ADVANCED REVIEW

WIREs DEVELOPMENTAL BIOLOGY WILEY

Methods for the study of innate immunity in *Drosophila melanogaster*

Katia Troha [ID] | Nicolas Buchon [ID]

Department of Entomology, Cornell Institute of Host-Microbe Interactions and Disease, Cornell University, Ithaca, New York

Correspondence
Katia Troha and Nicolas Buchon, Department of Entomology, Cornell Institute of Host-Microbe Interactions and Disease, Cornell University, Ithaca, NY.
Emails: kas536@cornell.edu (K. T.); nicolas.buchon@cornell.edu (N. B.)

From flies to humans, many components of the innate immune system have been conserved during metazoan evolution. This foundational observation has allowed us to develop *Drosophila melanogaster*, the fruit fly, into a powerful model to study innate immunity in animals. Thanks to an ever-growing arsenal of genetic tools, an easily manipulated genome, and its winning disposition, *Drosophila* is now employed to study not only basic molecular mechanisms of pathogen recognition and immune signaling, but also the nature of physiological responses activated in the host by microbial challenge and how dysregulation of these processes contributes to disease. Here, we present a collection of methods and protocols to challenge the fly with an assortment of microbes, both systemically and orally, and assess its humoral, cellular, and epithelial response to infection. Our review covers techniques for measuring the reaction to microbial infection both qualitatively and quantitatively. Specifically, we describe survival, bacterial load, *BLUD* (a measure of disease tolerance), phagocytosis, melanization, clotting, and ROS production assays, as well as efficient protocols to collect hemolymph and measure immune gene expression. We also offer an updated catalog of online resources and a collection of popular reporter lines and mutants to facilitate research efforts.

This article is categorized under:
    Technologies > Analysis of Cell, Tissue, and Animal Phenotypes

KEYWORDS
disease tolerance, *Drosophila*, gut repair, infection, innate immunity, oral infection, resistance, systemic infection

1 | INTRODUCTION

Over the last 25 years, the fruit fly, *Drosophila melanogaster*, has emerged as one of the leading models to study host–microbe interactions. Thanks, in part, to the high degree of evolutionary conservation between mammalian and fly signaling pathways and organ systems, studies using the *Drosophila* model have shed light not only on the basic molecular mechanisms of pathogen recognition and nuclear factor-κB signaling, but also on the nature of physiological responses activated in the host by infection and how dysregulation of these responses contributes to disease (Buchon, Silverman, & Cherry, 2014; Lemaitre & Hoffmann, 2007; Padmanabha & Baker, 2014). In the wild, *Drosophila* is naturally infected by viruses, bacteria, fungi, and parasites, and in the laboratory, flies can be experimentally infected with both *Drosophila* and human pathogens, making it an attractive model to study infectious diseases (Carpenter, Obbard, Maside, & Jiggins, 2007; Galac & Lazzaro, 2011; Panayidou, Ioannidou, & Apidianakis, 2014). *Drosophila* has played a role in identifying virulence factors of opportunistic human

pathogens (Kim, Park, Heo, & Cho, 2008). Notably, a number of microbes employ similar mechanisms to infect mammals and flies, and several virulence factors necessary for invasion and colonization of mammals are also effective against flies (Alarco et al., 2004; Chamilos et al., 2009; Fauvarque, 2014). The availability of a vast array of genetic tools is arguably the most attractive asset that *Drosophila* has over other model organisms for the study of immunity. This advantage has allowed for the fine manipulation of cells and tissues both spatially and temporally, as well as the generation of innumerable reporter lines that have facilitated the study of immune and immune-related processes in both a qualitative and quantitative manner (Venken & Bellen, 2014). In combination with genomic technologies, these tools have contributed to the advancement of our knowledge of the host response to infection in both flies and mammals.

To combat infection, *Drosophila* relies on both cellular and humoral innate immune responses. The cellular response consists of phagocytosis and encapsulation (Guillou, Troha, Wang, Franc, & Buchon, 2016; Kocks et al., 2005). The humoral response includes the prophenoloxidase cascade, which leads to the generation of reactive oxygen species (ROS) and clotting, and the production of antimicrobial peptides (AMPs) primarily by the fat body, an organ functionally analogous to the liver and adipose tissues of mammals (Boman, Nilsson, & Rasmuson, 1972; Buchon et al., 2014; Tauszig, Jouanguy, Hoffmann, & Imler, 2000). The Toll and Imd pathways are the principal signaling cascades responsible for AMP production (Lemaitre & Hoffmann, 2007). Although *Drosophila* can mount effective immune responses at all stages of development, some responses appear to be stage-specific. For example, proliferation of lamellocytes, large cells that primarily function in encapsulation, upon infection is only observed in the larval stage (Lanot, Zachary, Holder, & Meister, 2001). Immune reactions in *Drosophila* include both systemic and local responses. The hallmark of the systemic response is the inducible synthesis and secretion of AMPs into the hemolymph by the fat body and hemocytes (Lemaitre & Hoffmann, 2007). In contrast, local immune responses take place in barrier epithelia, such as the gut, which is capable of producing tissue-specific AMPs and ROS in response to microbes (Buchon, Broderick, Poidevin, Pradervand, & Lemaitre, 2009; Ha, Oh, Bae, & Lee, 2005). Although both the local and systemic responses include the generation of AMPs, the molecular mechanisms regulating AMP gene expression have been shown to differ between the two (Tzou et al., 2000). These systemic and local immune responses are complemented by other potent defense mechanisms, such as RNA interference (RNAi), which *Drosophila* employs to combat viral infection (X.-H. Wang et al., 2006; Figure 1).

The nature of the immune response in *Drosophila* can be affected by environmental, physiological, and genetic factors; therefore, special attention should be paid to control these experimental variables, whenever possible. For instance, starvation increases susceptibility to infection in both insects and humans, and changes in the ratios of specific dietary components, such as carbohydrates, proteins, and fat, have been implicated in shaping the immune response to infection (Cotter, Simpson,
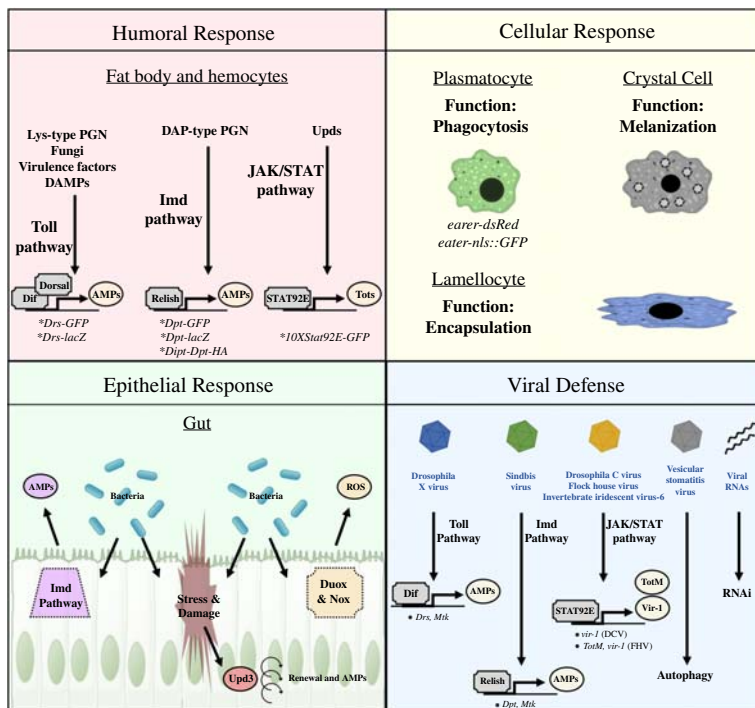

FIGURE 1 The response to infection in *Drosophila melanogaster*. Schematic overview of *Drosophila* host defense. Detection of an array of elicitors triggers a coordinated and synergistic activation of defense modules in the fly. * Denotes reporter lines associated with select pathways. ● Indicates reporter genes linked to individual pathways. Detailed information on these reporter lines and reporter genes can be found in Tables 2 and 3, respectively

Raubenheimer, & Wilson, 2010; Moret & Schmid-Hempel, 2000; Schaible & Kaufmann, 2007). Studies have also found that circadian rhythm mutants have immune phenotypes and that the temperature at which flies are maintained can affect host survival following infection (Linder, Owers, & Promislow, 2008; Stone et al., 2012). Variations in the composition of environmental microbes can have significant effects on host physiology and immune responsiveness; thus, standardization of microbiota is recommended at the beginning of every project (Fast, Duggal, & Foley, 2018; Gould et al., 2018; Rosshart et al., 2017). Basic protocols to homogenize environmental microbes have been described previously (Koyle et al., 2016). Special consideration should also be paid to the presence of viruses and endosymbionts in laboratory stocks, as some of these agents are reported to have significant effects on infection outcome (Palmer, Medd, Beard, & Obbard, 2018; Teixeira, Ferreira, & Ashburner, 2008; Xu & Cherry, 2014). Protocols for the eradication of both are described in subsequent sections of this study. *Drosophila* exhibits marked sexual dimorphism in response to infection; female flies customarily have lower survival rates compared to males after infection (Duneau et al., 2017; Shahrestani et al., 2018). Additionally, mating status has a substantial impact on the outcome of infection, with mated females typically presenting with increased susceptibility and higher pathogen loads compared to virgin females (Schwenke & Lazzaro, 2017; Short & Lazzaro, 2010). As aging flies are generally more susceptible to infection compared to their younger counterparts, age at infection also appears to play a role in the host response (Ramsden, Cheung, & Seroude, 2008). Finally, immune performance varies across genetic backgrounds. A recent study comparing five commonly used reference strains (*Canton^S*, *Oregon^R*, *w^{1118}*, *cinnabar brown*, and *yellow white*) detected sizeable differences upon infection in host survival, bacterial load, expression of antimicrobial peptide genes, number of circulating hemocytes, and levels of phenol-oxidase activity between all five strains (Eleftherianos et al., 2014). Because of potential interactions between mutations and specific genetic backgrounds, it is recommended that mutations of interest be tested in the context of different genetic backgrounds, particularly when the mutant phenotype is small and or subtle.

The host response to infection is also shaped by the type of pathogen used. For example, in flies, the transcriptional response to bacterial challenge differs from that to fungal infection (De Gregorio, Spellman, Rubin, & Lemaitre, 2001; De Gregorio, Spellman, Tzou, Rubin, & Lemaitre, 2002). Recent work has also shown that different bacterial pathogens elicit largely unique gene expression profiles in the host, with differences in the transcriptional magnitude of the host response and the type of peptidoglycan (PGN) found on bacteria (Lys-type vs. DAP-type PGN) predominantly accounting for the transcriptional differences observed (Troha, Im, Revah, Lazzaro, & Buchon, 2018). Selection of suitable microbes is a key consideration in all immune studies. Pathogen virulence, LT50 values (median time required to kill 50% of subjects after exposure to a known concentration of a pathogen), whether the microbe is cleared or enters a chronic infection, and the ability of the pathogen to suppress or avoid the immune response are all important factors to be considered. Lastly, infection route can determine the outcome of infection in flies. For instance, a number of microbes capable of causing mortality via systemic infection display no lethality during oral challenge (Martins, Faria, Teixeira, Magalhães, & Sucena, 2013).

After highlighting these key considerations for the study of immunity, we present below common methodologies to study the immune response in *Drosophila*.

2 | FREQUENTLY USED MICROBES AND IMMUNE ELICITORS

Drosophila is amenable to infection with a large variety of microbes. Both human pathogens and entomopathogenic microbes have been used in the study of immunity (Chakrabarti, Liehl, Buchon, & Lemaitre, 2012; Neyen, Bretscher, Binggeli, & Lemaitre, 2014; Troha et al., 2018). The availability of genetically tractable microorganisms is an important factor in the selection of microbes for immune investigation, as they allow for the parallel study of pathogen virulence factors and host immune genes. Here, we describe a selective group of microbes and immune elicitors commonly used across research groups for the study of *Drosophila* immunity. A more comprehensive list of the pathogens used in the field—including classification, culture conditions, typical dose, and usual route of infection—is provided in Table 1.

2.1 | Bacteria

The type of bacterial PGN is one of the most important factors shaping the host response to microbial challenge. Meso-diaminopimelic acid (DAP)-type PGN exclusively activates the Imd pathway, while lysine-type (Lys)-type PGN specifically induces the Toll pathway. Aside from shedding PGN during the course of infection, microbes also stimulate the immune response via the generation of damage signals. As a result, live infection with bacteria carrying different types of PGN (Lys-type vs. DAP-type PGN) activates both the Toll and Imd pathways but to quantitatively different levels (Lemaitre & Hoffmann, 2007; Troha et al., 2018).

TABLE 1 Frequently used microbes

Bacteria	Gram and peptidoglycan type	Growth conditions	Route of infection	Dose
Escherichia coli	Gram-negative, DAP-type	LB media, 29–37°C	Systemic	$OD_{600} = 100$ for needle pricking
				$OD_{600} = 0.1$ for microinjection
Serratia marcescens	Gram-negative, DAP-type	LB media, 29–37°C	Systemic	$OD_{600} = 1$ for needle pricking
				$OD_{600} = 0.1$ for microinjection
Pectinobacterium carotovora 15 (Ecc15)	Gram-negative, DAP-type	LB media, 29°C	Systemic and oral	$OD_{600} = 1$ for needle pricking
				$OD_{600} = 0.1$ for microinjection
				$OD_{600} = 200$ for oral infection
Salmonella typhimurium	Gram-negative, DAP-type	LB media, 37°C	Systemic	$OD_{600} = 1$ for needle pricking
				$OD_{600} = 0.1$ for microinjection
Providencia rettgeri	Gram-negative, DAP-type	LB media, 29–37°C	Systemic	$OD_{600} = 1$ for needle pricking
				$OD_{600} = 0.1$ for microinjection
Pseudomonas entomophila	Gram-negative, DAP-type	LB media, 29°C	Systemic and oral	$OD_{600} = 1$ for needle pricking
				$OD_{600} = 0.1$ for microinjection
				$OD_{600} = 200$ for oral infection
Micrococcus luteus	Gram-positive, Lys-type	LB media, 29–37°C	Systemic	$OD_{600} = 100$ for needle pricking
				$OD_{600} = 0.1$ for microinjection
Bacillus subtilis	Gram-positive, DAP-type	LB media, 37°C	Systemic	$OD_{600} = 1$ for needle pricking
				$OD_{600} = 0.1$ for microinjection
Listeria monocytogenes	Gram-positive, DAP-type	BHI media, 37°C	Systemic	$OD_{600} = 1$ for needle pricking
				$OD_{600} = 0.1$ for microinjection
Enterococcus faecalis	Gram-positive, Lys-type	LB media, 29-37°C	Systemic	$OD_{600} = 1$ for needle pricking
				$OD_{600} = 0.1$ for microinjection
Staphylococcus aureus	Gram-positive, Lys-type	LB media, 29-37°C	Systemic	$OD_{600} = 1$ for needle pricking
				$OD_{600} = 0.1$ for microinjection
Staphylococcus saprophyticus	Gram-positive, Lys-type	LB media, 37°C	Systemic	$OD_{600} = 1$ for needle pricking
				$OD_{600} = 0.1$ for microinjection
Fungi	**Growth conditions**	**Route of infection**		**Dose**
Candida albicans	YPG media, 29°C	Systemic		$OD_{600} = 200$ for needle pricking
Beauveria bassiana	Malt agar, 25–29°C	Cuticle breach (natural infection)		Roll flies in a sporulating plate for ~15 s
Metarhizium anisopliase	Malt agar, 25–29°C	Cuticle breach (natural infection)		Roll flies in a sporulating plate for ~15 s

Note. An injection volume of ~23 nL is recommended for both thorax and abdomen.

Abbreviations: BHI, brain heart infusion; LB, Luria Bertani; OD, optical density; YPG, yeast peptone glucose.

2.1.1 | DAP-type peptidoglycan bacteria

Providencia rettgeri is an opportunistic pathogen of hospitalized patients and a causative agent of traveler's diarrhea (Sagar, Narasimhaswamy, & D'Souza, 2017; Sharma, Sharma, & Soni, 2017; Yoh et al., 2005). This extracellular, Gram-negative bacterium also infects *Drosophila* in the wild (Corby-Harris et al., 2007; Juneja & Lazzaro, 2009; Galac et al., 2011). *P. rettgeri* activates a robust immune response in flies, as indicated by strong induction of the antimicrobial peptide genes *Diptericin* and *Drosomycin*. Systemic infection with *P. rettgeri* causes ~50% mortality in wildtype flies; half of the subjects die with a high pathogen burden, while the other 50% survive indefinitely with an asymptomatic, low-burden, chronic infection (Duneau et al., 2017; Galac & Lazzaro, 2011; Troha et al., 2018). This feature of *P. rettgeri* infection provides two advantages. First, the intermediate mortality allows researchers to observe not only host and bacterial mutations that increase susceptibility to infection, but also those that increase host survival. Second, as it establishes a persistent infection in surviving hosts, *P. rettgeri* can also be used to study the biology of chronic infection. The transcriptional profile of adult wildtype flies infected

with *P. rettgeri* has been published (Short & Lazzaro, 2013; Troha et al., 2018). Additionally, the *P. rettgeri* genome is fully sequenced, and the data is publicly available (Galac & Lazzaro, 2012; Marquez-Ortiz et al., 2017). *P. rettgeri* is grown as a shaking culture in Luria Bertani (LB) medium at 29°C. For faster growth, the bacteria can also be cultured at 37°C. To infect flies, an overnight culture (~16 hr) of 10 mL is pelleted by centrifugation (4 min at 1700*g*, 4°C) and resuspended in sterile phosphate buffered saline (PBS) buffer to reach an $OD_{600} = 1$ for the needle-prick method or an $OD_{600} = 0.1$ for the injector protocol (infection techniques are described in detail in subsequent sections of this study). Both approaches result in inoculation with ~3,000 CFU (colony forming units). Fresh cultures should be used for infection whenever possible, as they tend to give more consistent results; however, unpelleted cultures can be kept at 4°C and used for up to a week if necessary. *P. rettgeri* LB plates can be stored at 4°C for a month to inoculate liquid cultures. This bacterium is naturally resistant to the antibiotic tetracycline (10 μg/mL), which can aid to restrict the growth of other bacteria when plating fly homogenates.

Pectinobacterium (previously known as *Erwinia*) *carotovora carotovora* is a Gram-negative, extracellular bacterium employed in the study of both systemic and gut immunity in *Drosophila*. Naturally transmitted by insects, this phytopathogenic organism causes soft rot in fruits (Barras, van Gijsegem, & Chatterjee, 1994). Strain 15 (*Ecc15*) is genetically tractable and resistant to rifampicin (100 μg/mL). A spectinomycin-resistant green fluorescent protein (GFP)-transformed strain (25 μg/mL) is available to track the location of bacteria inside the host (Basset et al., 2000). *Ecc15* is also a strong inducer of the immune response (Buchon, Broderick, Poidevin, et al., 2009; Buchon et al., 2013; Troha et al., 2018). Unlike *P. rettgeri*, systemic infection with *Ecc15* causes little mortality (~15%) in healthy wildtype flies, which mostly clear the infection after ~5.5 days (Troha et al., 2018). This feature makes *Ecc15* a good choice for a screen of immune-deficient mutant flies. *Ecc15* is grown in LB broth as a shaking culture at 29°C; it will not grow at 37°C. For systemic infection, set up a 10 mL culture following the protocol outlined above for *P. rettgeri*. To orally infect flies, pellet an overnight culture (~16 hr) of 500 mL by centrifugation (10 min at 2400*g*, 4°C) and resuspend the pellet in sterile PBS to reach an $OD_{600} = 200$. Oral infection with *Ecc15* should cause little to no mortality in wildtype flies (Buchon, Broderick, Chakrabarti, & Lemaitre, 2009). The transcriptional profiles of flies infected systemically and orally with *Ecc15* are available online (Buchon et al., 2013; Troha et al., 2018).

For readers especially interested in gut infection models, two other Gram-negative bacteria commonly used in the field are *Pseudomonas entomophila* and *Serratia marcescens*. Ingestion of *P. entomophila* induces irreversible damage in the *Drosophila* gut, killing a majority of hosts. This infection is known to trigger a global translational blockage that impairs both immune and repair mechanisms in the fly gut (Chakrabarti et al., 2012; Dutta et al., 2015; Liehl, Blight, Vodovar, Boccard, & Lemaitre, 2006; Vodovar et al., 2005). *S. marcescens* Db11 strain is an entomopathogenic bacterium that opportunistically infects a wide range of hosts, including humans. Following oral infection, this bacterium crosses the gut barrier, resulting in a systemic infection. In this model, flies succumb to infection after only ~6 days (K.-Z. Lee et al., 2016; Nehme et al., 2007).

In terms of intracellular models, *Listeria monocytogenes* is capable of establishing lethal infections in the fruit fly. Although classified as a Gram-positive bacterium, *L. monocytogenes* carries DAP-type PGN. Upon infection, this microbe can be found in the cytosol of phagocytic cells. Similar to mammals, *L. monocytogenes* is able to direct host cell actin polymerization in order to spread to other cells (S2 cells in vitro) (Mansfield, Dionne, Schneider, & Freitag, 2003). This bacterium should be of interest to readers drawn to the study of autophagy, as it has been demonstrated that cytosolic recognition of *L. monocytogenes* by the pattern-recognition receptor PGRP-LE induces autophagy, which is important to inhibit intracellular growth of this bacterium (Yano et al., 2008). *Salmonella typhimurium* and *Francisella tularensis* infections have also been characterized in *Drosophila*. Both bacteria are pathogenic to flies (Ayres & Schneider, 2009; Vonkavaara, Telepnev, Rydén, Sjöstedt, & Stöven, 2008).

2.1.2 | Lys-type peptidoglycan bacteria

Enterococcus faecalis is a nosocomial, opportunistic human pathogen that also infects flies in the wild (Huycke, Spiegel, & Gilmore, 1991; Lazzaro, Sackton, & Clark, 2006). This extracellular, Gram-positive bacterium is a strong inducer of the Toll but not the Imd pathway during systemic challenge. Like *P. rettgeri*, this microbe causes an intermediate mortality (~60%) and leads to a persistent infection in surviving hosts (Troha et al., 2018). An advantage of *E. faecalis* is that transposon insertion mutant libraries are available for screening (Gao et al., 2013; Rigottier-Gois et al., 2011). The transcriptional profile of flies infected with *E. faecalis* is publicly available, and the bacterium's genome is fully sequenced (Brede, Snipen, Ussery, Nederbragt, & Nes, 2011; Troha et al., 2018).

One of the most classically used bacteria for the study of innate immunity in the fly is *Micrococcus luteus* (De Gregorio et al., 2001, 2002). Typically found in water, soil, and as part of the normal flora of human skin, this Gram-positive, extracellular bacterium is easily recognizable because of the small size and bright yellow color of the colonies it forms (Kloos &

Musselwhite, 1975; Tuleva et al., 2009). *M. luteus* is a strong inducer of the Toll pathway, and it causes almost no lethality (<5%) in flies. Some strains of this bacterium can be cleared by the fly in as little as ~6 hr (Troha et al., 2018).

Infections with a few intracellular, Gram-positive bacteria have been characterized in the fly. For example, the bacterium *Staphylococcus aureus* is pathogenic to flies and a particularly good choice to probe the role of melanization and phagocytosis during infection (this bacterium is almost exclusively cleared by phagocytosis and also tends to induce larger melanization spots) (Atilano, Yates, Glittenberg, Filipe, & Ligoxygakis, 2011; Ayres & Schneider, 2008; Binggeli et al., 2014; Chung & Kocks, 2011; Kocks et al., 2005; Nehme et al., 2011; Shiratsuchi et al., 2012).

2.2 | Viruses

Several viruses have been used to study antiviral immunity in *Drosophila*. Some are natural pathogens that infect *Drosophila* in the wild, such as *Drosophila* C virus ((+) single-stranded RNA) and Sigma virus ((−) single-stranded RNA), while others, like Invertebrate iridescent virus-6 (double-stranded DNA), are viruses originally identified in other insects. Several viruses trigger classical immune pathways in the fly. For example, *Drosophila* X virus induces the Toll pathway, while Sindbis virus stimulates the Imd pathway. Similarly, both *Drosophila* C virus (DCV) and Invertebrate iridescent virus-6 activate the JAK/STAT pathway (Avadhanula, Weasner, Hardy, Kumar, & Hardy, 2009; Dostert et al., 2005; Zambon, Nandakumar, Vakharia, & Wu, 2005; West & Silverman, 2018). DCV is reported to propagate in various tissues, including the fat body and gut, and it has been implied that Sigma virus infects the glial cells, which may account for the CO_2 sensitivity observed in these flies (Bussereau, 1970a, 1970b; Chow, Márka, Bartos, Márka, & Kagan, 2017). Sigma virus, Invertebrate iridescent virus-6, and DCV are capable of replication in Schneider 2 (S2) cells. Specific protocols to propagate and isolate these viruses have been described before (Merkling & van Rij, 2015). A comprehensive list of published *Drosophila* viruses can be found in the Obbard lab's website (http://obbard.bio.ed.ac.uk/data.html; Obbard, 2018).

2.3 | Fungi and yeasts

Beauveria bassiana and *Metarhizium anisopliae* are two naturally occurring entomopathogenic fungi used to probe the response to fungal infection in *Drosophila* (De Gregorio et al., 2001; Lu, Wang, Brown, Euerle, & St Leger, 2015). When fungal spores come into contact with the body of a fly, they germinate, penetrate the cuticle, and grow inside, killing the host in a matter of days. *B. bassiana* grows on malt agar plates at 25–29°C, preferentially in the dark. Culture plates should be kept dry at all times. If condensation droplets form on the lids of culture plates, either replace the lid with a new, dry lid or wipe the lid dry with a clean paper towel. To induce sporulation, switch culture plates to 29°C; this process may take up to a month. To check if a plate is sporulating, gently tap the plate while holding it upside down. A sporulating plate will release dust-like spores, which should now be visible on the lid of the plate. Culture plates can be stored for up to a month at 18°C. To propagate cultures, flip sporulating plates onto new malt agar plates (Neyen et al., 2014).

Candida albicans, a human pathogen, has also been used to infect *Drosophila* (Davis et al., 2011). A strong inducer of the Toll pathway, this yeast causes mild mortality in adult flies when administered systemically. For readers interested in studying a behavior-manipulating pathogen, the Eisen lab recently discovered a strain of *Entomophthora muscae* capable of such a feat. *E. muscae* is known to invade the nervous system, and flies infected with this fungus display unique behaviors. Hours before their death, the flies climb upward and extend their proboscides, affixing in place. This is followed by raising of their wings, which clears a path for infectious fungal spores to launch from their abdomens (Elya et al., 2018).

2.4 | Parasitoid wasps

Parasitoid wasps infect up to 70% of *Drosophila* larvae in the wild (Fleury et al., 2004). These wasps inject eggs into the bodies of fly larvae, which, upon hatching, slowly kill their hosts by feeding on their internal tissues. Some parasitoid wasps, such as *Leptopilina boulardi*, are specific to *Drosophila*, while others, like *Leptopilina heterotoma*, are generalists (Schlenke, Morales, Govind, & Clark, 2007). Wasps can be propagated in the laboratory using larvae of the permissive *Drosophila yellow white* genotype (grown at 24°C). On an average, new wasps eclose after 25–30 days. Adult wasps can be maintained on a honey diet. For detailed methods on wasp rearing, we refer the reader to the protocols developed by the Govind lab (Small, Paddibhatla, Rajwani, & Govind, 2012).

2.5 | Immune elicitors

Infection with live microbes produces a complex host response, part reaction to the presence of microbe-derived immune elicitors, such as PGN, and part response to microbial growth and virulence (Newton & Dixit, 2012). To separate the response to microbe-associated molecular patterns (MAMPs) from that to live pathogens, purified elicitors or heat-killed microbes can be used. Commonly used elicitors include: DAP-type PGN, TCT (monomeric PGN fragment capable of inducing the Imd pathway), Lys-type PGN, β-glucan, and proteases. Purified elicitors are available for purchase from Sigma or InvivoGen. It is important to note that LPS itself does not induce the Imd pathway (which detects the presence of Gram-negative bacteria) in *Drosophila* (Leulier et al., 2003). However, because commercial preparations of LPS are oftentimes contaminated with trace amounts of PGN, LPS has been recorded to induce immune activation on occasion (Kaneko et al., 2004). To heat-kill bacteria for injection, prepare a fresh bacterial solution, adjust it to the desired OD_{600}, and heat the bacteria to 70°C for 1 hr. To confirm that the bacteria are dead, plate 10 μL of this solution on the proper nutrient agar plate and incubate the plate overnight. If successful, no colonies should grow. To kill fungal spores, collect dust-like spores and incubate them in a 1.5 M NaOH solution for 30 min twice at 70°C. Immediately follow by washing the spores four times with PBS 0.01% Tween 20 (Gottar et al., 2006). A fraction of the spores should also be plated on nutrient agar plates to confirm the efficiency of the alkali treatment.

3 | ROUTES OF INFECTION

3.1 | Systemic infection

3.1.1 | Needle pricking

In this method, a 0.1-mm stainless steel needle (Fine Science Tools, Catalog #26002-10) is dipped into a concentrated bacterial, viral, or fungal spore solution and used to prick previously anesthetized adult flies in the thorax or abdomen (Figure 2a,b). Inoculation into either site results in systemic infection in the fly. Historically, thorax inoculation has been the preferred method to introduce microbes into the insect body cavity. However, compared to abdomen inoculation, inoculation via the thorax leads to higher mortality and bacterial load within the first few days of infection (Chambers, Jacobson, Khalil, & Lazzaro, 2014). Fly pricking should be gentle; it should breach the cuticle with minimal damage to internal tissues/organs, and the needle must never pierce through the entire fly. Typically, flies recover from this procedure within a few minutes. Infected flies should be laid down on the side of a new media tube while they recover from anesthesia to prevent them from sticking to the food. A dark spot, corresponding to the activation of the melanization cascade, will appear at the site of injury in a matter of hours. The presence of this spot can be used to ensure that the flies have been infected. To avoid scoring lethally injured flies (i.e., flies dying from lethal wounding as opposed to infection), dead subjects should be counted ~2 hr after inoculation and excluded from further analysis (Khalil, Jacobson, Chambers, & Lazzaro, 2015). Needle pricking is a fast and efficient method that allows for large numbers of flies to be tested in a short period of time (>250 adult flies can be infected per hour). While dosage is a little more variable with this method, it is surprisingly consistent. Following the procedure, subjects should be transferred to the appropriate temperature for experimentation.

Systemic infection of *Drosophila* larvae via the pricking method is associated with several technical difficulties, including high rates of premature death stemming from excessive loss of hemolymph and or tissue injury. Recently, Kenmoku, Hori, Kuraishi, & Kurata (2017) published a protocol that remedies these issues. Using a specific type of needle, they showed that >80% of larvae can survive sterile injury, making this method a viable approach to infect larvae. To infect larvae, wash larvae in sterile PBS buffer and place them in a small drop of PBS on a pre-chilled rubber pad (the cold temperature helps to immobilize them). Prick larvae on their posterior lateral side using a very fine tungsten needle (Kenmoku et al., 2017). Then transfer larvae to petri dishes containing apple juice agar or normal fly medium (Neyen et al., 2014).

3.1.2 | Microinjection

When inoculation with exact doses of microbes or immune elicitors is required, injection of a defined volume using a microinjector (Drummond, Catalog #3-000-204) is the preferred method (Figure 2c). An injection volume of ~23 nL is recommended for both thorax and abdomen injections (Khalil et al., 2015). Disadvantages of this method include slower speed compared to needle pricking and heavy injector equipment.
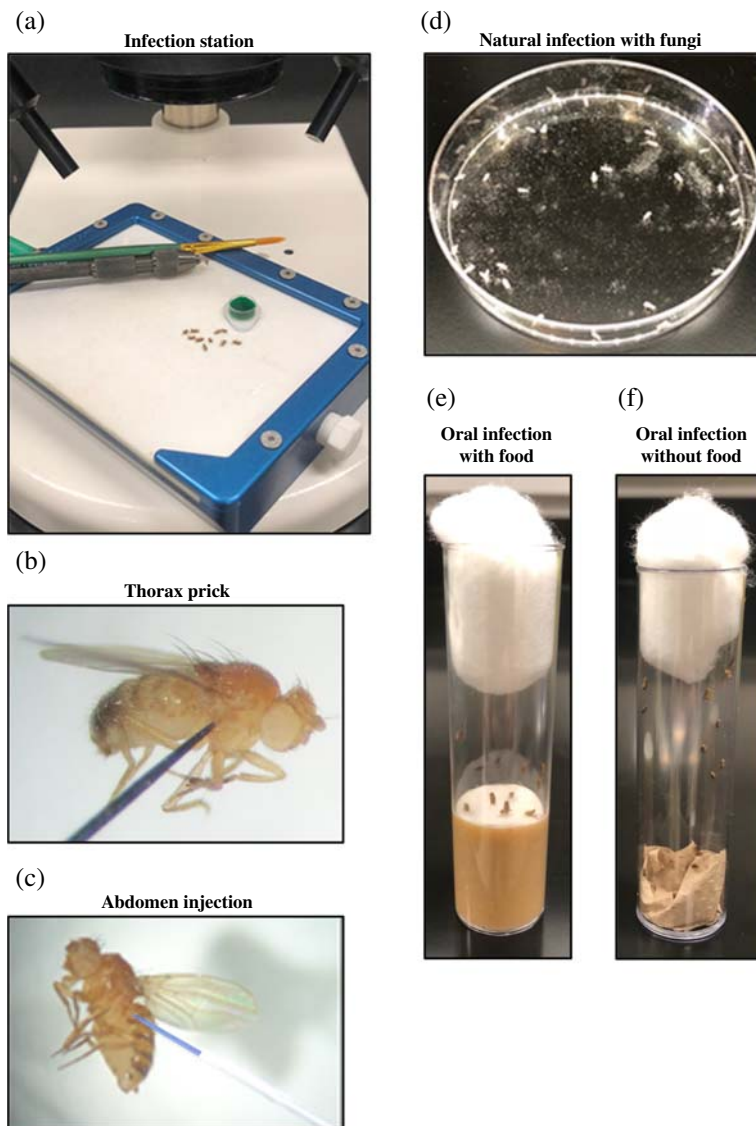
FIGURE 2 *Drosophila* infection techniques.
(a) Infection station setup. (b) Anesthetized fly pricked in the thorax with a stainless steel needle. (c) Fly injected in the abdomen with a defined volume using a microinjector. (d) Flies coated in fungal spores during natural infection. Two methods are available for oral infection. (e) In the first, previously starved flies are orally infected using a precut Whatman Filter Paper disk soaked in a bacterial solution. The paper disk sits atop food in a fly vial. This placement ensures that the flies receive the inoculum as well as nutrients, which are absorbed through the filter. (f) The second protocol places flies in a vial devoid of food, where they feed on a small piece of a Tork napkin that has been soaked in a bacterial solution. The availability of a nutrient source beyond the bacterial solution distinguishes the two methods

3.1.3 | Natural infection with fungi

While injection of fungal spores is a reliable method to challenge flies, natural infection is the preferred method to achieve systemic infection with fungi. In this method, CO_2-anaestethized flies are placed directly on the sporulating lawn of a fungal culture plate, and the plate is shaken for ~15 s to coat the flies in spores (Figure 2d). Flies are then transferred to a new, clean food vial to recover. Larvae can also be rolled on sporulating plates for infection. Flies and larvae will become infected as the spores germinate and breach the cuticle. A sporulating plate can be reused for multiple infections (Tzou, Meister, & Lemaitre, 2002).

3.1.4 | Sexual transmission

Although not commonly used, sexual transmission is a viable method to establish systemic infection in the fly. Briefly, male flies are anesthetized and laid ventral side up on the fly pad. Next, a pipette is used to place a large drop of a bacterial solution directly on the male genitalia, covering it fully. After treatment, each male is placed in a sterile food vial with a single virgin female to recover and mate. This arrangement leaves little, if no time for males to groom themselves before mating (Gendrin, Welchman, Poidevin, Hervé, & Lemaitre, 2009; Miest & Bloch-Qazi, 2008).

3.2 | Oral infection

Three model pathogens are commonly used to study the response to oral infection in the fly gut: *Ecc15*, *P. entomophila*, and *S. marcescens* Db11 (a description of these pathogens is provided in the section dealing with bacteria). To orally infect flies, 5- to 8-day-old adult flies are starved in empty vials for 2 hr at 29°C. This starvation treatment ensures that all flies are receptive to consuming a large amount of the oral inoculum. Next, the flies are transferred to infection vials and returned to 29°C, the optimal infection temperature for traditional *Drosophila* gut pathogens. Flies are left to feed on the infection vials for 4–12 hr, after which they are transferred to fresh food vials (Figure 2e,f). Infection vials should be prepared fresh while the flies undergo starvation. To prepare an infection vial, take a standard food vial and cover the media fully using a precut Whatman Filter Paper disk. No traces of food should be visible above the paper disk. This guarantees that the flies will only feed on the bacterial solution provided. Next, pipette 150 μL of either the control solution (5% sucrose) or the control solution mixed in a 1:1 ratio with the concentrated bacterial suspension (combine 75 μL of 5% sucrose with 75 μL of concentrated bacteria at $OD_{600} = 200$) onto the paper disk, making sure that the disk is completely and evenly covered by this mixture. Let the vials settle for ~15 min to allow for the entire solution to be absorbed into the filter disk before starting the infections. It is important that the surface of the media tubes used for infection be somewhat dry. If the vials are damp, the paper disks will become saturated before the addition of the infection solution. As a result, the inoculum will not be absorbed, and the flies will drown in these wet vials. This protocol can also be used to introduce viruses and fungi into the fly gut (Figure 2e). An alternative infection method places the flies in vials containing only a pile of folded papers (Tork) or cotton balls soaked in 2.5 mL of a concentrated bacterial solution (Figure 2f; Houtz & Buchon, 2014; Nehme et al., 2007). Unlike the former, this latter method does not provide access to nutrients (from the regular food source), thereby altering host physiology and susceptibility to infection.

To orally infect larvae, place larvae in an Eppendorf tube containing 400 μL of crushed banana mixed with 200 μL of control solution (×1 PBS, sterile) or with 200 μL of a concentrated bacterial suspension (the OD_{600} used should be ×3 the final desired OD_{600}, e.g., use 200 μL of an $OD_{600} = 300$ solution to achieve a terminal $OD_{600} = 100$ for infection). After adding the larvae, cap the tube with a foam plug and gently flick it to mix the contents. Let larvae feed on the bacteria for ~30 min at 29°C, then transfer them to fresh food vials by inverting the contents of the Eppendorf into the new vial. Optional: Ingestion of bacteria can be stopped immediately by washing infected larvae with ×1 PBS, but the extra handling might kill additional larvae (Neyen et al., 2014).

3.3 | Wasp infection

For wasp infection, place 6 female wasps in a 35-mm petri dish filled with 2 mL of fly food and ~50 fly larvae for 2 hr. Parasitized larvae are kept at 25°C. Capsules can be dissected 4–6 days after infection (Small et al., 2012).

3.4 | Generation of germ-free flies

Although flies feed on microbes and live in microbe-rich settings, the *Drosophila* gut lumen is an environment with relatively low bacterial diversity and bacterial numbers. Typically, only ~100 CFU/fly are found, with *Acetobacter* and *Lactobacillus* spp. comprising the majority of the associated species (Blum, Fischer, Miles, & Handelsman, 2013; Wong, Ng, & Douglas, 2011). The production of germ-free flies has allowed for the study of the effects of gut microbes on fly physiology.

Generation of germ-free flies begins with the collection of large numbers of fly eggs. A sizable number is required because many eggs will not survive the chemical treatment applied. Collected eggs (exclude larvae) are placed in a mesh sieve (Genesee Scientific, Catalog #46-102 for the mesh basket and FlyStuff.com, Catalog #57-102 for the 120 μm nitex nylon mesh). To surface sterilize the eggs, dip the mesh basket in a beaker of 70% ethanol for 2 min. Next, immerse the mesh basket in a separate beaker containing a 10% bleach solution for 10 min (longer treatments up to 30 min are possible, but they will result in increased mortality). The bleach treatment serves to dechorionate the eggs. This is followed by rinsing the eggs in sterile water three times to remove any residual bleach (dip the mesh basket in three separate beakers containing H_2O). Using a sterile pipette tip, transfer the eggs in a small amount of 70% ethanol to pre-autoclaved media vials (vial and food are sterile) and allow them to develop (Broderick, Buchon, & Lemaitre, 2014; Koyle et al., 2016). This procedure should be performed using sterile technique in a laminar flow hood. Bacterial sterilization can be confirmed by plating a homogenate of the resulting flies on De Man, Rogosa and Sharpe (MRS) media. Alternatively, germ-free status can be corroborated by performing 16S PCR on the flies using these universal primers: 5′-AGAGTTTGATCCTGGCTCAG-3′ (27F) and 5′-GGTTACCTTGTTACGACTT-3′ (1492R) and this PCR protocol: (a) 94°C for 10 min; (b) 30 cycles of 94°C for 1 min, 54°C

for 1 min, and 72°C for 2 min; and (c) 72°C for 5 min (Ren, Webster, Finkel, & Tower, 2007). Of note, some groups have reported using antibiotics (cocktail of kanamycin, ampicillin, rifampicin, streptomycin, and spectinomycin at 5 mg/mL each) in the food as a way to render flies germ-free. While an antibiotic cocktail will likely reduce the total number of microbiota present, it may not fully eliminate all microbes.

To clear *Drosophila* lines of the endosymbiont Wolbachia, flies are treated with the antibiotic tetracycline (0.05 mg/mL) for two generations, as previously described (Teixeira et al., 2008). Additionally, several viral infections can be eliminated using the protocols referenced here (Brun & Plus, 1980; Teixeira et al., 2008).

4 | TOOLS AND ASSAYS TO EVALUATE THE RESPONSE TO INFECTION

Over the past decade, it has become increasingly evident that a successful response to infection requires a combinatorial approach, involving both resistance mechanisms, which target the pathogen for elimination, and tolerance responses, which induce tissue-protective programs (Ayres & Schneider, 2012). Disease tolerance mechanisms involve many different biological processes, from metabolic adaptation and stress responses to tissue repair (Soares, Gozzelino, & Weis, 2014; Troha et al., 2018; Weis et al., 2017). In light of this, this section outlines methods to score both resistance and tolerance responses to infection.

4.1 | Tools

4.1.1 | Frequently used *Drosophila* lines

Commonly used fly stocks can be requested directly from laboratories or purchased from the principal *Drosophila* stock centers: Bloomington *Drosophila* Stock Center (https://bdsc.indiana.edu), Vienna *Drosophila* Resource Center (https:// stockcenter.vdrc.at), Kyoto Stock Center (https://kyotofly.kit.jp), and The Exelixis Collection at the Harvard Medical School (https://drosophila.med.harvard.edu).

Toll and Imd are the canonical immune signaling pathways in *Drosophila*, responsible for the production of AMPs (De Gregorio et al., 2002; Lemaitre & Hoffmann, 2007). Several mutants, RNAi lines, and overexpression stocks of key component genes of both pathways are publicly available (see Table 2). Among many nonimmune functions, Toll signaling plays a critical role during embryonic development; therefore, many Toll-deficient mutants exhibit some degree of lethality during the embryonic stage (Anderson, Bokla, & Nüsslein-Volhard, 1985). Nevertheless, viable mutants, such as spz^{rm7}, are amenable to experimentation (Michel et al., 2001). To activate the pathway in the absence of infection, recent studies have favored the use of gain-of-function mutations, such as $UAS\text{-}Toll^{10B}$ or *UAS-spz** (Buchon, Poidevin, Kwon, et al., 2009; DiAngelo et al., 2009). The current model of Toll pathway activation has three distinct upstream signaling cascades controlling the activation of Spz. Two of these cascades are initiated upon detection of Lys-type PGN by PGRP-SA and β-glucan by GNBP3, leading to the activation of ModSP, a modular serine protease that integrates signals from these sensors to activate Spz (Buchon, Broderick, Chakrabarti, & Lemaitre, 2009; Buchon, Broderick, Poidevin, et al., 2009; Buchon, Poidevin, Kwon, et al., 2009). A third cascade is mediated by the protease Psh, which senses virulence factors as well as damage-associated molecular patterns (Valanne, Wang, & Rämet, 2011). Mutants of all these genes are available, allowing for genetic dissection of the three branches controlling Toll activation. For the Imd pathway, the mutant Rel^{E20} exhibits complete abrogation of antimicrobial peptide induction, making it one of the best options available (Hedengren et al., 1999). Other mutants, such as the hypomorph imd^1, are suitable alternatives (Lemaitre et al., 1995). Induction of the Imd pathway can be achieved by overexpression of *imd*. However, there is at least one caveat associated with this approach; overexpression of *imd* in the fat body will not only activate the Imd pathway but also induce apoptosis (Georgel et al., 2001). Alternatively, the Imd pathway can also be activated via overexpression of the Imd receptors PGRP-LC and PGRP-LE (Gottar et al., 2002; Takehana et al., 2002).

Plasmatocytes are the primary phagocytic cells in *Drosophila*, and it is possible to generate viable flies that lack all phagocytes, named phagoless (Defaye et al., 2009). To generate these flies, *Hml-Gal4*, a hemocyte-specific driver, is crossed to *UAS-Bax*, which encodes a pro-apoptotic factor. Additionally, many mutants that affect phagocytosis, such as the CD36 homolog *crq*, have been described before (Guillou et al., 2016). To study melanization, a *PPO1*, *PPO2* double mutant, which lacks complete PO activity in the hemolymph, is available (Binggeli et al., 2014). In addition to the mutants described here, RNAi lines present a suitable alternative to target these genes. All mutants, overexpression, and RNAi lines mentioned in this section are listed in Table 2.

TABLE 2 List of frequently used *Drosophila* lines

Mutant	Line description	Recently used by
PGRP-SA^{seml}	Mutant (Toll pathway)	Michel, Reichhart, Hoffmann, and Royet (2001)
GNBP3^{Hades}	Mutant (Toll pathway)	Matskevich et al. (2010)
modSP^1	Mutant (Toll pathway)	Buchon, Broderick, Chakrabarti, et al. (2009); Buchon, Broderick, Poidevin, et al. (2009); Buchon, Poidevin, Kwon, et al. (2009)
psh^1	Mutant (Toll pathway)	Gottar et al. (2006)
spz^{rm7}	Mutant (Toll pathway)	Buchon, Broderick, Chakrabarti, et al. (2009); Buchon, Broderick, Poidevin, et al. (2009); Buchon, Poidevin, Kwon, et al. (2009)
UAS-Toll^{10B}	Expresses a constitutively active form of Toll	DiAngelo, Bland, Bambina, Cherry, and Birnbaum (2009)
Drs-GFP	Expresses *GFP* under the control of the *Drs* promoter. Toll pathway reporter	Wu et al. (2015)
Drs-lacZ	Expresses *lacZ* under the control of the *Drs* promoter. Toll pathway reporter	Foley et al. (2003)
Rel^{E20}	Mutant (Imd pathway)	Guillou et al. (2016)
imd^1	Mutant (Imd pathway)	Zerofsky et al. (2005)
UAS-imd	Overexpresses *imd*	Georgel et al. (2001)
Dpt-GFP	Expresses *GFP* under the control of the *Dpt* promoter. Imd pathway reporter	Flatt et al. (2008)
Dpt-lacZ	Expresses *lacZ* under the control of the *Dpt* promoter. Imd pathway reporter	Chakrabarti et al. (2012)
Dipt-Dpt-HA	Carries the *Dpt* CDS + HA tag under the control of the *Dpt* promoter. Imd pathway reporter	Chakrabarti et al. (2012)
10XStat92-GFP	Expresses *GFP* under the control of the *Stat92E* promoter. JAK/STAT pathway reporter	Bach et al. (2007)
PPO1$^{\Delta}$, PPO2$^{\Delta}$	Double mutant (melanization)	Binggeli, Neyen, Poidevin, and Lemaitre (2014)
crq^{ko}	Mutant (phagocytosis)	Guillou et al. (2016)
eater-dsRed	Plasmatocyte marker used to visualize hemocytes	Guillou et al. (2016)
eater-nls::GFP	Plasmatocyte marker used to visualize hemocytes	Guillou et al. (2016)
Hml-Gal4	Hemocyte driver	Guillou et al. (2016)
UAS-BAX	Induces apoptosis. Used in combination with *Hml-Gal4* to generate phagoless flies	Regan et al. (2013)

4.1.2 | Reporter lines

One of the biggest advantages of working with *Drosophila* is the availability of large numbers of reporter lines that allow for quick examination of various phenotypes. These reporters place immune-inducible promoter regions upstream of fluorescent proteins, such as GFP, or reporter enzymes, such as lacZ, and they provide a fast alternative to measurements of gene expression. In general, GFP reporters are best suited for qualitative analysis, while lacZ reporters are recommended for quantitative evaluations; lacZ encodes β-galactosidase, an enzyme whose activity can be measured (as described later in this section).

Fluorescent reporters, such as *Drs-GFP* (Toll pathway reporter) or *Dpt-GFP* (measures Imd pathway activity), can be used to assess antimicrobial peptide gene expression in the fly (Ferrandon et al., 1998; Vodovar et al., 2005). Similarly, in the gut, the reporters *10XStat92-GFP* and *esg$^{F/O}$* (*esg-Gal4, Gal80^{ts}, UAS-FLP, act > CD2 > Gal4, UAS-GFP*) can be used to track midgut renewal following enteric infection (Figure 3a; Buchon, Broderick, Chakrabarti, et al., 2009; Buchon, Broderick, Kuraishi, & Lemaitre, 2010; Houtz & Buchon, 2014). *10XStat92-GFP* is a reliable indicator of JAK/STAT activity, which is required for gut renewal, and *esg$^{F/O}$* is a tracing tool to monitor new epithelial cells generated during tissue repair. Flies or larvae carrying these reporters can be scored visually under a fluorescence dissecting microscope, where not only the presence
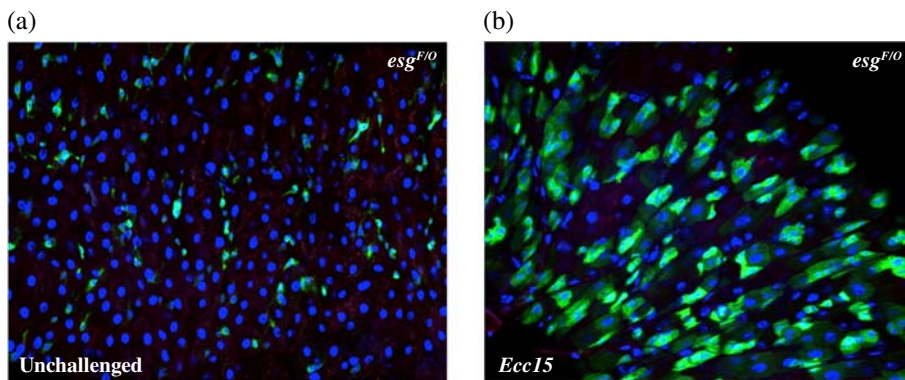
(a)

(b)

FIGURE 3 Tracking midgut renewal following enteric infection. Fluorescent reporter line $esg^{F/O}$, which strongly labels intestinal stem cells, enteroblasts, and newly synthesized enterocytes, can be used to detect midgut renewal following oral infection with a pathogen. (a) Unchallenged adult midgut. (b) Adult midgut after enteric infection with *Ecc15*

but also the specific location of fluorescent signal can be deduced. Typically, GFP can be detected in as little as 6 hr post-treatment; however, the amount of signal at this very early time point will be relatively weak. More time is normally required for a stronger GFP signal.

Enzymatic titration of a reporter enzyme, such as β-galactosidase, under the control of a promoter region belonging to a gene of interest is another classical method of assessing gene expression in the fly (quantitative method). P[lac-Z] insertion lines are available for a large number of genes at the Bloomington *Drosophila* Stock Center. To quantify gene expression using the β-galactosidase titration assay, dissected tissues (~20 guts or carcasses) or whole animals (~5 larvae or flies) are homogenized in Eppendorf tubes containing $100 \mu L$ of Buffer Z ($60 mM$ Na_2HPO_4, $60 mM$ NaH_2PO_4, $10 mM$ KCl, $1 mM$ $MgSO_4$, $50 mM$ β-mercaptoethanol, adjusted pH to 8 with NaOH). Samples are then centrifuged for 1 min at $4°C$ to pellet any debris. Clear supernatant of $40 \mu L$ per sample is then loaded onto a 96-well plate. Next, $250 \mu L$ of Buffer Z–*o*-nitrophenol-β-D-galactoside ($0.35 mg/mL$ ONPG in Buffer Z)—preheated to $37°C$ before addition—is added to each well. Plates are then incubated in a microplate reader at $37°C$, with measurements taken every minute at OD_{420} for 1 hr (Broderick et al., 2014; Houtz et al., 2017). Because melanization can interfere with this assay, it is recommended that samples be processed quickly. β-Galactosidase activity is typically normalized to the amount of protein present in each sample using the Bradford or BCA assays.

X-gal staining can also be used to evaluate gene expression in P[lac-Z]-expressing flies (nonquantitative method). In this protocol, tissues are dissected in PBS buffer and fixed in 0.5–1% glutaraldehyde in PBS at $4°C$. The size and thickness of the tissue will dictate the length of fixation. For example, hemocytes can be fixed in as little as ~20 s, while gut tissue requires ~4 min. Tissues are then washed ×3 in PBS buffer. Next, fixed tissues are incubated in staining buffer containing X-gal at room temperature or $37°C$. The staining buffer is composed of $150 mM$ NaCl, $3.5 mM$ K_3FeCN_6, $10 mM$ NaH_2PO_4/Na_2HPO_4, $1 mM$ $MgCl_2$, and $3.5 mM$ K_4FeCN_6. NaOH is used to adjust the pH to 7.2. X-gal stock is prepared as a 5% solution in dimethylformamide, and it is stored at $−20°C$. Prior to staining, $20 \mu L$ of 5% X-gal stock is added to each milliliter of staining buffer to be used. Incubation length is dependent on the amount of enzyme expressed (Neyen et al., 2014).

4.1.3 | Online resources

Microarray and RNA-seq studies have resulted in the creation of online databases that allow users to look up the expression value of any gene in response to both oral and systemic infection. FlySick-seq (http://flysick.buchonlab.com) is an interactive databank that provides gene expression data for every gene following systemic infection with 10 distinct bacterial pathogens (Troha et al., 2018). Flygut (http://flygut.epfl.ch) hosts an atlas of the *Drosophila* midgut, including an exhaustive description of the different regions of the gut and microarray data of flies orally infected with the pathogens *Ecc15* and *P. entomophila* (Buchon et al., 2013). Flygut-seq (http://flygutseq.buchonlab.com) provides cell- and region-specific transcriptomic data of the fly midgut, including cell-specific expression values under infection conditions (Dutta et al., 2015). The Lemaitre lab's website (http://lemaitrelab.epfl.ch/resources) also offers an assortment of microarray data profiling the host response to systemic and intestinal infection. Of particular interest, this resource includes expression data from *spz* and *rel* mutant flies, which can help to ascertain whether an immune gene is regulated in a Toll- or Imd-specific manner (De Gregorio et al., 2002). Finally, the *Drosophila* Interactions Database (DroID) is a comprehensive gene and protein interactions database that includes protein–protein, transcription factor–gene, miRNA–gene, and genetic interactions (http://www.droidb.org).

4.1.4 | Methods to identify novel genes involved in response to infection

Although ethyl methanesulfonate treatment was the standard approach for mutagenesis during the era of forward genetic screens in *Drosophila*, the advent of large collections of efficient RNAi lines has propelled a shift toward RNAi-based screens. Both in vivo and in vitro, RNAi screens have successfully identified novel components of immune responsive pathways, as well as other key regulators of the host response to infection (Echeverri & Perrimon, 2006; Kambris et al., 2006). Targeted expression of RNAi constructs using the Gal4/UAS system can be used for tissue- or cell-specific interrogation of gene function. Coupled with a thermosensitive version of the Gal80 inhibitor (Gal80ts), this technique can be used to block gene expression in a time-controlled manner, preventing developmental effects (McGuire, Mao, & Davis, 2004). However, the use of RNAi lines is not without its caveats. First, a number of RNAi lines have been shown to exhibit residual gene expression of 25% or more (Heigwer, Port, & Boutros, 2018; Perkins et al., 2015). Hence, RNAi is more likely to give rise to hypomorphic phenotypes, which can conceal the phenotypes of genes that only require a low level of gene expression to execute their function. Second, there is typically no information available on the half-life of the gene product of interest. Therefore, it is possible that targeted protein levels remain stable despite efficient knockdown, thus obscuring a potential phenotype. Lastly, off-target effects could lead to the erroneous attribution of phenotypes to specific genes (Kulkarni et al., 2006; Perrimon & Mathey-Prevot, 2007). Given the aforementioned drawbacks, we recommend verifying the results of RNAi studies through the following methods: using multiple RNAi lines to target the same gene, quantifying the level of knockdown via quantitative reverse transcription PCR (RT-qPCR) or similar method, and testing whether a mutant of the gene of interest (if available) replicates the RNAi phenotype observed. Of note, because genetic background can have a profound effect on the response to infection (Eleftherianos et al., 2014), it is crucial to set up proper controls for experiments involving RNAi lines. For example, the GAL4 line (driver) should be crossed not just to the RNAi line of interest, but also to a wildtype line of the same genotypic background as the RNAi line. This will ensure that both RNAi and control flies for the experiment share the same genetic background.

Another approach to identify novel genes involves the use of the *Drosophila* Genetic Reference Panel (DGRP; Mackay et al., 2012). The DGRP consists of >200 fully sequenced lines that have been inbred to homozygosity (originally derived from a single, wildtype population in Raleigh, North Carolina). These stocks have facilitated an expansion of genome-wide association studies. DGRP-based screens can identify genetic polymorphisms associated with differences in the host response to infection (Howick & Lazzaro, 2017; J. B. Wang, Lu, & St Leger, 2017). Moreover, this approach has the advantage that it can detect variants not only in protein-coding genes, but also in the regulatory regions that coordinate expression of immune-related genes.

4.1.5 | Generation of immune-deficient mutants

CRISPR/Cas9 is quickly becoming the most efficient method to engineer the genome of many organisms, including *Drosophila*. Several CRISPR/Cas9-derived knock-out mutants already exist, and the protocols used to generate them have been described extensively (Bassett & Liu, 2014; Bassett, Tibbit, Ponting, & Liu, 2014; Gratz, Harrison, Wildonger, & O'Connor-Giles, 2015). Transposon mutagenesis represents another approach to edit the genome. Newer generations of engineered transposable elements, such as the *Minos*-mediated integration cassette (MiMIC), have vastly expanded the capabilities of this tool. MiMIC, which integrates into the genome almost at random, carries a gene-trap cassette flanked by two inverted ΦC31 integrase *attP* sites. The *attP* sites allow for the replacement of the intervening sequence of the transposon with any other sequence through recombinase-mediated cassette exchange (Venken et al., 2011). In combination, these two features allow for virtually limitless gene modifications. A large collection of MiMIC lines is available at the Bloomington *Drosophila* Stock Center.

When loss of gene expression results in tissue or cell death, clonal analysis can be used to study the cell-autonomous function of a gene. For more information on both mosaic analysis with a repressible cell marker (MARCM) and twin-spot clonal analysis protocols, the reader is referred to the following studies Griffin et al. (2009) and J. S. Wu and Luo (2006).

4.2 | Assays

4.2.1 | Survival

Survival or death is the terminal outcome of infection. Therefore, survival analysis provides a comprehensive test to measure differences in the host response to infection. In a survival assay, 20 flies are placed together in a media vial (20 is a good

number in terms of vial density) following infection, and the number of dead flies is scored daily (Tzou et al., 2002). Only "healthy-looking" flies (no missing legs and no clipped wings) should be included in the experiment. Any subjects that die within the first 2 hr following infection should be excluded from further analysis, as these flies are dying from excessive injury rather than infection. A minimum of three biological replicates is required. It is important to transfer the flies to new vials every ~3 days. In the case of females, this is done because the food becomes gooey as their eggs develop which can trap and kill flies. Since a shiny, pinkish film, which signals microbial contamination, tends to develop on the surface of food vials with no larvae to churn the food (after a few days), it is recommended that male flies be flipped periodically into new tubes. The length of a survival assay depends mostly on how fast the pathogen can kill its host. For example, the fraction of subjects that succumb to *P. rettgeri* infection do so within the first 3 days following challenge. Therefore, survival is typically recorded over a period of no more than 7 days for this infection. Similarly, a pathogen that takes longer to kill flies will require a lengthier scoring window. If working with mutants, it is imperative to use a reference strain that matches the mutant background, as divergent wildtype lines (e.g., $Canton^S$ vs. w^{1118}) can display vastly different susceptibilities to the same pathogen (Eleftherianos et al., 2014). Similarly, the use of balancer-carrying stocks for experimentation presents unique considerations. For instance, *Curly*, is one of the most frequently used markers in fly genetics. Recently, it was shown that *Curly* mutations arise in the gene *duox*, which is best known for its role in the elimination of pathogens by generating bactericidal ROS (Hurd, Liang, & Lehmann, 2015).

4.2.2 | Quantification of microbial load

There are many techniques, both quantitative and qualitative, to measure microbial load following infection (Figure 4a). Below, we describe three distinct approaches.

Counting the number of CFUs per fly at defined time points postinfection is perhaps the most commonly used method, and it has the advantage of being both quantitative and representative of live bacteria. Briefly, individual flies (or larvae) are deposited into autoclaved Eppendorf tubes containing 500 μL of sterile PBS buffer. Flies are then manually homogenized using a Squisher (Zymo Research, Catalog #H1001). If working with a sizeable number of samples, an automated homogenizer that can simultaneously process large batches (MP Biomedicals, Catalog #116004500) is recommended. In this case, a single sterile metal bead (Omni International, Catalog #19-640) is added to the Eppendorf tube prior to loading the samples into the machine. Flies should be homogenized to the point where no large body parts are identifiable (Figure 4b). Next, samples are serially diluted in sterile PBS to reach countable dilutions and plated on agar plates of the appropriate media. To plate manually, pipette 50 μL of sample per plate and spread the solution using either sterile glass beads or a glass spreader. Plating can also be done via a semiautomated plater (Microbiology International, Catalog #WASP 2). It is recommended that at least two separate dilutions be plated the first time an experiment is performed (Figure 4c). On average, bacteria remain viable in PBS for up to 2 days at 4°C; therefore, if the initial dilutions prove to be uncountable once plated, it is possible to go back and replate a different dilution. Dilution factors will vary depending on the initial dose administered, the capacity of the microbe to grow within the host, the ability of the host to eliminate the microorganism, and the sampling time point. CFUs can be counted either manually or using an automated plate counter (Microbiology International, Catalog #ProtoCOL 3). This protocol can also be used to measure CFUs in specific tissues (with some adjustments to the volume of PBS used per sample). Lastly, bacterial quantification of orally infected flies presents an extra consideration. Because microbial contamination on the fly's appendages could, in principle, obfuscate the results, an additional step must be taken to eliminate surface microbes. Prior to homogenization, flies are quickly dunked in 70% EtOH, rinsed in sterile PBS, and dried by placing them on tissue paper (Neyen et al., 2014).

A second method to measure microbial abundance relies on the use of fluorescently labeled bacteria. For example, the presence or absence of *Ecc15*-GFP can be qualitatively scored visually using a fluorescence dissecting microscope (Figure 4d, e) (Acosta Muniz, Jaillard, Lemaitre, & Boccard, 2007). Additionally, several research groups have published protocols to quantify microbial genes of in fly extracts using PCR (Dionne, Pham, Shirasu-Hiza, & Schneider, 2006; Dostálová, Rommelaere, Poidevin, & Lemaitre, 2017).

4.2.3 | Bacterial load upon death

The bacterial load upon death (BLUD) represents the maximal quantity of bacteria that a fly can sustain while alive. Every bacterium has an associated BLUD for a given fly genotype and BLUD values differ across bacteria. BLUD can be used as a measure of disease tolerance (the ability of the host to withstand infection) (Duneau, Ferdy, et al., 2017; Troha et al., 2018). In

FIGURE 4 Qualitative and quantitative methods of pathogen detection. (a) Schematic representation of a model for bacterial growth within the host during the course of a chronic infection. In the early phase of infection, which precedes effective control by the immune system, bacteria grow exponentially inside the host. This is followed by the second stage of infection, called resolution, in which some of the hosts start to control bacterial proliferation lowering their total CFU (colony forming units) load. Hosts that fail to control their pathogen load early on entering the terminal phase of infection, where bacteria continue to divide until reaching a load that cannot be sustained by the host. Upon reaching this load, termed the bacterial load upon death or BLUD, the host dies. Hosts that survive the infection by controlling their bacterial burden enter the chronic phase of infection, where they sustain a persistent pathogen load called the set-point bacterial load (SPBL). To measure bacterial load, live flies can be sampled at points of interest during both the early and resolution phases of infection. To measure the BLUD, dead flies are collected within 15 min of their death. To quantify the SPBL, live flies should be sampled during the chronic phase of infection (approximately 5 days after infection is a good starting point for bacteria such as *P. rettgeri* and *E. faecalis*). (b) To quantify bacteria, individual flies are placed in single Eppendorf tubes containing 500 μL of sterile PBS buffer and a metal bead. The sample is then homogenized. (c) Following homogenization, samples are plated using the appropriate agar media. We advise that at least two dilutions of each sample are plated the first time an experiment is conducted so as to ensure that individual colonies are discernible and therefore countable. (d) *Drosophila* larvae orally infected with *Ecc15-GFP*, which is visible in the gut of the larvae. (e) Gut of an adult fly orally infected with *Ecc15-GFP*
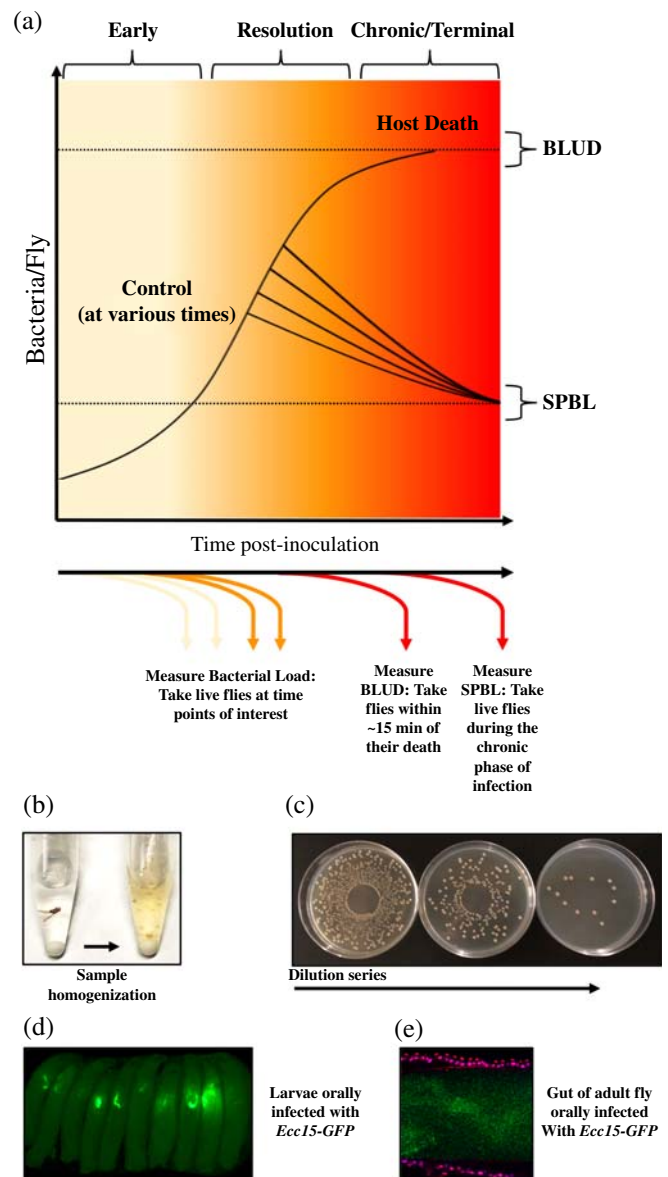
a BLUD assay, flies are collected within 15 min of their death, and CFU counting is used to determine the number of bacteria in each individual fly. For the purpose of this assay, flies that fall on their side and are unable to stand up again are considered dead (Figure 4a).

4.2.4 | Set-point bacterial load

Stereotypically, flies that survive bacterial infection rarely clear their infectious microbes. Instead, they often develop a chronic infection characterized by a persistent, low-level pathogen burden termed the set-point bacterial load (SPBL). The SPBL varies with both host and pathogen genotypes (Duneau, Ferdy, et al., 2017). To quantify the SPBL, collect live flies during the chronic phase of infection (approximately 7 days after infection is a good starting point for bacteria such as *P. rettgeri* and *E. faecalis*) and process them as described earlier to count CFUs (Figure 4a).

4.2.5 | mRNA quantification

Assessment of endogenous RNA transcript levels is fundamental for understanding transcriptional regulation and monitor the level of activation of immune pathways. It is also important for independent confirmation of data generated using techniques such as RNAi knockdown or RNA-seq. RT-qPCR is an accurate, sensitive, and quantitative method to measure gene

expression in the fly. Trizol extraction from a single fly can yield sufficient, albeit very low, levels of RNA for RT-qPCR. For convenience, most laboratories prefer to pool large numbers of larvae or adults per sample. Typically, 10–20 larvae or adult flies are combined for RNA extraction. If performing RT-qPCR on individual tissues, the number of necessary subjects will vary depending on the size of the tissue. For instance, while ~15–20 guts or fat bodies will produce an adequate amount of RNA, harvesting RNA from Malpighian tubules requires dissection of ~25–50 subjects. Of note, while column extraction often gives cleaner RNA compared to the Trizol method, it also yields considerably less RNA than Trizol extraction. For this reason, it is recommended that single fly and pooled tissue extraction be done via the Trizol protocol (Khalil et al., 2015).

FlyPrimerBank (http://www.flyrnai.org/flyprimerbank) is a searchable database that provides a list of predesigned qPCR primer pairs for each *Drosophila* gene (Hu et al., 2013). When validating RNAi knockdown, it is important to avoid amplifying the reagent itself. Instead, qPCR analysis should be performed on a different section of the transcript. One of the advantages of using FlyPrimerBank is that it includes the predicted overlap of each amplified sequence with RNAi constructs from various public resources (*Drosophila* RNAi Screening Center (DRSC), Vienna *Drosophila* Resource Center (VDRC), Transgenic RNAi Project (TRiP), and National Institute of Genetics (NIG)-Japan collections). In *Drosophila*, *RpL32* is the choice reference gene for normalization of RT-qPCR data (Neyen et al., 2014). A list of popular immune reporter genes (including qPCR primer pairs for their amplification) is available in Table 3.

An important consideration when measuring gene expression is circadian control of the immune system. It has been previously reported that immune genes display circadian rhythmicity, and differences in the time of day at infection significantly change survival outcomes in the fly (J.-E. Lee & Edery, 2008; McDonald & Rosbash, 2001). For these reasons, it is recommended that experimental flies be kept under a strict 12-hr light:12-hr dark cycle, and that infections be performed at the same time of day, whenever possible.

Although measuring mRNA provides an accurate representation of the transcriptional response in the fly, it does not offer much information on the availability of the encoded proteins. Recent studies have shown that translational inhibition is a property of some infections (Chakrabarti et al., 2012; Fontana et al., 2011). Therefore, it is strongly recommended that, whenever possible, protein levels also be measured in parallel. This can be done via Western blot using antibodies against the proteins of interest.

4.2.6 | Phagocytosis assays

Plasmatocytes, the most abundant hemocytes in flies, are very proficient phagocytic cells (Lemaitre & Hoffmann, 2007). Many protocols have been developed to both visualize and quantify phagocytosis in adult flies and larvae. These techniques benefit from the use of both fluorescent bacteria and transgenic plasmatocyte reporter lines, such as *Hml-Gal4>UAS-GFP*, *eater-dsRed*, and *eater-nls*::*GFP* (Guillou et al., 2016).

TABLE 3 List of commonly used primers

Gene name	Forward primer	Reverse primer	Pathway
RpL32	GACGCTTCAAGGGACAGTATCTG	AAACGCGGTTCTGCATGAG	Reference gene
Drs	CGTGAGAACCTTTTCCAATATGATG	TCCCAGGACCACCAGCAT	Toll pathway (antimicrobial peptide)
IM2	ACCGTCTTTGTGTTCGGTCT	TGCAGTCCCCGTTGATTACC	Toll pathway (antimicrobial peptide, Bomanin)
CG15067	GAGCCTGACGTTATTGGCG	CCTTTTCCACTTGTTGGCTTGT	Toll pathway (antimicrobial peptide, Bomanin)
Mtk	AACTTAATCTTGGAGCGA	CGGTCTTGGTTGGTTAG	Toll pathway (antimicrobial peptide)
Dpt	GCTGCGCAATCGCTTCTACT	TGGTGGAGTGGGCTTCATG	Imd pathway (antimicrobial peptide)
AttA	CCCGGAGTGAAGGATG	GTTGCTGTGCGTCAAG	Imd pathway (antimicrobial peptide)
CecA1	GAACTTCTACAACATCTTCGT	TCCCAGTCCCTGGATT	Imd pathway (antimicrobial peptide)
vir-1	GATCCCAATTTTCCCATCAA	GATTACAGCTGGGTGCACAA	JAK/STAT
upd3	GCGGGGAGGATGTACC	GTCTTCATGGAATGAGCC	JAK/STAT
TotA	CCCAGTTTGACCCCTGAG	GCCCTTCACACCTGGAGA	JAK/STAT
TotM	TCGACAGCCTGGTCACTTTC	ACCAAGACCACACGAGCATT	JAK/STAT
Socs36E	GCACAGAAGGCAGACC	ACGTAGGAGACCCGTAT	JAK/STAT
puc	TGGCTCTGTTCAAGCG	CCTTATCTCAGTCCCTCG	JNK

To visualize phagocytosis in vivo, flies (or larvae) are injected in the thorax with 69 nL of pHrodo red bacteria (Life Technologies, #P35361) using a microinjector (Drummond, Catalog #3-000-204). pHrodo red bacteria are nonfluorescent outside the cell, but fluoresce brightly red in acidic compartments such as maturing phagolysosomes. The fluorescence signal within the abdomen of flies or the body of larvae is then imaged using a fluorescence microscope and quantified using Image J (NIH) (Figure 5a; Guillou et al., 2016).

For ex vivo imaging of adults, reporter flies (e.g., *eater-dsRed*) are injected with 46 nL of PBS following infection with fluorescent bacteria (e.g., GFP-labeled bacteria to contrast with the dsRed reporter) to loosen the hemocytes. Next, the abdomens of ~10 flies are torn open with tweezers and mechanically scraped so as to release all hemocytes onto a drop of sterile PBS on a lysine-coated slide (the poly-L-lysine coat promotes adhesion). Hemocytes are then quickly dried and mounted using Citifluor AF1 Mountant Solution (Electron Microscopy Sciences, Catalog #17970-100). Slides are scanned using a confocal microscope, and the number of plasmatocytes as well as the average fluorescence signal per plasmatocyte is quantified (Guillou et al., 2016). This protocol is compatible with larval samples.

A different method for larval ex vivo studies involves collecting hemocytes, incubating them with fluorescent heat-killed bacteria, and then running the sample on a flow cytometer, which will both quantify the fraction of cells that phagocytosed bacteria and measure the intensity of the phagocytic uptake (Kurucz et al., 2007). Prior to bleeding, L3 larvae should be washed in PBS buffer to remove any remaining food. Larvae are then placed in a small drop of PBS on a pre-chilled rubber pad, which helps to immobilize them during the procedure. To bleed larvae, two pairs of tweezers are used to tear open chilled larvae on a glass slide containing 120 μL of cold Schneider's *Drosophila* media. The glass slide should be sitting atop a chilled block so the cells remain cold. Next, 100 μL of the media containing the plasmatocytes is transferred to a low binding 96-well plate (Corning, Catalog #3474) and incubated for 10 min at room temperature. *E. coli* or *S. aureus* Alexa Fluor 488 conjugate bacteria is then added, using doses ranging from 10^6 to 10^8 bacteria (Molecular Probes, Catalog #E13231). Plasmatocytes and bacteria are gently mixed by pipetting, and the cells are incubated together for 20 min at room temperature to allow for phagocytosis. After, 50 μL of 0.4% Trypan blue (Sigma, Catalog #T8154-20ML) is introduced to the wells to quench the fluorescence of extracellular bacteria. The sample is then immediately run on a flow cytometer, and the mean fluorescence intensity of the cell population is measured relative to a control sample that lacks fluorescent bacteria. The percentage of cells that have taken up bacteria is calculated by dividing the number of cells in the fluorescence positive gate by the number of cells in the fluorescence negative and positive gates (sum total) and then multiplying this number by 100.

The phagocytic index is computed by dividing the mean fluorescence intensity of bacteria-treated hemocyte samples over the mean fluorescence intensity of bacteria-free control samples. Of note, the number of circulating plasmatocytes in L3 larvae varies substantially by genotype. For instance, bleeding 20 *Oregon*[R] larvae will result in collection ~5,000 individual plasmatocytes, while 20 *w*[1118] larvae will yield ~8,000 cells. Therefore, the number of larvae bled for experimentation should be adjusted to compensate for any genotypic differences (Neyen et al., 2014).

Commonly used phagocytosis mutants are listed in Table 2. Phagocytosis can also be inhibited by injecting polystyrene beads into the hemocoel (Elrod-Erickson, Mishra, & Schneider, 2000).

4.2.7 | Melanization assays

Melanization is an immediate immune response in *Drosophila*, which results in the synthesis and deposition of melanin as well as oxidative byproducts, which are microbicidal. This reaction requires enzymatic cleavage of prophenoloxidase (PPO) into its active
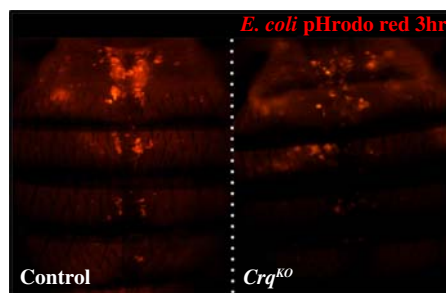

FIGURE 5 Tracking phagocytosis in vivo. Fluorescent images of the abdomen following injection with pHrodo red *E. coli*, which allows for visualization of phagocytosis in vivo. pHrodo red bacteria are nonfluorescent outside the cell, but fluoresce brightly red once inside phagosomes. Abdomen of wildtype fly and abdomen of phagocytosis-deficient *crq* mutant fly

form phenoloxidase (PO). Once activated, this enzyme catalyzes the oxidation of monophenols and diphenols to orthoquinones, which polymerize into melanin (Lemaitre & Hoffmann, 2007). Here, we describe commonly used assays to monitor PO activity.

Hemolymph collection

Collection of hemolymph is the first step to measure PO activity. To collect hemolymph, start by making a modified spin column. A typical spin column (Qiagen, Catalog #74104) is made up of the following parts: clear collection tube, pink plastic column, o-ring, two disks of a soft material, and a disk of hard, plastic-like material. To modify the column, first, take the components apart. Next, rinse the pink plastic column, o-ring, and disk of hard, plastic-like material in clean water. The two disks of soft material are discarded, and the clear collection tube is saved for the next step. After drying the individual parts with a clean napkin, assemble the column anew (keeping the same order for the components as before). To ensure that the modified spin column is fully dry before use, spin down the empty, reassembled column in the centrifuge at maximum speed for ~10 min using the saved collection tube. Discard the collection tube and replace it with a new, clean Eppendorf tube. This modified spin column is now ready to be used.

To collect hemolymph from adult flies, ~100 anesthetized flies are loaded into a modified spin column, and two small metal beads are placed on top of the flies. Flies are then centrifuged at $5,000g$ for 5 min at 4°C twice. The resultant hemolymph is immediately diluted in a 1:10 ratio using a protease inhibitor cocktail (Sigma, Catalog #11697498001, 1 tablet dissolved in 4 mL of PBS buffer), which prevents proteolytic activation of PO, and kept on ice. Finally, samples are normalized using protein concentration obtained via a Bradford assay or similar method (Park et al., 2014). Pricking adults with a sterile needle before centrifugation can increase hemolymph yield, but it is not necessary. Fly limbs are torn out during centrifugation and this is what allows the hemolymph to escape the fly. The same procedure can be used for larval hemolymph extraction using ~30 larvae, but pricking the larvae prior to centrifugation may be required. Samples must be kept on ice at all times.

DOPA assay

The DOPA assay is a robust method for quantification of PO activity in the insect hemolymph. In this assay, PO present in the hemolymph catalyzes the conversion of levodopa (L-DOPA) substrate into an orange to red pigment called dopachrome, whose absorbance can be measured at OD_{492}.

Briefly, combine 50 μL of your previously diluted hemolymph sample (5 μL of hemolymph in 45 μL of protease inhibitor cocktail) with 150 μL of a 5 mM $CaCl_2$ solution in a clean Eppendorf tube. Next, add 800 μL of L-DOPA (Sigma, Catalog #D9628-5G) reagent to each sample. L-DOPA reagent is prepared by dissolving 0.3944 g of L-DOPA in 130 mL of dH_2O using constant (~45 min) mixing. Because L-DOPA is rapidly oxidized by air and darkens upon exposure to air and light, the reagent should be prepared fresh each time. Following thorough mixing, load 200 μL of sample per well in triplicate into a 96-well plate. Using a spectrophotometer set to 29°C, perform a kinetic assay at OD_{492}. The intensity of the color observed will depend on the initial amount of cleaved PO in the sample. Due to the presence of protease inhibitors in the wells, the data from this assay conveys PO activity at the time of collection. To quantify both PPO and PO activity, protease inhibitors are replaced by chymotrypsin, which enables the cleavage of PPO into PO (Neyen et al., 2014).

Frequently used melanization mutants, which can be used as negative controls for this assay, can be found in Table 2.

PPO cleavage test

PPO1 and PPO2 both contribute to PO activity in the hemolymph of *Drosophila*. Performing Western blot analysis on hemolymph extracts using antibodies against PPO1 and PPO2 is another method to evaluate PPO activation (see Binggeli et al., 2014 for the full protocol). This method allows for the detection of both naive PPO (75 kDA) and mature PO (70 kDA) in samples following microbial challenge. The assay can be performed on both larval and adult extracts.

Imaging of crystal cells

Crystal cells are larval hemocytes involved in the melanization process; they are responsible for the synthesis and release of PPOs (Lemaitre & Hoffmann, 2007). Visualization of crystal cells is relatively easy. The cells can be observed using transgenic reporter lines, such as *lz-Gal4>UAS-GFP* (*lz-Gal4* is a crystal cell-specific driver), or by heating larvae for 10 min at

70°C in a water bath. The latter treatment induces the spontaneous activation of PPO within the crystal cells, blackening them. This change in color makes them easily visible through the cuticle (Neyen et al., 2014).

Scoring melanization spots

The simplest qualitative test to evaluate melanization in flies involves pricking flies in the thorax with a needle, similar to how systemic infection is performed. A black spot, corresponding to the activation of the melanization cascade, will appear at the site of wounding as early as 30 min postinjury. However, the final size and coloring of the spot will not be apparent for 4 hr. The characteristics (hue and dimensions) of the melanization dot can be recorded using a camera attached to a microscope. Of note, if scoring melanization spots following microbial challenge, careful consideration should be paid to the infecting agent, as infection with distinct bacterial species gives rise to different wound site melanization responses. For example, inoculation with *S. aureus* tends to produce a larger melanization dot (Ayres & Schneider, 2008; Neyen et al., 2014). Melanization spots can also be observed in larvae following needle pricking, with the first signs of a spot appearing in as little as ~5 min post-injury. Typically, the size and color of the dot is fully developed after 1 hr, at which point it can be recorded by a camera (Dudzic, Kondo, Ueda, Bergman, & Lemaitre, 2015). Finally, because the melanization response can vary substantially between subjects, it is crucial to repeat this experiment several times to have an accurate representation of the reaction.

4.2.8 | Quantification of ROS production

Several methods to measure ROS production have been developed over the past few years. Below, we describe three commonly used methods. Of note, when quantifying ROS production in gut tissue, the addition of live yeast to the diet of experimental stocks should be controlled because their presence can alter basal levels of ROS in the intestine (Neyen et al., 2014).

ROS production in *Drosophila* can be evaluated using 2′,7′-dichlorofluorescein-diacetate (DCF-DA) to sense H_2O_2, which can detect ROS levels in gut tissue (Chakrabarti et al., 2012; S.-C. Wu, Liao, Pan, & Juang, 2012). Briefly, adult female guts are dissected in a buffer solution containing 20 mM N-ethylmaleimide (Sigma, Catalog #E3876). Immediately following dissection, 100 μM DCF-DA fluorescent dye solution (Invitrogen, Catalog #C400) is added to the gut tissue, and the guts are incubated in this dye for 30 min. The tissues are then mounted in 70% glycerol and the anterior portion of the midguts are imaged. Analysis of DCF-DA fluorescent signal requires excitation at 488 nm and emission at 529 nm using a confocal microscope. To quantify signal intensity, average the signal measured on representative fields in a minimum of six guts (using Fiji) (Neyen et al., 2014). With small modifications, similar protocols can be easily adapted for use in any *Drosophila* tissue (Owusu-Ansah, Yavari, & Banerjee, 2008).

Another option for ROS quantification in the gut involves the use of R19S, a recently developed HOCl-specific rhodamine-based dye, which is unable to react with various other ROS. A detailed description of this technique was previously published (K.-A.Lee et al., 2013).

Finally, transgenic flies carrying genetically encoded redox probes to visualize redox differences are also available. Glutathione peroxidases reduce H_2O_2 to water by oxidizing GSH to GSSG (glutathione exists in reduced (GSH) and oxidized (GSSG) states). The Dick group has generated transgenic flies carrying redox-sensitive GFPs (roGFPs) targeted to the cytosol or mitochondria that can be used to measure the GSSG/GSH ratio and H_2O_2 levels in various *Drosophila* tissues (Albrecht, Barata, Grosshans, Teleman, & Dick, 2011).

4.2.9 | Clotting assay

Numerous protocols to evaluate clotting have been developed, including the bead aggregation test and the draw-out assay among others. A thorough methodological guide specific to clotting assays can be found here (Lesch & Ulrich, 2008).

CONFLICT OF INTEREST

The authors have declared no conflicts of interest for this article.

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ORCID

Katia Troha https://orcid.org/0000-0001-9389-4952
Nicolas Buchon https://orcid.org/0000-0003-3636-8387

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