**Drosophila versus Mycobacteria: A model for mycobacterial host–pathogen interactions**

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**Abstract**
Animal models have played an essential role in understanding the host–pathogen interactions of pathogenic mycobacteria, including the *Mycobacterium tuberculosis* and emerging nontuberculous mycobacteria (NTM) species such as *M. avium* and *M. abscessus*. *Drosophila melanogaster* has become a well-established model for the study of innate immunity and is increasingly being used as a tool to study host–pathogen interactions, in part due to its genetic tractability. The use of *D. melanogaster* has led to greater understanding of the role of the innate immune system in response to mycobacterial infection, including in vitro RNAi screens and in vivo studies. These studies have identified processes and host factors involved in mycobacterial infection, such as those required for cellular entry, those required to control or resist non-pathogenic mycobacteria, or factors that become dysregulated as a result of mycobacterial infection. Developments in genetic tools for manipulating mycobacterial genomes will allow for more detailed studies into how specific host and pathogen factors interact with one another by using *D. melanogaster*; however, the full potential of this model has not yet been reached. Here we provide an overview of how *D. melanogaster* has been used to study mycobacterial infection and discuss the current gaps in our understanding.

**KEYWORDS**
autophagy, *Drosophila melanogaster*, ESCRT, host–pathogen interactions, innate immunity, mycobacteria

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**1 | INTRODUCTION**

**1.1 | Drosophila melanogaster as a model for host–pathogen interactions**

*Drosophila melanogaster* (fruit fly) has become a well-established model for the study of innate immunity during infection, beginning with in vivo infection experiments identifying an inducible antibacterial defence system following injection with *Pseudomonas aeruginosa*, *Enterobacter cloacae*, and *Escherichia coli* (Boman et al., 1972). The subsequent identification of the immune function of *Drosophila Toll* (Lemaitre et al., 1996) and mammalian Toll-like receptors (Medzhitov et al., 1997; Poltorak et al., 1998; Rock et al., 1998) showed the existence of fundamental molecular similarities in innate immune detection between *D. melanogaster* and vertebrates, including humans and other mammals. Ensuing work has identified
multiple pathogen detection pathways in *D. melanogaster*, defined several cytokine-like immunomodulatory signals, and explored the regulation and activities of different effector arms of the *D. melanogaster* immune response (Lemaitre & Hoffmann, 2007).

With increasing knowledge of *D. melanogaster* immunity, there has been growing interest in using the fruit fly as a model host for the exploration of host–pathogen interactions. As with other invertebrates, *D. melanogaster* lacks an adaptive immune response, allowing for study of the role of the innate immune response in host–pathogen interactions. Highly conserved components involved in innate immune signaling pathways are frequently targeted by pathogens during infection. *D. melanogaster* can serve as a host for a wide range of pathogens and is easily experimentally infected via injection, wounding, or feeding. Additionally, the ease of use, accessibility, low cost, and genetic tractability of *D. melanogaster* allows for a combination of both prokaryotic and eukaryotic genetic manipulation to probe host–pathogen interactions. Alongside in vivo studies, the use of *D. melanogaster* cell lines in high-throughput RNAi screens has led to the identification of cell-autonomous factors interacting with a range of pathogens, including mycobacteria (Cherry, 2008).

### 1.2 | Mycobacteria

Mycobacteria are a unique and diverse group of bacteria, ranging from obligate pathogens such as *Mycobacterium tuberculosis* and *M. leprae* to numerous non-pathogenic environmental species such as *M. smegmatis*. *M. tuberculosis*, the causative agent of tuberculosis (TB), remains the leading cause of death from a single infectious agent, with 10 million people newly infected and 1.4 million deaths in 2019 (WHO, 2019). Beyond *M. tuberculosis*, many nontuberculous mycobacteria (NTM)—referring to over 180 mycobacterial species but excluding those within the *M. tuberculosis* and *M. leprae* complexes (Hasworth et al., 2017)—are increasingly being recognized as important emerging pulmonary pathogens. These species, such as *M. abscessus* and *M. avium*, can be especially dangerous for individuals with conditions such as cystic fibrosis (CF) or bronchiectasis. They are extremely challenging to treat, in part due to the high levels of intrinsic antibiotic resistance characteristic of these organisms (Shen et al., 2018).

The unique properties of mycobacteria that make these species so successful also make them inherently difficult to study. While more closely related to Gram-positive bacteria, the membrane structure of mycobacteria resembles that of Gram-negative bacteria due to the presence of the mycomembrane (Touchette & Seeliger, 2017). This is a double bilayer outer membrane (Bansal-Mutalik & Nikaido, 2014), with the inner leaflet formed of long-chain mycolic acids covalently anchored to an arabinogalactan-peptidoglycan complex (Daffé et al., 2017). The structure of the outer leaflet is debated and possibly varies across mycobacterial species, with some studies suggesting the presence of mycolates, phospholipids, or triacylglycerols (Bansal-Mutalik & Nikaido, 2014; Chiaraidia et al., 2017). This highly impermeable membrane contributes to the high intrinsic resistance of mycobacteria to antibiotics and other environmental stresses (Jarlier & Nikaido, 1994; Kirschner et al., 1999; Schulze-Robbecke & Buchholtz, 1992; Taylor et al., 2000) as well as their slow growth. The impermeable nature of the mycomembrane contributes to mycobacterial survival in intracellular compartments; many of its components are also bioactive, as targets of recognition by host receptors, as virulence factors, or both (Alderwick et al., 2015; Bernet et al., 2014; Lavollay et al., 2011; Mahapatra et al., 2008; Squeglio et al., 2018).

The focus of this review is on studies of interaction between mycobacteria and *D. melanogaster*. Many such studies have focused on using NTM species such as *M. marinum* as a model for tuberculosis. However, *M. tuberculosis* is a host-adapted pathogen: the biology of its interaction with humans has been shaped by coevolution, possibly limiting the tuberculosis-specific insights to be gained by study of interactions of NTM’s with nonhuman hosts (Bryant et al., 2021). In contrast, NTM species are found widely in the environment and are often not adapted to specific hosts, suggesting that *D. melanogaster* should be used to study more general mechanisms of mycobacterial pathogenesis shared across many of these species. This approach, in combination with studies using other animal models used to study NTM such as zebrafish (Bernut et al., 2014), is providing important insights into the fundamental shared biology of mycobacterial disease. This is especially timely due to the increasing prevalence of NTM disease (Haworth et al., 2017). We would suggest that this is where the true value of these studies is to be found. Here we provide an overview of *D. melanogaster* as a model for mycobacterial infection, including both pathogenic species such as *M. marinum* and *M. abscessus*, and non-pathogenic species such as *M. smegmatis*. We highlight findings into the role of the innate immune response in controlling mycobacterial infections, as well as factors found to be important in intracellular survival of mycobacteria identified from *D. melanogaster* studies. Finally, we discuss the broad potential *D. melanogaster* studies have in further understanding mycobacterial host-pathogen interactions.

### 2 | DROSOPHILA HOST DEFENCE

Humoral and cellular defence mechanisms comprise the innate immune response of *D. melanogaster* (Lemaitre & Hoffmann, 2007). An inducible humoral response leading to the secretion of antimicrobial peptides (AMPs) and production of reactive oxygen species (ROS) is mediated by two core microbe-detection pathways, termed IMD and Toll after their best-known components (Lemaitre et al., 1996). Cellular immunity is mediated via phagocytes that appear closest in their biology and function to mammalian macrophages. The cellular and humoral immune compartments communicate via several known cytokine signaling systems. Additionally, microorganisms can be sequestered by coagulation, which also contributes to wound healing. Epithelial immunity ensures the integrity of the gut lumen with local production of AMPs and ROS (Lemaitre & Hoffmann, 2007). The role of each of these host defense systems will now be discussed in turn relating to their role in controlling mycobacterial infection.
2.1 | Humoral response

*Drosophila melanogaster* has two known signaling pathways that are directly activated by bacterial components. These are the Toll and IMD pathways, both of which are activated by bacterial peptidoglycan (Govind, 2008). The IMD pathway is generally responsive to peptidoglycan containing m-DAP, while the Toll pathway responds to peptidoglycan containing l-lysine. The structure of peptidoglycan in mycobacterial species contains meso-diaminopimelic acid (m-DAP) in the peptide stem, rather than lysine as is more common among Gram-positive bacteria, suggesting a role for the IMD pathway. These allow *D. melanogaster* to roughly differentiate between Gram-negative and Gram-positive bacteria (Buchon et al., 2014). The IMD pathway can also be activated by dmSTING, the *D. melanogaster* ortholog of STING, which acts as an intracellular detector of cyclic dinucleotides (Martin et al., 2018). Both IMD and Toll pathways act primarily via activation of NF-κB-related transcription factors (Relish for the IMD pathway, Dif and Dorsal for the Toll pathway) (Hoffmann & Reichhart, 2002; Tzou et al., 2002). These trigger the expression of a large suite of AMPs, with specific AMP expression controlled by specific pathway activation induced by different pathogens, however, wounding can also induce expression of AMPs. Some target AMP genes are preferentially induced by one pathway or the other. For example, the AMP Drosomycin is primarily a Toll pathway target, whereas Dipterin is primarily an IMD pathway target (Buchon et al., 2014; Lemaître et al., 1997). In addition to AMPs, a melanization system functions downstream of Toll pathway activation. This system relies on secreted prophenoloxidases to produce ROS and is the primary ROS-generating bactericidal system in *D. melanogaster* humoral immunity, responsible for clearance of bacterial species such as *Staphylococcus aureus* (Binggeli et al., 2014; Dudzic et al., 2019).

In the case of mycobacteria, in vivo studies of different mycobacterial species in *D. melanogaster* have found differing results about the activation and role of these signaling pathways during infection, particularly with regards to the Toll pathway. Dionne et al. reported that injection of pathogenic *M. marinum* failed to induce production of any AMPs at levels higher than a wounding control (*D. melanogaster* injected with sterile PBS), up to 50 hr post-infection. However transcriptomic studies did show signs of Toll pathway activation at a later time point, 96 hr post-infection (Dionne et al., 2006). Nonetheless, flies with mutations in the IMD or Toll pathways were no more susceptible to lethal *M. marinum* infection compared with wild-type flies (Dionne et al., 2003), suggesting that either the induction of Toll pathway activity is too late to alter the progression of *M. marinum* infection, or that once the Toll pathway is activated (albeit late in infection), *M. marinum* has an intrinsic resistance to the Toll-induced killing mechanisms.

The absence of IMD and Toll pathway activity during infection with *M. marinum* differs from observations of infection of *D. melanogaster* with other mycobacteria, such as *M. smegmatis* which is non-pathogenic in humans. Infection with *M. smegmatis* is non-lethal to wild-type and IMD mutant *D. melanogaster*, but Toll pathway mutants are susceptible to *M. smegmatis*, dying approximately one-week post-infection with higher bacterial burdens compared with wild-type flies (unpublished data, Dionne Lab). The Toll pathway was also implicated in infection of *D. melanogaster* with *M. abscessus*, a highly pathogenic mycobacterium that causes (relatively) rapid death in *D. melanogaster*. Oh et al. found infection of *D. melanogaster* with *M. abscessus* elicited strong Toll pathway activation. They reported a 26- to 28-fold increase in *drosomycin* expression 4 days after *M. abscessus* infection, and that flies with mutations in the Toll pathway showed increased mortality compared with wild-type flies following *M. abscessus* infection (Oh et al., 2013b). Importantly, the timing of induction of the Toll pathway is not obviously different between these three mycobacterial infections: induction of *drosomycin* expression or death of Toll mutant flies occurs several days post-infection, but *M. abscessus* and *M. smegmatis* may be more susceptible to Toll-induced killing mechanisms than *M. marinum*. This might reflect differences in growth rates or differences in anatomical localization within the fly: *M. marinum* thrives within fly macrophages, while *M. smegmatis* is killed by phagocytosis, and the location of *M. abscessus* is as yet unresolved. That said, the findings of Oh et al. are limited, partly due to the use of mixed male and female flies—which are known to have differing immune activity—in experiments (Belmonte et al., 2020; Vincent & Dionne 2021).

Several puzzles remain. The apparent delay in recognition of mycobacteria by the humoral immune system relative to other bacteria suggests that the mycomembrane may shield the mycobacterial peptidoglycan from detection due to reduced efficiency in detection, though this has not yet been determined. It is also unclear why these mycobacteria appear to activate the Toll pathway rather than the IMD, despite mycobacterial peptidoglycan containing m-DAP, not l-lysine peptidoglycan typically associated with Toll-pathway activation (Buchon et al., 2014; Lavollay et al., 2011). An alternative mycobacterial microbe-associated molecular pattern (MAMP) such as a glucan, Glycopeptidolipid, or other factors in the mycomembrane may be recognized by receptors that trigger activation of the Toll pathway. Alternatively, it may be activated by the widespread tissue damage caused by the *M. abscessus* and *M. marinum* infections (Dionne et al., 2003; Oh et al., 2013b)—though this would not explain the requirement for the Toll pathway in the clearance of *M. smegmatis*. It is also possible that mycobacterial cyclic dinucleotides are recognized by dmSTING, and that there is some synergy between both Toll and IMD pathway signaling (Martin et al., 2018; Tanji et al., 2007). Further work will be required to understand the nature of these interactions.

2.2 | Cellular response

The cellular defence of *D. melanogaster* plays an important role in controlling bacterial infection via phagocytosis. *D. melanogaster* have professional phagocytes which are responsible for the majority of phagocytosis, specialized hemocytes (also called plasmatocytes or macrophages). These engulf debris, apoptotic cells, and can also produce AMPs and cytokines to trigger other signaling pathways as part of the immune response (Martinek et al., 2008; Melcarne et al., 2019; Samakovlis et al., 1990).
2.3 | In vitro cell culture screening

*Drosophila melanogaster* tissue culture cells have numerous features that have made them particularly suitable for pseudo-genetic RNAi screens; many of these screens have focused on the interaction of cultured *D. melanogaster* phagocytes (usually the S2 cell line, and derivatives) with bacterial pathogens, including mycobacteria. In vitro studies of this kind have proved highly informative in identifying key host responses in controlling mycobacterial infection. S2 cells recapitulate in vivo behavior of various mycobacteria species, such as restricting growth of non-pathogenic *M. smegmatis* whereas pathogenic species such as *M. fortuitum* and *M. marinum* are capable of growing intracellularly and lead to cell death, mirroring their in vivo lethality (Philips et al., 2005, 2008). Additionally, the ideal culture temperature of S2 cells at approximately 28°C closely matches the optimum growth temperature of many nontuberculous mycobacteria, such as *M. marinum*, which are typically found in environmental sources. However, S2 cells—and intact *Drosophila*—are limited in the study of *M. tuberculosis* and other obligate human pathogens, as the optimum growth temperature for those bacteria is closer to 37°C, which causes cytotoxic heat shock response in S2 cells (Koo et al., 2008).

The initial step in phagocytosis of bacterial pathogens is recognition and entry via phagocytic receptors. Such receptors required for mycobacterial entry have been identified via a RNAi screen using *D. melanogaster* S2 cells. Intracellular growth of *M. fortuitum* was detected using Green Fluorescent Protein (GFP) expression under the control of *map24* and *map49* promoters. These *map* genes are induced when *M. marinum* and other mycobacteria able to survive within macrophages are phagocytosed (Chan et al., 2002). The combination of *map24/map49*-based GFP reporters, automated microscopy, and simple, highly-effective RNAi protocols enabled early genome-wide screens for host genes that affected intracellular growth of *M. fortuitum* in phagocytic *Drosophila* S2 cells (Philips et al., 2005). In total, 81 host factors were found to be required for *M. fortuitum* infection of S2 cells. 54 of these host factors were found to be required for general phagocytosis of both *M. fortuitum* and *E. coli*, most of which were involved in vesicle trafficking and the actin cytoskeleton such as the Arp2/3 complex, Arp14D, Rab5, Cdc42, coflin, and actin capping proteins. Several host factors targeted by dsRNA, such as Rab5 and Rac2, were found to cause a severe defect in intracellular *M. fortuitum* growth, despite only causing a mild defect in general phagocytosis of other bacteria such as *E. coli*, suggesting that these factors play an additional role in mycobacterial infection. Other categories of host factors were also identified to be essential for *M. fortuitum* growth in S2 cells, despite having no direct role in bacterial phagocytosis in general, such as chromatin factors and components of the vacuolar ATPase (Philips et al., 2005). Further characterization was performed on one host factor specifically required for *M. fortuitum* uptake, a CD36 family scavenger named *Pest* (*Pes*). *Pes* was found to be required for *M. fortuitum* infection of S2 cells, as well as entry of *M. smegmatis* and *Listeria monocytogenes*, but played no role in uptake of *S. aureus* or *E. coli*. Transformation of human embryonic kidney 293 (HEK293) cells to express *Pest* allowed them to be infected with *M. fortuitum*, to which they are normally resistant (Philips et al., 2005).

Following entry into cells, many pathogenic mycobacteria can resist intracellular killing by inhibiting phagosome maturation and fusion with lysosomes (Clemens & Horwitz, 1995). So, somewhat unexpectedly, an RNAi screen in *D. melanogaster* S2 cells revealed lysosomal β-hexosaminidase to play a role in controlling *M. marinum* infection (Koo et al., 2008). *M. marinum* was shown to be internalized by S2 cells within 2 hr of inoculation, then showed steady intracellular growth for 3–4 days before lysis of the S2 cells. Next, S2 cells were treated with dsRNA directed against a range of *D. melanogaster* genes and infected with *M. marinum* to identify genes that, when knocked down, led to increased *M. marinum* growth. RNAi knockdown of HEXO2 in S2 cells, a homolog of mammalian lysosomal enzyme β-hexosaminidase β-subunit, led to a clear increase in intracellular *M. marinum* (Koo et al., 2008). This finding was replicated in murine bone marrow-derived macrophages (BMDMs), with intracellular growth of *M. marinum* being significantly increased in HexB-deficient mice, the HEXO2 homolog, compared to wild-type mice (Koo et al., 2008). It was further found that β-hexosaminidase was secreted by BMDMs in response to *M. marinum*, and not as a general phagocytic response, and this secretion led to extracellular killing of *M. marinum* at the cell surface (Koo et al., 2008). Interestingly, Tay-Sachs disease, an inherited neurodegenerative disorder, is caused by genetic loss of the α-subunit of hexosaminidase; instead of producing the αβ-heterodimer, Tay-Sachs disease patients exhibit increased production of the β-subunit (the HEXO2 homolog) (Koo et al., 2008; Okada & O’Brien, 1969; Utsumi et al., 2002). These patients display increased resistance to tuberculosis (Koo et al., 2008; Spyropoulos, 1988). This is consistent with the findings from Koo et al. that RNAi knockdown of HEXO1, the *D. melanogaster* homolog of the α-subunit, had no effect on *M. marinum* infection of S2 cells. The authors also noted that the bacterial effect of β-hexosaminidase was not seen against *L. monocytogenes* and *Salmonella typhimurium*, suggesting a specific mycobacterial action, possibly due to the far slower replication rate of mycobacteria or to specific interactions with a mycobacterial factor, such as a glycopeptidolipid within the mycomembrane (Koo et al., 2008).

*Drosophila melanogaster* S2 cells have also contributed to understanding the modulation of phagosomes, and the role of the endosomal sorting complex required for transport (ESCRT) systems in restricting intracellular growth of mycobacteria. S2 cells were inoculated with *M. smegmatis*, which constitutively expressed GFP, and treated with dsRNA targeting *D. melanogaster* host factors in order to identify genes normally responsible for the restriction of *M. smegmatis* within S2 cells by measuring intracellular growth (Philips et al., 2008). The genes identified that led to an increase in the percentage of *M. smegmatis* infected S2...
cells included Rab7, involved in vesicle maturation and a marker of late endosomes (Philips et al., 2008; Rink et al., 2005; Via et al., 1997), dVps28, a component of the D. melanogaster ESCRT I complex (Philips et al., 2008; Sevrioukov et al., 2005), and CG8055, a homolog of CHMP4B/SNF7, an ESCRT III complex protein (Philips et al., 2008). Other components of ESCRT complexes were then targeted with dsRNAs. Vps4, Vps20, Vos24, Vps25, Vps36, and dTsg101 knockdown all led to deficiencies in ESCRT function, shown by the impact on ubiquitin trafficking, and also led to increased intracellular growth of M. smegmatis. The dsRNAs that led to the most disruption to ESCRT function (those targeting Vps4, Vps28, dTsg101, and CG8055) were also found to alter the phagosome environment of the S2 cells, shown by decreased map24 and map49 induction by M. fortuitum (Philips et al., 2008). Immunofluorescence imaging of M. smegmatis in S2 cells treated with dsRNAs targeting these ESCRT factors showed that ESCRT machinery acts directly on the bacterial compartment (Philips et al., 2008). The inhibition of a single host activity (ESCRT machinery) leading to a profound change in the intracellular survival of non-pathogenic M. smegmatis highlighted host vulnerabilities, and subsequently led to an understanding of how pathogenic mycobacteria can target and manipulate such vulnerabilities for their own intracellular survival and growth. Following on from this work, it has been shown in a Dictyostelium discoideum model of infection that ESCRT machinery is recruited to phagosomes containing both M. marinum and M. tuberculosis in an ESX-1 dependent manner, likely as a host response to repair the small membrane perforations caused by the ESX-1 system (López-Jiménez et al., 2018; Mittal et al., 2018). As a countermeasure, M. tuberculosis secretes virulence factors EsxG and EsxH via ESX-3 to inhibit both ESCRT function and phagosomal maturation (Koo et al., 2008; Mehra et al., 2013; Mittal et al., 2018; Philips et al., 2005). As a result of ESCRT inhibition by EsxH, M. tuberculosis was found to undermine the recognition of infected macrophages by CD4+ T-cells, potentially due to a role of ESCRT in antigen presentation (Portal-Celhay et al., 2016).

2.4 In vivo studies

While cultured D. melanogaster cell lines allow for high-throughput genetic screening, they do not permit analysis of infection in the in vivo physiological context, and it is difficult to model interactions between phagocytes and other cell types in culture. In vivo studies in D. melanogaster have contributed to understanding host responses to mycobacterial infection; a significant focus of work in this area has been the role of autophagy-related proteins. Autophagy is a key intracellular degradation pathway; autophagy-related proteins are important in cellular and systemic defence against intracellular pathogens such as M. tuberculosis and other pathogenic mycobacteria, though the precise nature of this involvement is still unclear (Kimmey et al., 2015). Many studies have found that virulent mycobacteria, predominantly M. tuberculosis, actively impair autophagic function and phagosomal maturation with secreted virulence factors such as ESAT6/EsxA, EsxG, and EsxH (Mehra et al., 2013; Mittal et al., 2018; Romagnoli et al., 2012). It seems increasingly likely that individual autophagy genes do not significantly contribute to protection from virulent mycobacteria, and that many of these genes have roles beyond autophagy that act to control mycobacterial infection. A notable example using a mouse model of M. tuberculosis infection found autophagy-related gene (ATG) Atg5 uniquely protected against M. tuberculosis by ameliorating excessive immunopathology mediated by polymorphic mononuclear cells. This study by Kimmey et al. also found that autophagic capacity did not correlate to the outcome of M. tuberculosis infection, and other canonical autophagy genes were not as protective against M. tuberculosis (Kimmey et al., 2015). In vivo studies with D. melanogaster, supplemented by in vitro work, have contributed to understanding the unique and complex role of ATGs in controlling mycobacterial infection.

ATG2 is an example of an ATG found to have a role in mycobacterial infection beyond its normal role in autophagy. Infection of D. melanogaster with M. marinum was found to trigger macrophages to produce upd3, a cytokine that shows a similar four-helix bundle structure to mammalian cytokine IL-6. upd3 mutant flies were found to survive for longer and have reduced bacterial load following infection with M. marinum when compared to wild-type D. melanogaster. The upd3 mutant flies were also found to have elevated Atg2 expression, due to a conserved JAK/STAT signaling cascade within hemocytes. upd3 binds domeless, a GP-130-like receptor, activating the sole D. melanogaster STAT, Stat92E, which subsequently inhibits Atg2 expression (Peán et al., 2017). Overexpression of Atg2 in D. melanogaster S2R+ cultured phagocytes halved levels of intracellular M. marinum and prevented cell death, without changes in the uptake of the bacteria. The protective effect of Atg2 on mycobacterial infection is likely due to its effect on intracellular triglyceride levels. Increased Atg2 expression was found to reduce the number of large lipid droplets, preventing lipid accumulation that is highly beneficial for the survival of intracellular ycobacteria (Peán et al., 2017). Additionally, disruption of Atg2 by mycobacteria may alter the lipid composition of the autophagosome. The role of Atg2 in autophagy is to mediate lipid transfer to aid autophagosome formation (Osawa et al., 2019). Lipid profiles of Atg2 mutant D. melanogaster showed the autophagic membranes became enriched in phosphatidylinositol (PI) species (Laczkó-Dobos et al., 2021). Accumulation of phosphatidylinositol-3-phosphate (PI3P) within autophagosomal membranes is important for the recruitment of downstream effectors, however, mycobacterial species have several tools to prevent this. Examples include SapM, a PI3P phosphatase (Fernandez-Soto et al., 2019), and lipoarabinomannan (LAM) which intercalates into the autophagosomal membrane and blocks recruitment of PI3P kinase hVPS34, thus preventing the initial production of PI3P (Vergne et al., 2003; Vergne & Deretic, 2010).
Atg7 is another autophagy factor investigated for its role in mycobacterial infection using D. melanogaster models. In vivo experiments using Atg7 mutant D. melanogaster also showed decreased survival when infected with M. marinum. Induction of autophagy by INH encoding the sole E1-like enzyme in D. melanogaster which is responsible for the activation of Atg8 (LC3) and Atg12. These are ubiquitin-like protein conjugation systems that are vital for the formation and closure of the membrane structures of autophagosomes (Juhász et al., 2007; Nagy et al., 2014). In mammals, these ATG factors play essential roles in LC3-associated phagocytosis (LAP), a noncanonical autophagy pathway linking pathogen recognition receptors and phagosomal maturation. The role of LAP in mycobacterial clearance is unclear, though a recent study using mice found that M. tuberculosis infection, however, chloramphenicol and tetracycline did not (Kim et al., 2012). INH and PZA are both pro-drugs, converted into their active forms by the action of mycobacteria (Kim et al., 2012; Rozwarski et al., 1998; Zhang & Mitchison, 2003). Induction of autophagy by INH and PZA was shown to require the production of cellular and mitochondrial ROS by the host cell initially induced by the production of bacterial ROS following treatment with INH and PZA (Kim et al., 2012). These findings were supported by an in vivo study, which showed increased autophagy marker Atg8a in dissected fat body of larvae administered amikacin or rifampicin following M. marinum infection. GFP-tagged Atg8a also showed colocalization with lysosomes in the antibiotic-treated larvae, suggesting that treatment with these antimycobacterial drugs was promoting the degradation of Atg8a labeled vesicles (Kim et al., 2012). This study continued by infecting adult Atg7 mutant D. melanogaster with M. marinum, and then administered amikacin or rifampicin in the food. Wild-type flies showed increased survival following the M. marinum infection, however, there was only a mild protective effect of the antibiotics in the Atg7 mutant flies, showing the importance of autophagy in the action of antimycobacterial drugs (Kim et al., 2012).

In addition to roles in antimycobacterial defence, there may be a relationship between autophagy-related factors and systemic pathology induced by mycobacterial infection. Autophagic mechanisms are controlled by endocrine signals that regulate systemic metabolism, in particular, the insulin signaling pathway (O’Farrell et al., 2013; Scott et al., 2004). M. marinum infection impairs systemic insulin signaling, leading to systemic metabolic pathophysiology; animals in which the insulin-inhibited transcription factor FOXO is mutated survive longer after infection and exhibit reduced metabolic pathology (Dionne et al., 2006). The loss of insulin signaling and potential changes in autophagy are almost certainly connected, though the precise nature of this connection is not yet clear.

3 | MYCOBACTERIAL VIRULENCE FACTORS

Drosophila melanogaster as a host model has led to many developments in understanding host defence factors important in the control of mycobacterial infection, however, its potential to study important mycobacterial virulence factors has not been exploited. Diverse screening assays using the D. melanogaster host model have identified key virulence factors in other pathogens such as P. aeruginosa (Boonna et al., 2017; Broderick et al., 2008; Erickson et al., 2004). Bacillus anthracis (Guichard et al., 2010), Vibrio cholerae (An et al., 2009), and S. aureus (Atilano et al., 2011), highlighting the usefulness of D. melanogaster as a tool for such screens. Currently, there is a single example of D. melanogaster being used to study factors involved in mycobacterial pathogenesis. mag24, a M. marinum promoter, was induced by phagocytosis of M. marinum following infection of adult D. melanogaster. This was confirmed by blocking phagocytosis with a prior injection of 0.2-µm-diameter polystyrene beads and finding that GFP expression induced by mag24 was not activated. M. marinum strain L1D, in which the mag24 locus is deleted, was then found to be less virulent in infected D. melanogaster compared with type-strain M. marinum. This shows that the process of phagocytosis and subsequent induction of the mag24 promoter enhances the virulence of M. marinum (Dionne et al., 2003).

Recent developments in CRISPR interference (CRISPRi) now allows for targeted gene silencing in mycobacteria, including species such as M. tuberculosis and M. marinum (Choudhary et al., 2015; Meijers et al., 2020; Rock et al., 2017). This system will allow for specific gene repression in these pathogenic mycobacteria, and the D. melanogaster host model will allow for broad screening of these strains to identify key virulence factors and genes essential to the intracellular survival of these mycobacteria. The genetic tractability of D. melanogaster then provides further opportunities to probe deeper into the specific host–pathogen interactions between the mycobacteria and the innate immune response.

4 | CONCLUDING REMARKS

Drosophila melanogaster is widely recognized as a powerful tool in studying host–pathogen interactions and has already contributed to a better understanding of how the host innate immune system responds to infection with mycobacteria. Initial studies have also made use of D. melanogaster as a model for mycobacterial infection to screen new antimycobacterial drug compounds (Oh et al., 2013a). The key outstanding questions in the field currently are what mycobacterial factors, either within the mycomembrane or secreted, are being detected by the innate immune system and how; how are pathogenic mycobacterial species evading or manipulating the host cell defences in order to survive; what host responses are most effective in controlling and clearing mycobacterial infection. There is a great deal of underutilized potential of the D. melanogaster host model in understanding mycobacterial infection, which hopefully will lead to improvements in preventing and treating mycobacterial disease.
| References          | Drosophila model                                      | Mycobacterial species | Major conclusion                                                                 |
|---------------------|-------------------------------------------------------|-----------------------|----------------------------------------------------------------------------------|
| Dionne et al. (2003)| In vivo adult and larval D. melanogaster              | M. marinum            | - M. marinum kills flies in a dose-dependent manner                               |
|                     |                                                       | M. smegmatis          | - M. marium infects hemocytes prior to spreading systemically, causing severe tissue damage |
|                     |                                                       |                       | - M. marinum is capable of blocking phagosomal acidification in hemocytes         |
|                     |                                                       |                       | - M. marinum strain LD1 has reduced virulence in vivo, in which the mag24 locus is deleted |
|                     |                                                       |                       | - Neither M. marinum nor M. smegmatis induced production of AMPs up to 50 hr post-infection |
|                     |                                                       |                       | - ind or Toll mutant flies were no more susceptible to M. marinum infection than wild-type flies |
| Phillips et al. (2005)| In vitro S2 cells                                      | M. fortuitum          | - A CD36 family scavenger receptor, peste, is required for uptake of M. fortuitum and M. smegmatis by S2 cells |
| Dionne et al. (2006)| In vivo adult D. melanogaster                         | M. marinum            | - Mild induction of Toll and IMD genes 72 hr post infection relative to PBS-injected controls |
|                     |                                                       |                       | - foxo mutant flies die more slowly when infected but have similar bacterial loads |
|                     |                                                       |                       | - Insulin signaling is impaired in M. marinum infected flies                      |
|                     |                                                       |                       | - Wasting caused by M. marinum infection in flies is caused by failure of Akt activation and subsequent activation of transcription factor FOXO |
| Koo et al. (2008)   | In vitro S2 cells                                      | M. marinum            | - HEXO2 is involved in restricting M. marinum growth in S2 cells                 |
|                     |                                                       |                       | - HEXO2 is secreted in response to M. marinum and has a bactericidal effect       |
| Phillips et al. (2008)| In vitro S2 cells                                      | M. fortuitum, M. smegmatis | - ESCRT machinery (including Rab7, dTsg101, SNF7/CHMP4B, Vps4, and Vps28) acts to restrict intracellular growth of non-pathogenic mycobacteria. |
| Kim et al. (2012)   | In vivo adult and larval D. melanogaster              | M. marinum            | - Atg7 mutant flies died faster than wild-type flies following infection with M. marinum |
|                     |                                                       |                       | - Treatment of Atg7 mutant flies with antimycobacterial drugs could not rescue fly survival rates of that of wild-type flies when infected with M. marinum, indicating autophagy is required for effective antimycobacterial drug action |
| Oh et al. (2013a)   | In vivo adult D. melanogaster                         | M. marinum            | - Treatment of flies with antimycobacterial compounds such as rifampicin, amikacin, isoniazid, and ampicillin, resulted in increased fly survival and decreased bacterial loads following infection with M. marinum |
| Oh et al. (2013b)   | In vivo adult D. melanogaster                         | M. abscessus          | - M. abscessus kills flies                                                       |
|                     |                                                       |                       | - Increased expression of drosomycin was induced in flies following expression with M. abscessus |
|                     |                                                       |                       | - PGRP-SA and Dif mutant flies were more susceptible to infection with M. abscessus |
|                     |                                                       |                       | - M. abscessus induced widespread tissue damage in infected flies               |
| Peán et al. (2017)  | In vivo adult D. melanogaster In vitro S2R+ cells     | M. marinum            | - M. marinum triggers the production of upd3 in infected flies, causing activation of Stat92E and subsequently inhibiting Atg2 |
|                     |                                                       |                       | - Inhibition of Atg2 results in the formation of large, irregular lipid droplets, promoting intracellular survival and growth of M. marinum |
|                     |                                                       |                       | - Overexpression of Atg2 reduces M. marinum survival in S2R+ phagocytes due to changes in neutral lipid metabolism |
|                     |                                                       |                       | - Flies lacking upd3-Stat92E signaling live longer than wild type flies following M. marinum infection |
|  Kim et al. (2017)  | In vivo adult D. melanogaster                         | M. marinum            | - Atg7 mutant flies show decreased survival and increased bacterial loads following infection with M. marinum |

**SUMMARY TABLE**
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CONFLICT OF INTEREST
The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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