in this thesis show that contrary to the olfactory attraction to pathogenic bacteria, flies prefer feeding on harmless bacteria to the corresponding pathogenic strains, but do not necessarily avoid feeding on pathogens per se if the alternative is a less nutritive food source (Figure 23 - Figure 27). Exposure to both *Ecc15* and *Pe* has been reported to reduce food intake in *Drosophila* larvae, with feeding cessation being only transient in the case of *Ecc15* (Liehl et al., 2006; Keita et al., 2017). However, these studies did not clarify whether larvae learn to avoid detrimental food or how their feeding behaviour would develop if offered an alternative. In two-choice feeding assays, too, adult flies were shown to avoid food sources containing pathogenic *Pe* WT or LPS (Soldano et al., 2016; Keesey et al., 2017). It should be noted that the master student I supervised, Irina Petcu, saw an opposite feeding preference for both PGN and LPS over sucrose, however at a lower

concentration (data not shown). In the two aforementioned studies, starved flies chose between pathogenic bacteria or bacterial components mixed with sucrose and a sucrose or medium control, so it cannot be excluded that the feeding decision was influenced not only by the virulence of the bacterial strain, but also by the protein content and nutritional value of the respective food substrate. This is supported by the observation that *Drosophila* larvae reduce their aversion to a food source containing pathogenic *Pe* WT when starved (Surendran et al., 2017). Accordingly, it is not surprising that hungry flies preferred pathogenic *Ecc15* to LB medium in the flyPAD (Figure 27) and in several instances even to mere sucrose in the CAFE towards the end of the assay (e.g. wild-type CS in Figure 24). In most cases however, hungry flies were indifferent to a choice between pathogenic *Ecc15* and sucrose. In the case of *Ecc15* and contrary to what has been reported for *Pe* feeding (Keesey et al., 2017), flies never avoided feeding on pathogenic *Ecc15* if the alternative was less nutritive sucrose, a behaviour that can most likely be explained by the higher nutritional value of the bacteria-sucrose mixture.

The relationship between nutritional needs and consumption of a detrimental food source is illustrated even better by the initial and only transient preference for sucrose over a *Pe*-sucrose mixture in the CAFE (Figure 23). It is likely that with increasing starvation time, flies rate the nutritive value of pathogenic bacteria higher than the threat they pose. Hunger not only changes olfactory sensitivity in *Drosophila*, but also decreases bitter sensitivity in order to allow starved flies to approach less optimal food sources (Inagaki et al., 2014; Devineni et al., 2019). Similarly, flies might accept food contaminated with pathogenic bacteria and the accompanying negative post-ingestive effects if starved for extended periods of time.

In addition, wild-type and control flies preferred a sucrose solution supplemented with harmless *Ecc15* to mere sucrose in most cases, which was expected due to the higher nutritive value of the bacteria-sucrose solution. Surprisingly, a recent study reported that hungry flies were indifferent to a feeding choice between beneficial microbes and mere growth medium (Qiao et al., 2019). Yet bacteria and their metabolites constitute a valuable food source, and *Drosophila* depends on the ingestion of beneficial bacteria to both establish and maintain its microbiome (Broderick et al., 2012; J. E. Blum et al., 2013). As both *Ecc15 evf* and *Pe gacA* neither trigger the activation of the immune response nor otherwise negative post-ingestive

effects, a mixture of those bacteria and sucrose should constitute an attractive food source for hungry flies.

The best possible option to determine whether flies actually avoid food containing detrimental bacteria due to their pathogenicity while at the same time limiting other confounding factors is a two-choice assay involving food sources of similar nutritive value and composition. Such two-choice feeding assays showed that flies prefer a sucrose solution supplemented with harmless *Ecc15* or *Pe* strains to one mixed with corresponding pathogenic strains (Figure 23 - Figure 26). It has been reported previously that flies prefer bacteria-laced food to standard food irrespective of the pathogenicity of the used *Pe* strains (Babin et al., 2014). However, this study did not measure preferences for a direct choice between harmless and pathogenic *Pe* and did not record food intake but merely the number of flies on the different food substrates for the duration of two hours. Since bacterial odours are innately attractive for flies (see 3.3), it is unclear which part of the attraction to food supplemented with pathogenic *Pe* shown by Babin and colleagues is due to olfaction and which due to feeding. By contrast, the pathogenic *Ecc15* and *Pe* bacterial strains and their corresponding harmless mutant counterparts used in the present study only differed regarding their pathogenicity. Accordingly, testing feeding preferences of flies for a direct choice between pathogenic and harmless *Ecc15* or *Pe* allows excluding a difference in nutritive value as a reason for the observed preference for harmless over pathogenic bacterial strains in both the CAFE and the flyPAD (Figure 23 - Figure 26).

4.4.2 Delayed feeding aversion to pathogens and the role of taste

Why do flies prefer feeding on harmless instead of pathogenic bacterial strains? One possibility might be an innate attraction or aversion to the taste of the bacteria. In the flyPAD, innate taste preferences for one of the two food substrates are reflected in an immediate divergence of the number of sips taken. As expected and as previously reported (Itskov et al., 2014), this was the case for a feeding choice between substrates with higher and lower sucrose concentrations (Figure 29). Since flies also immediately preferred harmless to pathogenic *Pe* in the flyPAD (Figure 25), I can currently not exclude a contribution of taste to the feeding preference for pathogenic over harmless *Pe*. By contrast, flies readily fed on both pathogenic and harmless *Ecc15* strains initially, suggesting that they are not innately

averse to the taste of pathogenic *Ecc15*. Flies shifted their preference to the harmless strain only later in the CAFE assay, but most strikingly in the flyPAD, where they started consuming significantly more harmless bacteria after 15-20 min (Figure 24 & Figure 26). This indicates that at least in the case of *Ecc15*, taste is not the major determinant of feeding preference for harmless as opposed to pathogenic bacterial strains and that this aversion is instead due to other, potentially post-ingestive mechanisms.

Nevertheless, we did not test flies deficient for specific taste receptors for their preferences in an *Ecc15* or *Pe* feeding choice. It is difficult to pinpoint which taste receptors would be necessary for flies to differentiate harmless and pathogenic strains – provided they can differentiate them at all by means of taste. The bacterial strains are grown in the same culture medium and adjusted to the same optical density, and both are supplemented with sucrose. Thus, the food substrates containing harmless and pathogenic bacteria are highly similar regarding their protein content and nutritional value, with the only apparent difference being their virulence, while potentially bitter tasting compounds in both strains will be at least partially masked by sweet taste. Given that flies immediately distinguish between harmless and pathogenic *Pe* strains, it is plausible that *Pe* WT and *Pe gacA* strains can be differentiated by taste. The GacS/GacA system regulates a variety of processes such as secondary metabolite production or the expression of pore-forming toxins (Opota et al., 2011), which could be recognized by the gustatory system. By contrast, *Ecc15 evf* and *Ecc15 pOM1* only differ regarding Evf, which is not a toxin and confers infectious properties by promoting persistence in the gut (Muniz et al., 2007).

Still, *Drosophila* does possess gustatory mechanisms to detect bacterial components prior to ingestion. Flies strongly avoid food containing the bacterial membrane component LPS, and this avoidance depended on TRPA1 in GRNs expressing the bitter receptor *Gr66a* (Soldano et al., 2016). In addition, feeding cessation of larvae following exposure to *Ecc15* was also reported to require TRPA1 (Keita et al., 2017). Nevertheless, LPS is obviously not what distinguishes harmless *Ecc15 evf* from pathogenic *Ecc15 pOM1*, as both strains possess LPS in their membranes. Thus, even if TRPA1 contributed to a putative gustatory-mediated avoidance of pathogenic *Ecc15*, flies lacking TRPA1 would consequently be unable to detect LPS in harmless as well as pathogenic strains; and the same applies to taste receptors detecting protein. Due to the similarity of the two *Ecc15* strains, it is thus questionable how

flies would be able to differentiate between harmless and pathogenic *Ecc15* merely by means of external taste receptors. However, this does not exclude the possibility that gustatory receptors in the gut could be involved in the post-prandial detection of pathogens (J.-H. Park et al., 2011).

Combined with the observation that it takes flies more than 15 minutes to start distinguishing harmless from pathogenic *Ecc15*, this puts forward an acquired aversion of pathogenic bacteria due to post-ingestive mechanisms as the most likely explanation for the feeding aversion to this pathogenic bacterial strain. In the CAFE, it is more difficult to assess choice behaviour directly after feeding onset due to methodological constraints and a lower resolution of feeding measurements, yet feeding in the CAFE showed the same delayed preference for harmless over pathogenic *Ecc15* strains after an initial period of equal feeding. Contrary to the innate attraction to the odour of pathogenic bacteria, feeding thus reverses this preference behaviour and induces a lasting avoidance of food containing detrimental bacteria.

4.4.3 Feeding aversion to pathogens relies on an associative learning mechanism

The initially equal consumption of harmless and pathogenic bacteria and the delayed preference for harmless over pathogenic *Ecc15* seen in the flyPAD (Figure 26) already suggested an acquired avoidance due to the post-ingestive consequences of bacteria consumption instead of a mere effect of taste. That this acquired feeding aversion could indeed rely on an associative learning mechanism was supported by experiments showing that synaptic output from the MB as well as the Ca^{2+}/calmodulin-stimulated adenylyl cyclase rutabaga were required to differentiate between food containing harmless and pathogenic bacteria (Figure 30 & Figure 31). The MB is the main centre for associative learning and memory formation in the fly brain and is involved in the modulation of behaviours by internal states (Heisenberg, 2003; Owald et al., 2015; Grunwald Kadow, 2019). Rutabaga is mainly, but not exclusively, expressed in the MB where it is thought to act as a coincidence detector during conditioning that is involved in the molecular changes required for synaptic plasticity and thus learning (Zars et al., 2000; Tomchik et al., 2009; Gervasi et al., 2010). In classical shock conditioning paradigms, the MB serves as a site of integration of the information about the odour (the CS), which is transmitted by KCs, and the information

about punishment (the aversive US) conveyed by dopaminergic neurons, and subsequently generates the appropriate behavioural output. Learning to avoid a contaminated food source requires animals to form associations between a particular character of the food such as its smell or taste, and the presumably negative post-ingestive consequences of pathogen ingestion occurring much later, e.g. infection and malaise. The observation that both the MB and rutabaga were necessary for flies to distinguish between harmless and pathogenic bacteria during feeding suggests that the avoidance behaviour towards pathogen-infested food identified in this thesis could constitute such a learned food aversion (see also discussion in 4.6.2). Yet further investigations are needed for example as to how stable and long-lasting this acquired feeding aversion or 'negative food memory' is. Rutabaga has mostly been implicated in the formation of short-term memories (Zars et al., 2000); however, this does of course not exclude the additional formation of a long-term memory of the contaminated food source.

4.5 Immune signalling and the avoidance of contaminated food

Ingestion of pathogenic bacteria triggers the activation of the innate immune response. Apart from a cellular response, the main pillar of the *Drosophila* immune defence against intruding pathogens is the inducible expression and synthesis of AMPs. While the different signalling pathways and effectors of the immune response have been studied extensively, less is known about how the immune system directly influences the behavioural adaptations ensuing infections. The data presented in this thesis support the notion that select components of a signalling pathway of the innate immune response, namely the Imd pathway, are required for the acquired feeding aversion of *Drosophila* to pathogenic bacteria and thus provide experimental evidence for a role of immune signalling beyond the induction of direct antimicrobial strategies.

Feeding aversion to pathogenic *Ecc15* required several components of the Imd pathway, namely the two immune receptors PGRP-LC and PGRP-LE as well as the NF-κB transcription factor Relish and AMPs, but surprisingly not the caspase Dredd (Figure 32 - Figure 36). Dredd is necessary for the cleavage of Relish and was moreover shown to be the only caspase triggering this cleavage (Stöven et al., 2000; Stöven et al., 2003; C.-H. Kim et al., 2014). In *dredd* mutant flies, Relish is thus not cleaved and cannot translocate into the

nucleus to induce the transcription of AMPs and other immune effectors. However, since both Relish and AMPs were necessary for flies to distinguish between pathogenic and harmless bacterial strains and as Dredd acts upstream of Relish, it seems unlikely that Relish and AMPs, but not Dredd are required for this behaviour. In addition, AMP expression upon Imd pathway activation does not exclusively rely on Relish, but might likewise depend on JNK signalling via TAK1 (Delaney et al., 2006), which at the same time has been reported to downregulate NF-κB signalling (J. M. Park et al., 2004; T. Kim et al., 2005). To determine if Dredd is indeed dispensable for distinguishing harmless from pathogenic bacteria, it would be advisable to test a fly line with a different mutation of *dredd* or to downregulate Dredd via RNAi during pathogen feeding. It is worth mentioning that the reverse is conceivable as well, namely that Relish and AMPs are in fact not needed for avoiding contaminated food. Yet since both *relish* and *AMP*-deficient flies were unable to distinguish between harmless and pathogenic bacteria, this latter possibility seems less likely.

Interestingly, Relish not only induces the transcription of immune effectors such as AMPs, but has also been implicated in behavioural adaptations following infection other than the adaptation of feeding reported in this study. Relish has been shown to be upregulated following sleep deprivation and promotes sleep during an ongoing immune response (Williams et al., 2007; Kuo et al., 2010). Immunological challenges are typically accompanied by increased sleep and rest phases to facilitate combating pathogens (see also 4.2). Directly coupling the regulation of sleep to an immune-inducible transcription factor could constitute an efficient way to guide a behavioural adaptation aimed at accelerating recovery. To get a clearer picture of the role Relish plays in acquired feeding aversions to pathogenic bacteria, it would be informative to narrow down the bodily regions where it is required.

Furthermore, one factor that could potentially confound the results regarding a contribution of AMPs to acquired feeding aversions to pathogens is that ΔAMP flies are mutant for all AMPs but for the four inducible cecropins. Cecropins exhibit antimicrobial activities mostly against Gram-negative bacteria and some fungi (Hultmark et al., 1980; Ekengren et al., 1999; Imler et al., 2005), leaving room for the possibility that ΔAMP flies could partially mediate feeding aversion to pathogenic bacteria via cecropins. However, even though ΔAMP flies still possess wild-type cecropins, they were previously shown to be

highly susceptible to an infection with *Ecc15* via pricking (Hanson et al., 2019). As ΔAMP flies did not distinguish between food sources containing harmless and pathogenic *Ecc15* (Figure 36), cecropins appear to be dispensable for acquired feeding aversions to contaminated food. Interestingly, two specific AMPs were shown to be required for the formation of long-term associative appetitive memories as well as memories of an unsuccessful mating experience (Barajas-Azpeleta et al., 2018), suggesting that AMPs could have been repurposed in the nervous system to guide memory formation. Given that the immune receptor PGRP-LC was required in octopaminergic neurons (Figure 43 and discussion in 4.6.2), AMPs might contribute to the observed acquired feeding aversion to pathogenic bacteria by acting in the same type of neurons as PGRP-LC.

The data presented here show that *Drosophila* relies on the two main receptors activating downstream Imd signalling upon recognition of bacterial PGN, namely cytosolic PGRP-LE and in particular the transmembrane PGRP-LC, to differentiate between food sources containing harmless and pathogenic bacteria (Figure 32 & Figure 33). The requirement for PGRP-LC was confirmed via whole-body RNAi-mediated downregulation (Figure 37), while a comparable approach for PGRP-LE would surely lend further support to the observed contribution of PGRP-LE to the acquired feeding aversions to pathogens. Moreover, it is possible that other PGRPs not tested in the present study are involved in suppressing feeding on detrimental food. Negative regulators of the Imd pathway such as PGRP-LB detect and cleave extracellular PGN and thereby prevent an excessive, detrimental activation of the immune response (Zaidman-Rémy et al., 2006). PGRP-LB seems to constitute a link between the activation of the immune response and behavioural adaptations, as a specific PGRP-LB isoform modulates NF-κB pathway activation in octopaminergic neurons, which in turn triggers a reduction in egg-laying post-infection (Kurz et al., 2017; Masuzzo et al., 2019). It will certainly be of interest to determine whether PGRP-LB similarly contributes to the feeding aversion to pathogenic *Ecc15* observed in this study.

However, as *PGRP-LC* mutant flies were most evidently unable to distinguish between harmless and pathogenic bacterial strains among all Imd pathway mutants tested, our further efforts concentrated on the role of PGRP-LC in pathogen-modulated feeding behaviour. Little is known about potential additional functions of PGRP-LC beyond the Imd

and JNK signalling cascades it triggers primarily in the fat body and the gut upon recognition of DAP-type PGN of Gram-negative bacteria. For example, PGRP-LC was not needed for the NF-κB-mediated drop in oviposition following injection of *E. coli* or PGN (Kurz et al., 2017), but has been associated with hygienic grooming behaviours in decapitated flies induced by LPS (Yanagawa et al., 2017). Nonetheless, PGRP-LC is mostly expressed in the gut and in the fat body, where it triggers the activation of the local and systemic immune response upon sensing bacterial PGN, respectively (Neyen et al., 2012). The *Drosophila* fat body is functionally equivalent to the mammalian liver and adipose tissue in that it senses and stores nutrients or for example synthesizes glycogen, but is also the main site of the systemic immune response (Buchon et al., 2014; Droujinine et al., 2016). Following ingestion of pathogenic *Ecc15*, bacterial PGN reaching the haemolymph induces the activation of the systemic immune response via PGRP-LC. Yet while *Drosophila* did not require PGRP-LC in midgut enterocytes to distinguish between harmless and pathogenic bacteria, PGRP-LC was potentially necessary in the fat body (Figure 39 & Figure 40), even though the role of PGRP-LC in the fat body would need further confirmation due to the low overall consumption of these flies. Together with the absence of an effect of midgut PGRP-LC downregulation, this suggests that local, but not systemic immune signalling via PGRP-LC seems to be dispensable for avoiding contaminated food.

Apart from losing the ability to distinguish between harmless and detrimental food sources, it is worth mentioning that *PGRP-LC*- and *Relish*-deficient flies seemed to increase their total food consumption in comparison to controls (Figure 32 & Figure 35). However, this cannot be generalised, as this was not the case for flies lacking AMPs or PGRP-LE. Disruption of Imd signalling has been previously reported to have metabolic consequences such as increased weight and lipid as well as glucose storage (Davoodi et al., 2019). The observation that PGRP-LC could also be involved in regulating appetite thus points to a potential link between the immune response and metabolic adaptations during an immune challenge.

The experimental evidence presented in this study clearly shows that PGRP-LC is required in the nervous system, more specifically in octopaminergic neurons, to distinguish between harmless and detrimental food sources (Figure 41 & Figure 43, see discussion in 4.6), suggesting that an immune receptor acts directly in the nervous system to mediate the

behavioural adaptations following the ingestion of contaminated food. It has previously been reported that PGRP-LC is expressed in the nervous system, where it was shown to control homeostatic synaptic plasticity in the absence of an infection (Harris et al., 2015).

At this point, there are still many open questions as to which Imd signalling components aside from PGRP-LC are indeed necessary to trigger acquired feeding aversions to pathogenic bacteria, which will be the subject of further investigations. With the Imd pathway bifurcating into JNK and NF-κB signalling at the level of TAK1 and thus downstream of PGRP-LC and PGRP-LE, there is room for possible redundancy in the pathways leading to the expression of AMPs, which were likewise implicated in pathogen-modulated feeding behaviour (Figure 36). Similarly, the control of presynaptic homeostatic plasticity by PGRP-LC relied on diverging pathways downstream of PGRP-LC via TAK1 on the one hand and Relish on the other hand (Harris et al., 2018).

Finally, the Imd pathway shares conserved signalling molecules with the mammalian TNFR pathway (Myllymäki et al., 2014), pointing to the possibility that immune signalling could mediate behavioural adaptations following ingestion of contaminated food in animals other than *Drosophila*.

4.6 From gut to brain: how intestinal pathogens change behaviour

4.6.1 How does the information about an infection reach the brain?

Pathogens that are taken up with food enter the body via the digestive system, where they trigger the activation of the immune response and cause an infection. Apart from direct antimicrobial strategies, detrimental microbes also trigger behavioural adaptations and memory formation to alleviate the impact of the infection and to avoid repeated infections by ingesting the same (or a similar) contaminated food source. Reducing or avoiding feeding on a contaminated food source and instead feeding on non-hazardous food, as I have reported in this thesis, constitutes such an acquired behavioural adaptation following the ingestion of pathogenic bacteria. However, the mechanisms underlying these conditioned food aversions or pathogen-modulated feeding behaviours are unclear in particular regarding the necessary communication between the periphery and the brain. The evidence presented here suggests a model wherein the ingestion of pathogenic bacteria induces

feeding aversion via PGRP-LC signalling in octopaminergic neurons as well as via the MB (Figure 44), thus directly linking the ingestion of pathogens and the immune system with the behavioural adaptations regulated by the brain.

It will be the subject of further investigations to determine how the information about pathogen ingestion and the ongoing infection reaches the nervous system and higher brain centres such as the mushroom body. One possibility is that bacterial components or metabolites traverse the gut epithelium and activate PGRP-LC directly in the nervous system. If bacteria accumulate in the gut, PGN fragments such as the monomeric PGN tracheal cytotoxin can cross the epithelial barrier, reach the haemolymph and activate the systemic immune response via the fat body (Zaidman-Rémy et al., 2006; Neyen et al., 2012), and could hence travel to the nervous system to initiate PGRP-LC signalling and inhibit pathogen feeding. Bacterial PGN from the gut microbiota of mice seems to cross the blood-brain barrier and translocate into the brain, where it is detected by PGRPs, one of which was moreover shown to regulate social behaviour (Arentsen et al., 2017). In mammals, PGRPs in the brain have thus been suggested to act as mediators of the microbiota-gut-brain communication (Tosoni et al., 2019).

Yet PGRP-LC in *Drosophila* can not only be activated by PGN, but potentially also by means of bacterial metabolites. The microbial fermentation product acetate was shown to increase Imd signalling in enteroendocrine cells via PGRP-LC to regulate lipid homeostasis in enterocytes (Kamareddine et al., 2018). Accordingly, bacterial products other than PGN could travel to the nervous system and trigger PGRP-LC signalling or its expression and thereby subsequent feeding suppression on pathogen-contaminated food. Interestingly, the reported regulation of lipid homeostasis in enterocytes depended on the upregulation of tachykinin in enteroendocrine cells via PGRP-LC-mediated Imd pathway activation (Kamareddine et al., 2018). Tachykinins constitute a large family of neuropeptides that are conserved across species and modulate diverse functions in the central nervous system and the gut as well as during the immune response (Nässel et al., 2019). Substance P is for instance a vertebrate member of the tachykinin family that serves a multitude of roles in response to stressors, e.g. in infection, inflammation or nociception, and is thought to act as a key mediator of interactions between the nervous system and the immune response (Mashaghi et al., 2016). In *Drosophila*, tachykinins are involved in odour perception,

locomotion, pheromone-induced courtship suppression and promote aggressive behaviour in males (Winther et al., 2006; Asahina et al., 2014; Shankar et al., 2015). Tachykinins are important modulators of metabolic processes, as they regulate lipid metabolism in the gut as well as insulin-producing cells in the brain (Birse et al., 2011; Song et al., 2014). Tachykinin release in the AL moreover affects odour-guided behaviour (Ignell et al., 2009), and the enhanced attraction of starved flies to otherwise aversive high concentrations of vinegar was shown to be partially mediated by an increase in tachykinin signalling, which suppressed the activity of an AL glomerulus conveying food odour aversion (Ko et al., 2015). Given the diverse roles of tachykinin in feeding-related behaviours and metabolism, it is conceivable that similar to the PGRP-LC-mediated tachykinin expression in enteroendocrine cells and the subsequent modulation of metabolic processes, PGRP-LC could also activate tachykinin (or another neuropeptide) signalling in the brain in order to suppress consumption of detrimental food following infection with pathogenic bacteria. In *Drosophila* larvae, evasion behaviour towards pathogenic bacteria depended on the neuropeptide hugin, as avoiding pathogens required the activation of hugin bitter taste interneurons in the larval brain by *Pe* (Surendran et al., 2017).

Another way of communication between the immune system, the gut and the brain are cytokines. Cytokines serve as long-distance or intercellular messengers during immune or inflammatory processes and can signal from the periphery to the brain. Major cytokines in vertebrates such as interleukin-1 (IL-1) or TNF-α, which are produced by immune cells following the detection of pathogens, induce sickness behaviours, e.g. increased sleep or a loss of appetite, via the hypothalamus (Tizard, 2008). Factors other than pathogens might trigger cytokine expression, too: in mice, food intake was reported to lead to the expression of the pro-inflammatory cytokine IL-17 in the gut, which in turn acts on the hypothalamus to reduce food intake (Nogueira et al., 2019). In *Drosophila*, infection with pathogenic *Ecc15* or *Pe* damages gut cells and thereby induces for instance the release of the cytokine unpaired 3 (Upd3), which promotes stem cell proliferation via the JAK-STAT pathway and thereby stimulates epithelium renewal and repair processes (Buchon et al., 2009a; Buchon et al., 2009b; Jiang et al., 2009). However, it is currently not known if cytokines induced by intestinal infection are in addition involved in gut-brain signalling to alter neural processing and thus adapt behaviours. Yet the cytokine Upd3 can serve as a long-distance cue, as

expression of Upd3 by haemocytes following infection was reported to activate JAK-STAT signalling in the fat body and the gut and thereby remotely activates intestinal stem cells (Chakrabarti et al., 2016). Moreover, JAK-STAT signalling activated by the cytokine Upd seems to be required in the MB specifically for long-term aversive olfactory memories (Copf et al., 2011). Altogether, further investigation will be needed to determine whether cytokines contribute to pathogen-modulated feeding behaviours and if they interact with PGRP-LC in suppressing pathogen feeding, or if there is redundancy in the pathways guiding behavioural adaptations to intestinal infections.

Instead of bacterial components, metabolites or specific neuropeptides, another mechanism of transmitting the information about an infection from the periphery to the central nervous system could be a neuronal pathway. The mammalian vagus nerve is a prime example of such a major neural connection between the internal organs and the brain and has been suggested to be utilized by intestinal microbiota to modulate behaviour (Fülling et al., 2019). The dorsal vagal complex, where vagal afferents terminate in the brainstem, is thought to act as an immune-behaviour interface in mice, since its inactivation abolished sickness behaviours normally observed after injection of LPS (Marvel et al., 2004). Similar to higher animals, the fly gut is innervated by neurons that regulate peristalsis and gut physiology (Miguel-Aliaga et al., 2018). In *Drosophila* larvae, a cluster of serotonergic neurons in the brain, which innervate the enteric nervous system and modulate the motor patterns underlying food ingestion, could serve as a functional analogue of the vagus nerve (Schoofs et al., 2014; Schoofs et al., 2018). Serotonin is not only an important neurotransmitter in the enteric nervous system and in the gut-brain axis, but – as mentioned previously – has also been implicated in conditioned food aversions in *C. elegans* and the honeybee (Y. Zhang et al., 2005; Wright et al., 2010), and is therefore another potential candidate for gut-brain signalling in *Drosophila* following ingestion of contaminated food.

Thus, while it is currently not known how the information that pathogens have entered the digestive system is transmitted to the brain, earlier studies of gut-brain signalling provide cues regarding possible mechanisms. It will be the subject of further investigations whether upon ingestion of pathogenic bacteria, PGRP-LC signalling in the nervous system is activated directly by bacterial components or metabolites to suppress feeding on

pathogen-infested food, if a neural gut-brain connection is involved and if this behaviour is additionally mediated by cytokines or neuropeptides.

4.6.2 Processing in the nervous system and associative learning mechanisms

Flies that lack PGRP-LC in the nervous system, and interestingly more specifically in octopaminergic neurons, lose the ability to distinguish between harmless and pathogenic bacteria (Figure 41 & Figure 43) and thus cannot protect themselves from ingesting detrimental food. The additional requirement for rutabaga and synaptic output from the MB (Figure 30 & Figure 31) indicates that the acquired aversion to feeding on pathogenic bacteria requires an associative learning mechanism. It is unclear, however, if PGRP-LC signalling was also required directly in the MB. Even though downregulation of PGRP-LC in the KCs of the MB rendered flies unable to distinguish between harmless and pathogenic bacteria (Figure 42), the poor health of these flies and their very low overall consumption should be considered when interpreting these results. At this point, we can thus not state with certainty whether PGRP-LC was necessary in the MB to avoid feeding on pathogen-contaminated food, and it will be the subject of further studies to confirm these data.

PGRP-LC initiates the immune response primarily in the gut and the fat body, while the data presented in this study implicate PGRP-LC in octopaminergic neurons and potentially in the fat body, yet not in the gut. So far, it is not known how the information about an infection reaches the nervous system (see discussion in 4.6.1) or which neural circuits are responsible for guiding the behavioural adaptations upon pathogen ingestion. Nonetheless, it has become increasingly acknowledged that the immune and nervous system share abundant reciprocal connections and interact on many levels, e.g. via hormones or cytokines (reviewed in Dantzer, 2018). In *Drosophila*, AMPs as major immune effectors have for example been implicated in memory formation (Barajas-Azpeleta et al., 2018).

PGRPs have been reported to be expressed in the nervous system of the fruit fly, where some of them were additionally involved in behavioural adaptations following an immune challenge. PGRP-LC is required for presynaptic homeostatic plasticity at the neuromuscular junction, where it was shown to control the homeostatic expansion of the readily releasable pool of synaptic vesicles in the absence of an infection (Harris et al., 2015). Other components of the Imd pathway essential for presynaptic homeostatic plasticity were Imd, IKKβ, TAK1

and Relish, with TAK1 modulating presynaptic release locally and immediately, and Relish controlling the long-term maintenance of the homeostatic response (Harris et al., 2018). Interestingly, an artificial activation of the immune response via upregulation of PGRP-LC in the fat body might decrease memory abilities in flies (Mallon et al., 2014), however, it cannot be excluded that this effect is due to potential detrimental effects of excess immune signalling. Moreover, the amidase PGRP-LB and PGRP-LE – but not PGRP-LC – induce NF-κB pathway activation in a group of brain octopaminergic neurons upon infection with Gram-negative bacteria or stimulation with PGN, which in turn triggered a drop in egg-laying (Kurz et al., 2017; Masuzzo et al., 2019). Together with the data presented in this study, this points to a non-traditional role of PGRP-LC-mediated Imd signalling in octopaminergic neurons in guiding the behavioural adaptations to pathogen uptake.

Octopamine is expressed by neurons throughout the *Drosophila* nervous system that innervate diverse bodily regions such as reproductive organs, skeletal muscles, antennae, legs and wings and a variety of brain regions including the MB and the SEZ (Busch et al., 2009; Pauls et al., 2018). Octopamine is involved in a broad range of different behaviours relating for example to aggression, motivation or feeding as well as in reinforcing short-term appetitive memories during olfactory conditioning or in mediating microbial cues to regulate locomotion (Hoyer et al., 2008; Burke et al., 2012; Schretter et al., 2018; Youn et al., 2018; Sayin et al., 2019). Octopaminergic neurons of the VPM4 cluster in the SEZ, which directly connect to a particular output neuron of the MB (MBON-γ1pedc>αβ), are thought to relay information about food or feeding to higher brain centres, as they promote feeding initiation and suppress food odour tracking once food is found (Youn et al., 2018; Sayin et al., 2019). Moreover, the PGRP- and NF-κB-mediated decrease in egg-laying following infection is initiated by PGN sensing in octopaminergic neurons of the VM III cluster in the SEZ (Masuzzo et al., 2019). As the SEZ additionally receives taste input, houses motor neurons regulating feeding and is a site of abundant neuromodulation (Thorne et al., 2004; Z. Wang et al., 2004; Gordon et al., 2009; Marella et al., 2012), it is worth exploring the possibility that the PGRP-LC-mediated suppression of pathogen feeding likewise occurs in octopaminergic neurons of the SEZ. Analogous to the vagus nerve in the mammalian brain stem, the SEZ could thus serve as an immune-brain interface, which transmits the information about pathogens taken up with food to higher brain centres.

Similar to conveying the presence of food to the MB (Sayin et al., 2019), one possibility is that octopaminergic neurons, upon pathogen uptake and activation of PGRP-LC signalling, propagate the information about an infection to the MB, which was likewise shown to be necessary for the observed acquired aversion to pathogen-contaminated food in this thesis (Figure 30). The MB is mostly studied for its role in associative olfactory learning, yet receives multisensory information, e.g. from visual projection neurons, about the presence of water or from taste organs (Lin et al., 2014; Kirkhart et al., 2015; Vogt et al., 2016). In addition, the MB is the target of post-ingestive signals. Feeding and conditioning flies with a bitter-sugar mixture induces immediate avoidance of the conditioned odour via particular dopaminergic neurons, which later turns into aversion, indicating the formation of a long-term nutrient-dependent memory (Das et al., 2014). Sweet taste and nutrient value reinforce memory through distinct dopaminergic neuron subpopulations innervating the MB (Huetteroth et al., 2015), and the long-term association between an odour and nutritive value required delayed post-ingestion signalling of energy levels to the MB via specific dopaminergic neurons (Musso et al., 2015). Thus, the MB can integrate sensory information with delayed post-ingestion signals regarding the energetic value of food to form memories of food quality.

At this point, it is not known how exactly the MB contributes to differentiating between non-hazardous and pathogen-infested food. Given the role of the MB in integrating post-ingestion signals during associative memory formation, it is conceivable that the information about the post-ingestive consequences of pathogen ingestion together with the chemosensory cues of the food source are integrated at the level of the MB. This is further supported by the observed requirement for rutabaga in avoiding pathogenic bacteria (Figure 31). While rutabaga is implicated in the formation of short-term memories (Livingstone et al., 1984), it will be insightful to determine whether the feeding aversion to pathogenic bacteria constitutes a short-term or more long-lasting behavioural adaptation. In addition, rutabaga is required in particular in the MB for olfactory memory formation, and flies lacking *rutabaga* seemingly perform less well in a two-choice assay between food substrates with different sucrose concentrations (Zars et al., 2000; Motosaka et al., 2007). Hence, it is likely that the adjustment of feeding behaviour to avoid a contaminated food source relies on an associative learning mechanism at the level of the MB.

It will be the subject of future studies to unveil the neural circuits guiding the behavioural adaptations following pathogen ingestion, not only regarding the identity of the involved octopaminergic neurons as well as their potential connections to the MB, but for example also regarding the role of dopaminergic neurons. Data obtained from a similar bacterial feeding choice assay suggest that dopaminergic neurons of the PPL1 cluster are required to distinguish between a non-hazardous and a *Pe*-containing proteinaceous food source (data not shown, Irina Petcu, master thesis "Investigating the Role of a Memory Centre in Pathogen-Modulated Feeding Behaviour in *Drosophila melanogaster*", 2019). PPL1 dopaminergic neurons convey information about aversive stimuli and punishment and signal aversive reinforcement during olfactory learning (Riemensperger et al., 2005; Claridge-Chang et al., 2009; Aso et al., 2010). In addition, PPL1 neurons were reported to be both necessary and sufficient for aversive (yet pre-ingestive) taste memories (Masek et al., 2015). Thus, it will be interesting to see if PPL1 dopaminergic neurons contribute to choosing between harmful and pathogenic bacteria as well. However, it should be noted that we have not yet investigated what the decisive stimulus for the fly's feeding decision is. While assuming that the negative post-ingestive consequences of pathogen ingestion and the ensuing infection prevail, there is room for the possibility of additional positive post-ingestive feedback regarding the innocuousness of the second food source containing harmless bacteria.

4.7 Learning to avoid spoiled food as a common survival strategy

Food that has caused negative post-ingestive effects and malaise in the past is typically rejected in the future. Being able to associate the taste or smell of a detrimental food source with its delayed post-ingestive effects or a later occurring sickness is an essential defence mechanism against the ingestion of toxins or pathogens. This acquired food aversion is part of a larger sequence of defence strategies to protect against harm, which includes the immune response and more immediate sickness behaviours such as vomiting or anorexia, and which is crucial to reduce the risk of repeated infections. Acquired food aversions thus constitute an important survival strategy across the animal kingdom and are thus potentially based on conserved mechanisms. The behavioural responses upon exposure to food that has caused malaise in the past vary greatly across species: rats for example bury the spout of a

solution previously paired with malaise-inducing lithium chloride, mice do not show obvious sickness signs but similarly avoid the solution associated with malaise, and *C. elegans* leaves spaces with pathogenic bacteria (Parker, 1988; Welzl et al., 2001; Y. Zhang et al., 2005). What is common to all these behaviours is an avoidance reaction to the stimulus associated with intestinal malaise and/or other post-ingestive effects and thus the necessity for communication between the periphery and the brain.

For instance, while it is so far not known if and how immune signalling in the nervous system directly contributes to sickness-induced food avoidance in mammals, pattern recognition receptors are also present in the mammalian brain, where they were reported to regulate specific behaviours (Arentsen et al., 2017). Due to the survival value of conditioned food aversions across species, it has been suggested that in mammals, this type of learning may initially require relatively simple associative mechanisms in sub-cortical structures of the brain such as the brain stem (Scalera, 2002). Following the integration of taste as well as visceral input at the level of the brain stem, higher brain centres such as the insular cortex and the amygdala are thought to be crucial for immune learning (Pacheco-López et al., 2011; Molero-Chamizo et al., 2017). In particular the insular cortex seems to be required for the acquisition, retention and extinction of CTA learning (Yiannakas et al., 2017). However, due to the complexity of the involved neural circuits and the interactions between the periphery and the brain, it has proven difficult to disentangle the detailed mechanisms underlying acquired aversions to pathogens. While lithium chloride as a common inducer of CTA has been shown to activate the vagus nerve in mammals (Niijima et al., 1994), it is unclear in any organism how pathogens that enter the body via the digestive system signal to the brain to induce behavioural adaptations. Thus, owing to their simpler nervous systems and an abundance of genetic tools, undertaking this challenge in invertebrates such as *Drosophila* is much more promising. *Drosophila* offers the crucial advantage that its nervous system is simple enough to disentangle the means of gut-immune-brain interactions as well as the neural circuits responsible for adapting behaviour in response to pathogens. Given that acquired aversions to contaminated food sources are common defence strategies, it is likely that some of the underlying mechanisms of immune-gut-brain communication are conserved, and that immune signalling directly in the nervous system serves as an efficient response across species to adapt behaviours to intruding pathogens and ensure survival.

5. Conclusion and outlook

When evaluating whether a food source is innocuous or potentially detrimental, animals cannot solely rely on identifying contaminants by means of olfaction or gustation. Pathogens in a food source may easily evade detection by the external sensory system and once ingested, cause intestinal malaise, damage to internal organs or even jeopardise survival. In that case, the upregulation of the immune response is essential to eliminate infectious microbes from the body. Apart from direct antimicrobial strategies, behavioural adaptations help limit the exposure to the pathogen and alleviate the consequences of an infection. In addition, associating the sensory perception of the contaminated food source with the post-ingestive consequences of pathogen uptake allows animals to avoid said food source and thus prevent repeated infections in the future.

The aim of this thesis was to identify neural and immune mechanisms underlying these acquired food aversions following the ingestion of pathogenic bacteria. The data presented here provide evidence how such an interaction between the gut, the immune system and the brain guides adaptive behaviour. Flies that were able to choose between food sources containing harmless or pathogenic bacteria preferred the innocuous food source, yet only after a period of equal feeding, suggesting an acquired aversion to detrimental food. Deficiency for the immune receptor PGRP-LC in octopaminergic neurons abolished this behaviour and rendered flies unable to differentiate between good and bad food. Moreover, this acquired aversion to pathogen-infested food might rely on an associative learning mechanism, as it required the memory centre of the fly brain, the MB, as well as the adenylyl cyclase rutabaga, which mediates synaptic plasticity. PGRP-LC is known to trigger the immune response to Gram-negative bacteria in the fat body and the gut. The necessity for PGRP-LC in the nervous system for a particular behavioural adaptation following the ingestion of pathogens indicates a role for the immune system beyond direct antimicrobial strategies. This study thus provides insights into how immune mechanisms are utilized directly in the central nervous system to adapt behaviours upon exposure to pathogens.

Going forward, it will be crucial to identify the neural circuits guiding this behaviour in particular with respect to the identity of the involved octopaminergic neurons as well as a presumptive connection to the MB. Moreover, determining which regions of the MB as well

as which MB-associated neurons – in particular dopaminergic neurons – are implicated in the observed acquired feeding aversion to pathogenic bacteria would bring us a big step closer to understanding how pathogen-modulated feeding behaviour is regulated in the brain. This could be achieved by further behavioural screens as well as functional studies, once candidate neurons have been established.

In addition, it will be crucial to investigate how the information about pathogen ingestion reaches the brain. However, it will be challenging to narrow down which of the possible scenarios (see 4.6.1) leads to the activation of PGRP-LC signalling in octopaminergic neurons, as there are for example no PGN-tracing methods available to date.

Finally, it is not yet known if the observed feeding aversion to pathogens constitutes a short-term behavioural adaptation or a long-lasting memory. While the conditioned avoidance of pathogens was for instance only transient in *C. elegans* adults, exposure in the larval stage induced a much stronger memory, indicating the formation of an imprinted memory (Y. Zhang et al., 2005; Jin et al., 2016). Similarly infecting *Drosophila* larvae with pathogenic bacteria and investigating their feeding behaviour towards those pathogens as adults could elucidate whether *Drosophila* forms such strong imprinted memories, too.

As learning to avoid detrimental food is a behaviour of great ecological significance in that it ensures the well-being and survival of an animal, ingestion of a detrimental food source commonly induces lasting avoidance. Animals need to constantly evaluate a potential food source and decide whether to take the risk of ingesting it or continue feeding despite potentially harmful consequences. When balancing nutritional needs and the quality of a food source, post-ingestive consequences or memories of food-borne infections can tip the scales to avoidance.

6. References

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7. Appendix

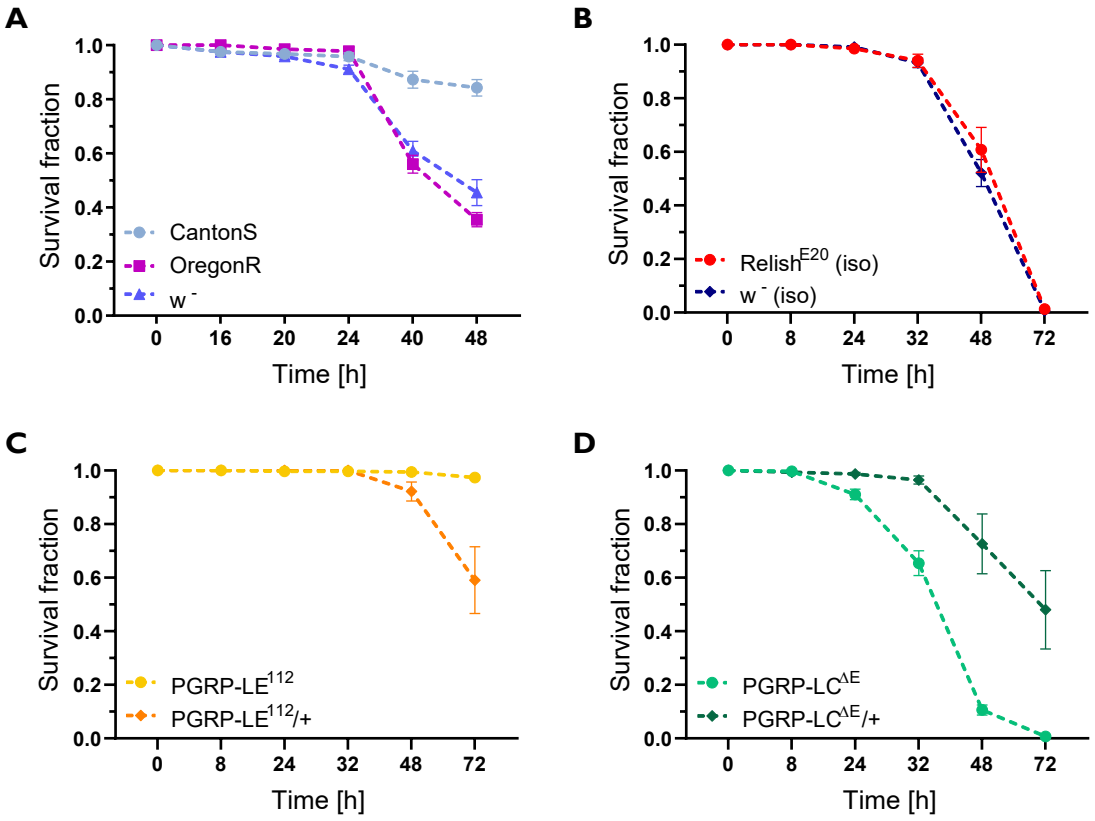

Figure 45 - Survival during wet starvation

Survival fractions of different fly lines on water only. **(A)** Survival of wild-type CS (n = 4), OrR (n = 4) and w $^{-}$ (n = 6) fly strains. **(B)** Survival of Relish^{E20} (isogenized, n = 5) and w $^{-}$ (isogenized, n = 7) flies. **(C)** Survival of PGRP-LE^{112} mutant flies (n = 9) and heterozygous PGRP-LE^{112}/+ control (n = 8). **(D)** Survival of PGRP-LC$^{\Delta E}$ mutant flies (n = 9) and heterozygous PGRP-LC$^{\Delta E}$/+ control (n = 9). 1 n represents one bottle with 100 female flies.

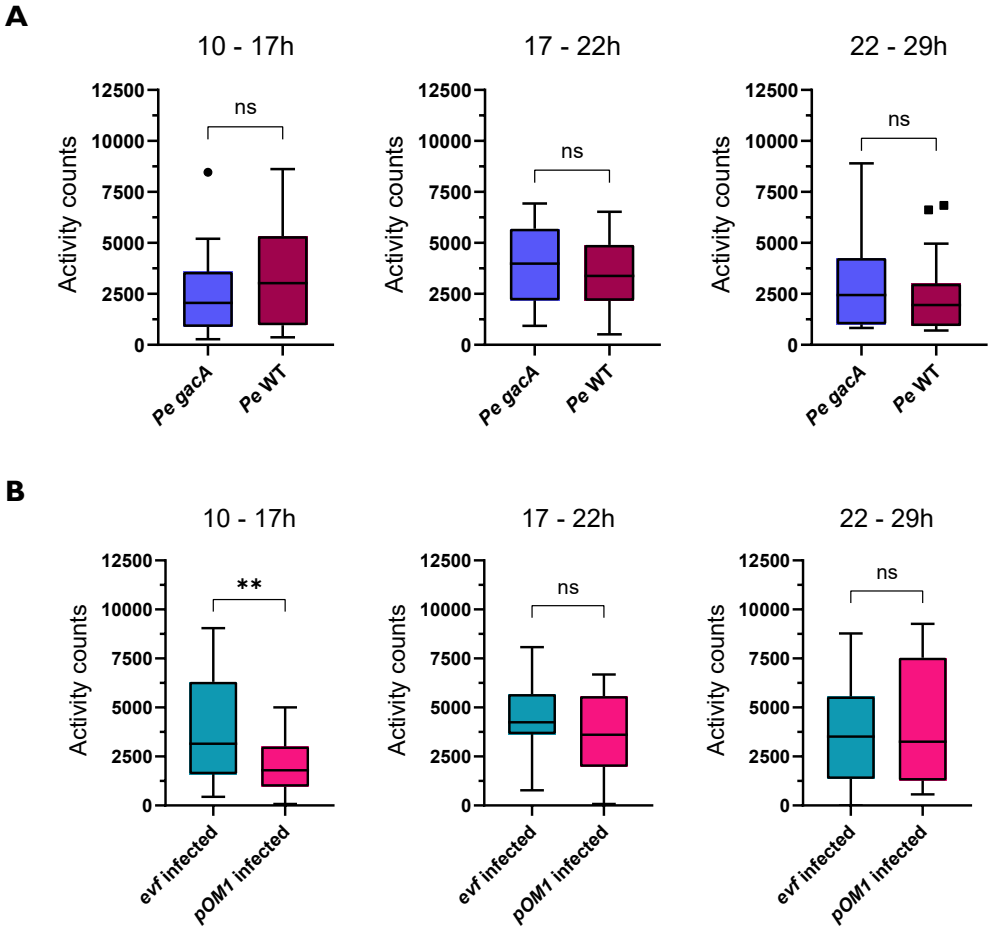

Figure 46 - Locomotor activity during specific time windows after bacteria feeding

(A) Total activity counts of wild-type OrR flies infected with *Pe gacA* or *Pe* WT during three specific time windows post-infection. n = 23 (10-17 h and 17-22 h), n = 23/21 (*gacA*/WT 22-29 h). **(B)** Total activity counts of wild-type OrR flies infected with *Ecc15 evf* or *Ecc15 pOM1* during three specific time windows post-infection. n = 24/22 (*evf*/*pOM1* 10-17 h), n = 24/20 (*evf*/*pOM1* 17-22 h), n = 24/17 (*evf*/*pOM1* 22-29 h). Infected flies were excluded from the time of death onwards. p-values calculated via unpaired t-test with Welch's correction. Figure relates to experiment in Figure 12.

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Publications and talks

Submitted article

Immune receptor signalling and the mushroom body mediate post-ingestion pathogen avoidance

Johanna M. Kobler, Irina Petcu, Ilona C. Grunwald Kadow

Articles in peer-reviewed journals

Revisiting the developmental and cellular role of the pigmentation gene *yellow* in *Drosophila* using a tagged allele

Hélène Hinaux, Katharina Bachem, Margherita Battistara, (…), **Johanna M. Kobler**, Ilona C. Grunwald Kadow, Lisa Rodermund, Benjamin Prud'homme, Nicolas Gompel

Developmental Biology 2018, 438(2), 111-123, doi 10.1016/j.ydbio.2018.04.003

Internal state dependent odor processing and perception - the role of neuromodulation in the fly olfactory system

Sercan Sayin*, Ariane C. Böhm*, **Johanna M. Kobler***, Jean-François De Backer, Ilona C. Grunwald Kadow (*equal contribution)

Frontiers in Cellular Neuroscience 2018, 12(11), doi 10.3389/fncel.2018.00011

Genetically driven brain serotonin deficiency facilitates panic-like escape behavior in mice

Jonas Waider, Sandy Popp, Maren D. Lange, Raphael Kern, Jann F. Kolter, **Johanna M. Kobler**, (…), Angelika Schmitt-Bohrer, Christopher A. Lowry, Hans-Christian Pape, Klaus-Peter Lesch

Translational Psychiatry 2017, 7(10), e1246, doi 10.1038/tp.2017.209

Preview article

Innate Behavior: Flies spring a surprise

Johanna M. Kobler & Ilona C. Grunwald Kadow, eLife 2019, 8, e47720, doi 10.7554/eLife.47720

Conference talks and posters

2020, Mar	Poster presentation at Cold Spring Harbor "Neuronal Circuits" meeting, New York, United States (virtual meeting)
2019, Dec	Talk at ZIEL conference "Nutrition & Microbiome", Weihenstephan, Germany
2019, May	Talk at DFG FOR2705 retreat, Teistungenburg, Germany
2018, Nov	Talk at "State of Olfaction" retreat, Raitenhaslach, Germany
2018, Nov	Talk at the Leibniz Institute for Neurobiology, Magdeburg, Germany
2018, Oct	Talk at ZIEL retreat, Weihenstephan, Germany
2018, Sept	Poster presentation at Neurofly conference, Krakow, Poland
2017, May	Poster presentation at symposium "Neuronal control of appetite, metabolism and weight", Copenhagen, Denmark

Eidesstattliche Versicherung / Affidavit

Hiermit versichere ich an Eides statt, dass ich die vorliegende Dissertation „Neural mechanisms of pathogen-modulated feeding behaviour in *Drosophila*“ selbstständig angefertigt habe, mich außer der angegebenen keiner weiteren Hilfsmittel bedient und alle Erkenntnisse, die aus dem Schrifttum ganz oder annähernd übernommen sind, als solche kenntlich gemacht und nach ihrer Herkunft unter Bezeichnung der Fundstelle einzeln nachgewiesen habe.

I hereby confirm that the dissertation “Neural mechanisms of pathogen-modulated feeding behaviour in *Drosophila*” is the result of my own work and that I have only used sources or materials listed and specified in the dissertation.

München, den 31. März 2020
Munich, 31 March 2020

Johanna Kobler
Unterschrift / *signature*

Declaration of author contributions

I designed and carried out all experiments with the exception of Figure 25.

Irina Petcu and myself designed the *Pseudomonas* feeding choice experiment for the flyPAD. Irina Petcu carried out the *Pe* feeding choice experiment in the flyPAD (Figure 25). Some of these data are also published in her master thesis at the Technical University of Munich "Investigating the Role of a Memory Centre in Pathogen-Modulated Feeding Behaviour in *Drosophila melanogaster*" (2019).

Sophie Aimon carried out additional analyses for the activity monitoring experiments, i.e. the burstiness calculations and the analysis of periodicity (Figure 14).

Author _____

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Signature Ilona C. Grunwald Kadow