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Deciphering the role of innate immune response in the model of tuberculosis in *Drosophila melanogaster*

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“Marcher en ligne droite, on ne peut pas aller très loin”

“Caminant en línia recta, un no pot arribar massa lluny”

Antoine de Saint-Exupéry

Abstract

Nowadays it is estimated that a third of the world population is infected with *Mycobacterium tuberculosis* (Mtb). However, just a reduced percentage will develop the active disease. It has been shown that this progression is related to massive neutrophil infiltration of lesions infected with Mtb and subsequent induction of a Th17 type immune response. Previous studies from this Unit have shown that the induction of regulatory T cells (Tregs) by repeated oral administration of a low dose of heat-inactivated *Mycolicibacterium manresensis* (khMm) has the ability to stop this process. However, the innate mechanisms capable of inducing the initial neutrophil response in only some infected individuals are not known.

The fruit fly *Drosophila melanogaster* is an animal model that has been highly used for the understanding of fundamental principles of genetics and regenerative biology, as well as for the study of several human diseases and drug discovery for over a century. The resemblance of this model with higher organisms in the pathways and transcriptional regulators that are crucial for development, metabolism and immunity, together with the late increasing interest in finding new animal models that might help reduce, refine and replace the current mammal models, makes of *Drosophila melanogaster* a great candidate for the study of infectious diseases, including mycobacterial infections.

The characterisation of the innate immune response triggered by mycobacterial infections in *D. melanogaster* has been shown to be species-specific and strongly linked to the metabolic status of the host, showing that a metabolic increase helps to eliminate innocent mycobacteria, while pathogenic mycobacteria are able to attenuate the response. Furthermore, both the sex and reproductive status of the host also have a major impact on the regulation of the immune response against infection by the pathogenic species *Mycobacterium marinum*. The evaluation of the protective effect of oral administration of hkMm in both sexes has demonstrated the induction of a non-specific innate immune response that protects flies against infection by other pathogenic bacteria.

Finally, the evolutionary adaptive capacity of the innate immune response of D. melanogaster has been studied in flies exposed to infection by M. marinum and/or to oral administration of hkMm for 10 generations, revealing that both stimuli are able to induce tolerance in the host to new infections with *M. marinum*, again revealing the existence of a sexual dimorphism in the adaptation mechanisms. Subsequent studies have shown that host-pathogen co-evolution reduces the virulence of the pathogen without affecting host survival, while the addition of oral treatment with hkMm to the equation improves host response.

Resum

Actualment, s'estima que una tercera part de la població mundial està infectada per *Mycobacterium tuberculosis* (Mtb). Tot i això, només un petit percentatge desenvoluparà la malaltia activa. S'ha demostrat que aquesta progressió està relacionada amb una infiltració massiva neutròfils a les lesions infectades per Mtb i la posterior inducció d'una resposta immune de tipus Th17. Estudis anteriors d'aquesta Unitat han demostrat que la inducció de cèl·lules T reguladores (Tregs) mitjançant l'administració oral repetida de una dosis baixa de *Mycolicibacterium manresensis* inactivat por calor (khMm) té la capacitat de reduir aquest procés inflamatori. Malgrat això, es desconeixen els mecanismes innats capaços d'induir la resposta neutrofílica inicial en alguns individus infectats i en d'altres no.

La mosca de la fruita *Drosophila melanogaster* és un model animal que ha estat àmpliament utilitzat per comprendre els principis fonamentals de la genètica i la biologia regenerativa, així como l'estudi de diverses malalties humanes i nous fàrmacs durant més d'un segle. La similitud d'aquest model amb organismes superiors en les vies i reguladors transcripcionals crucials pel desenvolupament, el metabolisme i la immunitat, juntament amb el creixent interès per buscar nous models animals que ajudin a reduir, perfeccionar i substituir els actuals models de mamífers, fan de *D. melanogaster* un gran candidat per l'estudi de les malalties infeccioses, incloent les causades per micobacteris.

La caracterització de la resposta immune innata desencadenada per infecciones micobacterianes en *D. melanogaster* ha demostrat ser específica per cada espècie i estar molt vinculada a l'estat metabòlic de l'hoste, mostrant que un increment metabòlic ajuda a l'eliminació de micobacteris innocus, mentre que els patogènics són capaços de atenuar la resposta. A més a més, tant el sexe com l'estat reproductiu de l'hoste també tenen un gran impacte en la regulació de la resposta immune contra la infecció pel micobacteri patogènic *Mycobacterium marinum*. L'avaluació de l'efecte protector de l'administració per via oral de hkMm en ambdós sexes, ha demostrat la inducció d'una resposta immune innata inespecífica que protegeix les mosques davant la infecció per altres bacteris patogènics.

Finalment, s'ha estudiat la capacitat d'adaptació evolutiva de la resposta immune innata de *D. melanogaster* en mosques exposades a la infecció per *M. marinum* i/o a l'administració oral de hkMm durant 10 generacions, revelant que ambdós estímuls són capaços d'induir tolerància en l'hoste en front a noves infeccions amb *M. marinum*, novament revelant l'existència d'un dimorfisme sexual en els mecanismes d'adaptació. Posteriors estudis han mostrat que la coevolució hoste-patogen redueix la virulència del patogen sense afectar a la supervivència de l'hoste, mentre que l'adició del tractament oral amb hkMm a l'equació millora la resposta de l'hoste.

Abbreviations

AM	Alveolar macrophages
AMP	Antimicrobial peptides
APCs	Antigen presenting cells
ATB	Active tuberculosis
Atg	Autophagy-related
BCG	bacille Calmette-Guerin
Cec	Cecropin
CFU	Colony forming units
DCs	Dendritic cells
DEGs	Differentially expressed genes
Dif	Dorsal-related immunity factor
dILPs	Drosophila insulin-like peptides
Dpt	Diptericin
Droso	Drosomycin
Duox	Dual oxidase enzyme
EcR	Ecdysone receptor
ESAT-6	6kDa early secretoy antigenic target
FM	Foamy macrophages
Foxo	forkhead box, sub-group O
GALT	gut-associated lymphoid tissue
GNBP	Gram negative binding protein
GSEA	Gen Set Enrichment analysis
HDT	Host-directed theraphy
HIF	hypoxia-inducible factor
hkMm	heat-killed Mycolicibacterium manresensis
IIS	Insulin/Insulin-like signalling
IL	Interleukin
Imd	Immune deficiency
IMpl2	antagonist Imaginal morphogenesis protein late 2
INF-γ	Interferon-γ
InR	Insulin receptor
IPCs	Insuling producing cells
ISC	Intestinal stem cells

JH	Juvenile hormone
JNK	Janus Kinase
LB	Luria-Bertani
LD	Lipid droplets
LN	Lymph nodes
LPS	Lipopolysaccharides
LTBI	Latent tuberculosis infection
MAPK	Mitogen-activated protein kinase
MLNs	Mesenteric lymph nodes
Mtb	Mycobacterium tuberculosis
mTOR	mammal Target of rapamycin
NETs	Neutrophil extracellular traps
Nox	NADPH oxidase
NSAID	Non-steroidal anti-inflammatory drugs
OD	Optical density
p.i.	post-infection
PBS	Phosphate buffered saline
PCA	Principal component analysis
PGN	Peptidoglycan
PGRP	Peptidoglycan recognition protein
PO	phenol oxidase
PPO	pro-phenol oxidase
ROS	Reactive oxygen species
SIFs	Selfish immune factors
SOCS	Supressors of cytokine signalling
Spn	Serpin
TB	Tuberculosis
TGF-β	Transforming growth factor beta
Th	T helper
TLR	Toll-like receptor
Tot	Turandot
Tregs	regulator T cells
Upd	Unpaired
WHO	World Health Organisation
Wnt	Wingless

1| General Introduction

1.1| Tuberculosis

1.1.1| Global thread

Tuberculosis (TB) is an airborne disease caused by Mycobacterium tuberculosis (Mtb) bacillus. Although many people think of it as an eradicated disease, the truth is that is one of the top 10 causes of death worldwide and the leading cause of death from a single pathogen. The World Health Organization (WHO) reported that 10 million people fell ill and approximately 1.4 million people died from TB in 2019, and that about a quarter of the world's population might be infected with Mtb (1). This underestimation of the importance of this disease might be because only 30 high TB burden countries account for almost 90% of those who fall sick with TB each year, and are mainly developing countries (Figure 1), although TB can affect anyone anywhere.

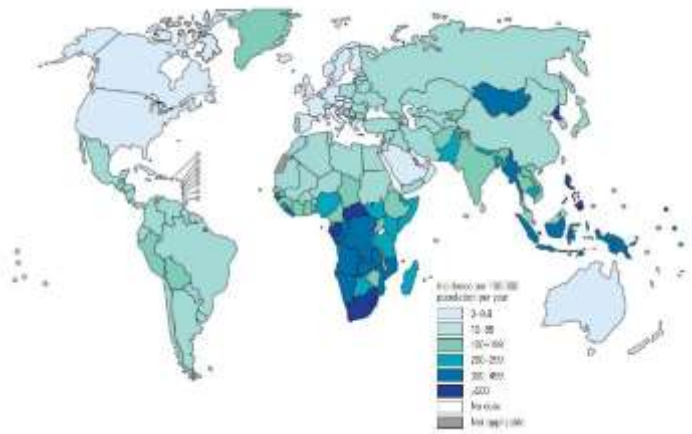


Figure 1| Global estimated TB cases in 2019. Estimated TB incidences rates, as reported by the WHO. Extracted from Global Tuberculosis report, 2020.

1.1.2| Infection and disease

Although Mtb can infect almost every organ in the human's body, pulmonary affection is the most common and contagious form of the disease. Its dissemination happens when aerosol droplets containing the bacilli from active TB patients are released (i.e. by coughing or speaking) and are inhaled by another individual (Figure 2-1). After an infection, only 5 to 10% of infected people will develop an active pulmonary disease (ATB) while most of them will remain latently infected (LTBI) and only a small part of the population will be capable of clearing the infection (2). However, the immune mechanisms that determine which outcome an individual will follow remain unclear.

1.1.2.1| Immunity of TB

Once the infected aerosols reach the new host, the bacilli have to overcome the first lines of defence, such as the bronchial epithelium and the mucosa, which activation may result in an unsuccessful infection process (3). After reaching the alveoli, Mtb enters the alveolar macrophages (AM) (Figure 2-2) and secrete the 6kDa early secretory antigenic target (ESAT-6), which inhibits the phagosome-lysosome fusion and the apoptosis of the infected cells, and the bacilli translocate to the cytoplasm where their replication starts. The infected cell, eventually necrotized releasing the bacilli that will be phagocyted by another AM, thus repeating the cycle (Figure 2-3a) (4). This modus operandi allows an unnoticed replication of the bacilli until enough chemokines are generated by the AM to induce an inflammatory focus, which will evolve until forming the granuloma.

Before any adaptive immune response is induced, the dissemination of the bacilli occurs (5). This is due to the constant drainage of the infected alveoli, which allows the circulation of the bacilli to the lymph nodes (LN) (Figure 2-4) where they can infect the dendritic cells (DCs) (6). The hematogenous dissemination of the bacilli from the LN, through the right atrium and ventricle, to the lungs again (7) (Figure 2-5) allows the bacilli to create new infection foci or to enter previous one since granulomas are highly vascularized structures (8). If the bacilli reach the left atrium and ventricle, the dissemination becomes systemic (7) (Figure 2-9).

In the LN, infected DCs present the Mtb antigens to the T CD4+ cells, which will migrate to the alveoli and induce a different response depending on the cytokine and chemokine profile they find in the alveoli. Generally, a T helper (Th) 1 response based on the production of interferon-gamma (INF-γ) that will activate the infected macrophages is induced. This response generates a so-called proliferative lesion, which is characterized by the destruction of the majority of the bacilli (Figure 2-6a), the attraction of monocytes to the infection loci (Figure 2-7a) and the encapsulation of the lesion by fibroblasts (Figure 2-8), thus controlling the granuloma formation. If a Th17 response is triggered instead, an exudative lesion is formed and this is related to the development of an active TB (Figure 2-6b). These types of lesions are characterized by a higher neutrophil population in which neutrophil extracellular traps (NETs) Mtb can grow extracellularly, thus preventing active AM to kill them and also the encapsulation of the lesion, which allows higher drainage of the bacilli and the generation of new lesions (Figure 2-7b).

A small portion of bacilli might remain dormant inside the macrophages, which also phagocyte cellular debris resultant from necrosis and accumulate fatty acids into the cytoplasm becoming foamy macrophages (FM). These cells are drained to the bronchial tree (Figure 2-10) and may end up being eliminated by the gastrointestinal

tract (Figure 2-11) or be destroyed and the dormant bacilli return to the lungs and re-infect the host (Figure 2-1).

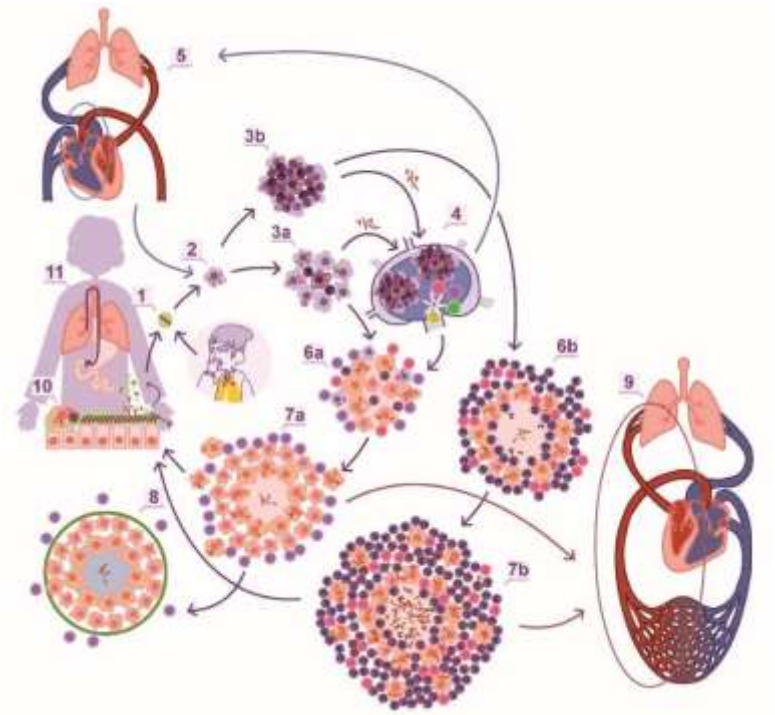


Figure 2| Mtb infection cycle. Summary of the stages form Mtb infection until development of TB. Infectious aerosols are inhaled and enter the alveoli (1). The alveolar macrophage phagocyte the Mtb and the bacilli start replicating (2). Depending on the milieu found in the alveoli two granulomas can be formed (3a or 3b). The bacilli are drained to the LN (4). If the initial granuloma is mainly formed by monocytes (3a) a Th1 response is triggered where the granuloma is controlled (6a), most of the bacilli are killed (7a) and the granuloma is encapsulated (8). If the initial granuloma is mainly formed by neutrophils, a Th17 response is induced instead (3b) with the infiltration of more neutrophils (6b) and the granuloma grows uncontrolled until the development of active TB (7b). The hematogenous dissemination of the bacilli can lead to reinfections in the lungs (5) or can become systemic (9). Foamy macrophages containing dormant bacilli are drained towards the bronchial tree (10) where they reach the gastrointestinal system and are eliminated (11) or return to the lung via new aerosols (1). Adapted from Cardona, 2018.

1.1.3| Importance of a balanced response in TB

Although the main Mtb antigens have been described and the cellular immune response is effective in protecting the majority of the population, the reason why some individuals will end up developing an active TB remains unclear. Here, the "damage-response" framework can help us better understand Mtb infections. This approach is based on three principles: the pathogenesis is the result of the host-pathogen interaction, the outcome of this interaction is determined by the damage caused to

the host and this damage can be done by the pathogen and/or the own host (9). Bearing this in mind, a weak immune response leads to Mtb dissemination, while an exacerbated pro-inflammatory immune response would lead to the destruction of the granulomas and enhanced tissue damage that result in the active pulmonary TB (10) (Figure 3). In between, we find the rest of the spectrum of Mtb infections, such as LTBI patients (11). This correlates with the findings that people with a pro-inflammatory environment, such as men (12) or type 2 diabetes mellitus patients (13), are more prompted to develop an active TB. This dichotomy, together with the presence of re-infections and the early infection being silent, are the reasons why no prophylactic vaccines worked to avoid infection, and therapies are focused on preventing the active disease instead.

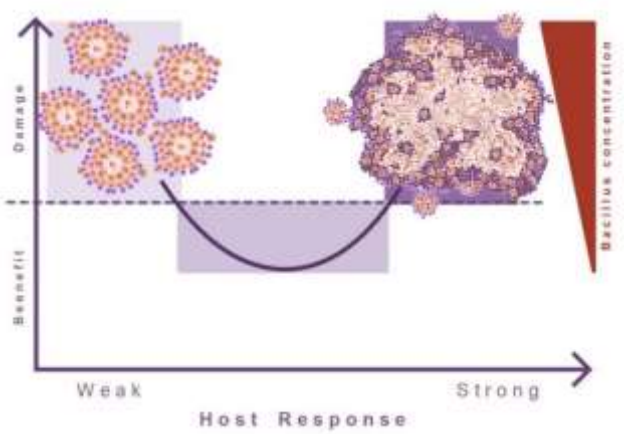


Figure 3| Evolution of TB according to the damage-response framework. The y-axis shows the damaged caused to the host, while the x-axis shows the intensity of the host response. In the case of TB, a weak response leads to the formation of multiple lesions and the dissemination of the bacilli. On the other hand, the induction of an excessive inflammatory response induces a massive infiltration of neutrophils to the infection foci and the production of exudative lesions causing massive tissue destruction. In between, the majority of the hosts are able to induce a balanced immune response that allows them to live with the infection without major consequences. Extracted from Cardona, 2017.

1.2| Tolerance to Infections

1.2.1| From plant ecology to animal infectious diseases

For many years, in the infectious diseases field, the hosts were defined either as resistant or susceptible to an infection and the dogma was to discover new antimicrobial drugs to attack directly the root of the problem (i.e., the pathogens), although this accelerated the selection and the spread of drug-resistant pathogens. However, in the past years, some authors have included the concept of tolerance into

the equation, a concept widely used in the plant ecology community. Plants have evolved many specialized defence mechanisms against a wide range of pathogens including the ability to tolerate some infections without affecting the pathogen load, naming this strategy as “disease tolerance” (14).

Although studies of infections in animals have been long focused exclusively on resistance, now it is increasingly understood that host defence against pathogens is a mix of both strategies. While being resistant is understood as the ability of a host to prevent the infection or to kill the pathogen, the concept of tolerance refers to the ability of the host to live with a certain concentration of a pathogen without affecting its fitness, focusing on reducing the tissue damage caused either by the pathogen or the immune response (10) (Figure 4). These two concepts are expected to have different effects on the epidemiology of infectious diseases and might help to better understand host-pathogen coevolution.

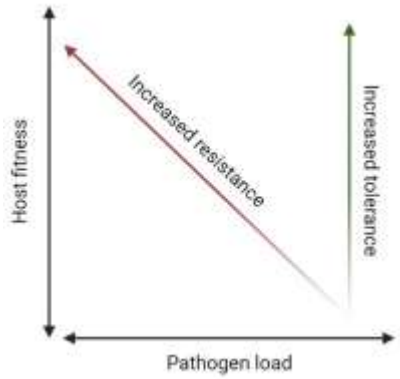


Figure 4| Tolerance and resistance concepts in infectious diseases. Changes in the degree of tolerance directly affects the fitness of the host, but does not affect the pathogen concentration. However, a decrease in resistance allows an increase in the pathogen load and diminishes the host fitness. Based on Ayres and Schneider, 2008.

Many authors have reviewed the ecological definition of tolerance and how it applies to infections in animals (15–19). Ecoimmunologists aim to understand the costs and benefits those different immune strategies have on the host fitness (i.e., the trade-offs) and agree that neither the strongest immune response nor the minimum pathogen concentrations necessarily maximize host fitness and that the evolutionary consequences of these two strategies should differ. On the other hand, in biomedical research, to distinguish between tolerance and resistance and to study their correlation might lead to a better understanding of the actual causes of pathology, and thus an improvement in treatments.

The statistical framework established in plant ecology defines resistance as the inverse of the pathogen concentration; when all the other variables are equal, a lower

concentration means the host is more resistant. Tolerance, on the other hand, is defined as the slope when plotting the maximum parasite load and the fitness of the host in 2-dimensional health-by-microbe space; the flatter the slope, the higher the tolerance (Figure 5) (20). In ecology and evolutionary biology, this description of how specific individuals respond to different environmental conditions is known as the "reaction norm" and considers the different pathogen burdens that can be applied to infections (21).

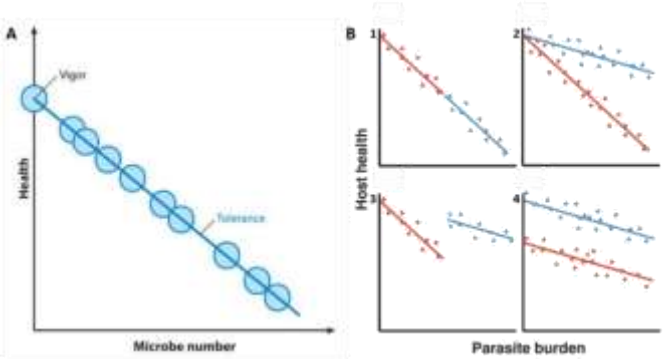


Figure 5| (A) Graphical definitions of resistance and tolerance. Vigor is defined as the health of the host in the absence of the parasite. Tolerance is defined as the slope of the curve, while resistance is defined as the inverse of the mean parasite load in the system – more resistant hosts have less parasites. **(B) Schematic representation of reaction norms of two different types of hosts. (B1)** Both types of hosts are equally tolerance but differ in resistance; the red host has lower parasitic burden and thereby maintains a higher health status. **(B2)** Both are equally resistant but the blue is more tolerant. **(B3)** The host types differ both in tolerance and resistance. **(B4)** The hosts differ in the general vigor when uninfected but not in tolerance nor resistance. Adapted from Ayres and Schneider, 2012 and Råberg, 2007.

1.2.2| Tolerance in TB

Mtb has coevolved with humans for more than 70,000 years and has achieved an evolutionary trade-off that, as mentioned in section 1.2, rarely affects the host survival (22). This trade-off has been long associated with the host resistance and its ability to avoid Mtb growth inside the host. Thus, mice and guinea pigs have been the most used animal models to study TB being considered resistant and susceptible, respectively (23). However, some years ago our unit postulated that mice should be considered as tolerant hosts instead (11) due to the observation that infected mice generate poorly structured granulomas which allow the systemic dissemination of the infection and the presence of a higher bacillary load but survive longer, whereas guinea pigs induce a human-like granuloma which delays the systemic dissemination but generate a stronger inflammatory response that ends up killing the host (11). Some studies also showed spatial compartmentalization of the immune response, being the pro-inflammatory response mainly found in the core of the granuloma while

anti-inflammatory signalling is found in the periphery (24). The balance of the pro- and anti-inflammatory signalling improves the granuloma's ability to contain and kill the bacilli and reduces the probability of developing the active disease (25).

Host-directed therapies (HDT) are currently under active development, setting the focus on reducing the tissue damage derived from the inflammatory response and not on killing the bacilli. Thus, previous work done in our unit has shown excellent results with the administration of non-steroidal anti-inflammatory drugs (NSAID), such as ibuprofen and aspirin, along with the common TB treatment (26,27). However, the possibility that NSAIDs may produce adverse effects related to dose and length of treatment should be taken into account, especially with long-term therapies like the one for TB (28,29). To prevent these safety issues, the unit started to explore the idea of using low-dose tolerance as prophylaxis based on the positive results obtained in other inflammatory diseases (30–33).

Lately, the importance of regulatory T cells (Tregs) in TB has increased. Tregs are a lymphocyte subset population responsible for the maintenance of immune homeostasis and peripheral tolerance, by suppressing the immune response against persistent self- and non-self-antigens (34). The key cytokines involved in the function of Tregs are Interleukin (IL)-10, TGF-β and IL-35 (35–37). Tregs immunosuppressive function is achieved by three mechanisms: they interfere with the T-cell activation by dendritic cells (38), induce apoptosis of target cells (39), and induce metabolic disruption by consuming available IL-2 (40). Tregs and Th17 cells are closely intertwined, as TGF-β induces the transcription factors essential for the development of both of these subsets, FoxP3 (41) and RORγt (42). If IL-6 is also present the balance is tipped toward Th17 cells (43), whereas in absence of IL-6 FoxP3 inhibits RORγt function (44). The loss of this counterbalance between Tregs and Th17 is considered to be the key to numerous inflammatory and autoimmune diseases.

In infectious diseases, the role of Tregs is controversial as their presence helps to limit the damage caused by the inflammatory response, but also promotes pathogen growth (45–47). The same happens in TB. Initially, the presence of Tregs would seem to be detrimental as an inflammatory response is necessary to prevent Mtb growth. However, this inflammation needs to be controlled to avoid excessive tissue damage. Thus, a balance between these responses seems to be key in preventing the progression of the infection towards active TB.

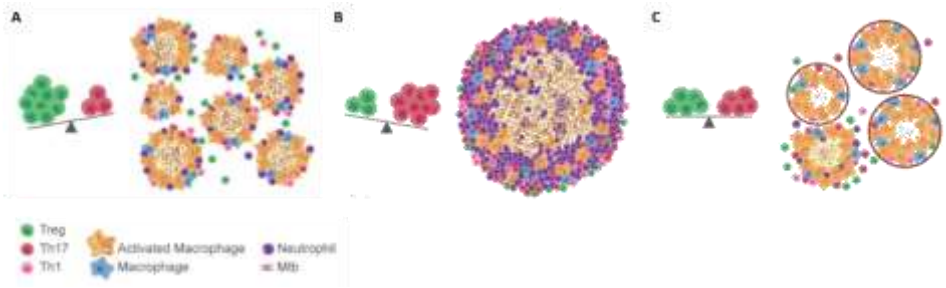


Figure 6| Hypothesis on the balance between Th17 and Tregs cells in the development of TB. In an immunosuppression context where Tregs are predominant, Mtb spreads more easily (A), whereas if there is a higher number of Th17 cells, inflammation and neutrophilic infiltration fuel the progression towards active TB (B). If a balance between these populations is achieves, the immune response is effective in encapsulating the lesions and stop Mtb spread (C). Edited from cardona P., 2019.

1.2.3| Oral tolerance

The gut-associated lymphoid tissue (GALT) is considered the largest immune organ in the body (48). It is constantly exposed to a large number of antigens and it needs to efficiently distinguish between beneficial and harmful stimuli, such as commensal or pathogenic bacteria, and respond accordingly. In this context, the role of the GALT is to maintain a tolerogenic environment that allows the presence of dietary and microbiome antigens and protects against pathogens (49).

The immune response in the gut is induced in the Peyer's patches, the mesenteric lymph nodes (MLNs) and the microfold cells (M cells). The MLNs serve as a communication path between the peripheral lymph nodes and the intestinal mucosa, while the M cells are involved in the transfer of material from the lumen to the GALT (50). For an antigen to induce a mucosal immune response, it needs to cross the mucus layer and the epithelial barrier to gain access to the dendritic cells (DCs), which can also sample the contents from the lamina propria (LP) through the epithelium without disrupting the tight junctions (51). The further migration of the DCs to the MLNs is crucial for the induction of oral tolerance (52). These gut-associated DCs never reach the circulation (53) and possess tolerogenic and immunoregulatory properties such as stimulating the expression of homing molecule CCR7 on T cells from the MLNs and the induction of Tregs (54,55). The production of retinoic acid (vitamin A metabolite) by DCs, together with the expression of the TGF-β, were shown to restore the balance between pro-inflammatory Th17 cells and Tregs (56,57), whereas intestinal inflammation impairs this tolerogenic function of the GALT (58).

One of the prime determinants of oral tolerance is the dose of antigen fed. Continuous feeding of low doses favours the induction of Tregs (49), while higher doses induce

unresponsiveness or anergy mechanisms in which T cells are defective in immunologic synapse formation with antigen-presenting cells (APCs) (59). The expression of homing molecules that keep the newly generated Tregs in the gut as well as regular stimulation by gut antigen are both essential for maintaining oral tolerance (60). Another characteristic feature of oral tolerance is that it can involve the whole animal. One potential organ that may participate in the induction of oral tolerance is the liver. This organ receives large amounts of gut-derived antigens through the portal vein, which drains blood from the gut. The induction of antigen-specific tolerance due to direct administration of antigens into this vein has been observed (61), although certain shreds of evidence were seen that systemic dissemination of fed antigens is not important for oral tolerance (52,62,63) and that tolerance is mainly induced locally in the MLNs. Several studies performed in the murine model showed that both mice lacking MLNs and mice whose DCs had their migration to the MLNs impaired were not able to induce oral tolerance (64,65). The mechanisms of induction of oral tolerance in the gut are illustrated in Figure 7.

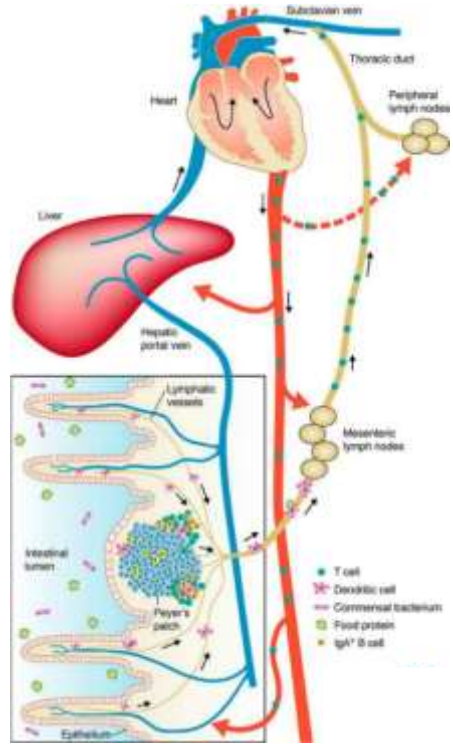


Figure 7| Functional anatomy of systemic induction of immune responses by intestinal antigens. Antigens present in the intestine can pass into the bloodstream through the hepatic portal vein or be taken up by DCs and carried to the MLNs via the afferent lymphatics. Although it is possible for circulating antigens to tolerize T cells in the liver, presentation in the MLNs is the dominant tolerogenic pathway. Because very few commensal microbes can be carried to the MLNs by DCs, systemic tolerance to these live organisms is not induced. Extracted from Macpherson and Smith, 2006.

1.2.3.1| Microbiota and probiotics

The microbiota is the sum of the commensal microorganisms that help the organism in key physiological processes in exchange for nutrients and a niche to grow. In humans, the Firmicutes and the Bacteroidetes are the most prevalent phylogenetic types (66). The presence of this microbiota is crucial for creating the proper environment in which oral tolerance can be developed (67). Thus, the recognition of intestinal bacteria teaches the GALT to induce a stimulatory immune response against pathogens and a tolerogenic response in the case of commensal microbes, and cells from the innate immunity are the principal ones responsible for this recognition.

Pathogen-sensing molecules, such as Toll-like receptors are down-regulated in the apical membrane of the gut epithelial cells (enterocytes), compared to the basolateral side. Thus, these receptors prime tolerance to cell wall components such as lipopolysaccharides (LPS) and peptidoglycans (PGN) of commensal bacteria that are detected in the apical membrane, but respond to basolateral detection of these antigens (68). Another mechanism that induces tolerance to LPS is the concentration of alkaline phosphatase close to the apical membrane, which dephosphorylates LPS and prevents LPS-dependant induction of Toll-like receptor 9 (TLR9). Thus, in the presence of normal levels of LPS from commensal microbiota, the alkaline phosphatase inhibits LPS recognition inducing a tolerogenic response (69). A growing number of studies show that intestinal microbiota also modulates the Treg-mediated response (70–73).

Gut microbiota and its metabolites are highly related to lung microbiota through the gut-lung axis. In TB, gut microbiota-derived metabolites butyrate and propionate, have been shown to reduce IL-17 production, thus suppressing Th1 response and increasing the number of Tregs (74–76), while propionic acid could inhibit Mtb growth by disturbing tryptophan biosynthesis (77,78). Also, there is a strong correlation between gut and lung microbiota; microbiota diversity increases or decreases simultaneously in both organs and directly affects the local immune response and granuloma formation (79,80). Thus, several studies have compared gut microbiota between TB patients and healthy people, as well as the effect that anti-TB treatment has on gut microbiota (81) and have shown that TB patients have a reduced microbiota diversity and numbers, mainly affecting Bacteroidetes phylogenetic type, compared to healthy individuals and that this microbiota changes along with the progression of the disease.

In this sense, the use of probiotics and postbiotics as a preventive strategy against infectious diseases has raised and they could also be used in TB prevention and treatment. Probiotics are defined as “live microorganisms which when administrated

in adequate amounts confer a health benefit on the host" (82), while postbiotics are inactivated microbial cells and/or their components that also confer beneficial effects on host health (83). These products modulate the host microbiota, regulate the innate immune response, stimulate epithelial cell growth and improve barrier function (84–86). Some examples could be the antimicrobial activity against Mtb that several *Lactobacillus* species have shown in vitro (87), the restorage of the functions of DCs and T cells against Mtb in dysbiotic mice by the administration of *Lactobacillus plantarum* (88), or the strong inhibition that antimicrobial agents produced by *Lactococcus lactis* have on Mtb growth *in vitro* (89,90).

1.2.3.1.1| Heat-killed mycobacteria as oral tolerance inducer

As Th17 lymphocytes and Tregs are in counterbalance in Mtb infections, this led to the idea of increasing Tregs to reduce the presence of Th17 lymphocytes. The oral administration of heat-killed mycobacteria in the C3HeB/FeJ murine model, showed a reduction in the bacillary load and a decrease in the damaged area in the lungs of infected mice, compared to untreated individuals, which lead to an increase in survival. Several mycobacteria were tested, ending up considering the environmental *Mycliciobacterium manresensis* as the best candidate. This is a fast-growing environmental mycolicibacterium isolated from the Cardener river in Manresa, belonging to the *Mycolicibacterium fortuitum* complex and had shown low toxicity in mice (91).

Further characterization of the effect of the treatment on the immune system showed the induction of a population of purified protein derivative (PPD)-specific memory Tregs cells and a reduced inflammatory milieu in the lungs of treated infected mice, which protects against progression towards active TB and relapse after treatment (91). Currently, clinical trials are being carried out to demonstrate the safety, non-toxicity and effectiveness of the oral administration of low doses of heat-killed *M. manresensis* (hkMm) against TB (92).

1.3| *Drosophila melanogaster* as a model for infectious diseases

The fruit fly *Drosophila melanogaster* is a whole-animal model system that has been highly used for the understanding of fundamental principles of genetics (93) and regenerative biology (94), as well as for human diseases and drug discovery (95,96) for over a century. One of the principal advantages of this model is the rapid life cycle and the possibility to control it through temperature: a single fertile mating pair can produce hundreds of genetically identical offspring within 10 days at 25ºC and twice as long at 18ºC. In addition, every developmental stage has its advantages for specific fields. The embryo is often used for studying organogenesis, neuronal development

and cell fate determination, while the larva is mainly used to track the evolution of undifferentiated epithelium, named imaginal discs, which contain the future structures of adult flies, providing significant insight to both fly and human biology (95,97) (Figure 8). Finally, the adult fly is not so different from higher organisms and has conserved structures that perform the equivalent function of the mammalian organs and also conserves several key pathways and transcriptional regulators that are crucial for development, metabolism and immunity (Figure 9). Lately, with the increasing interest on finding new animal models that might help reduce, refine and replace the current mammal models, *Drosophila melanogaster* has begun to be considered an essential tool for the study of several human infections. However, very few studies have focused on mycobacterial infections.

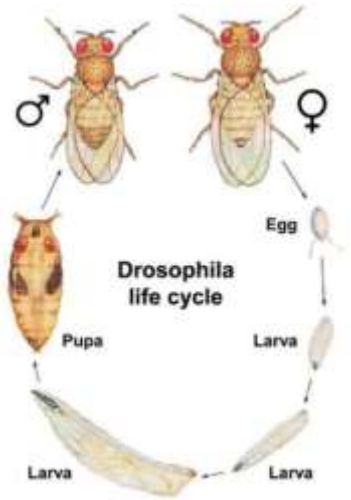


Figure 8| The Drosophila melanogaster life cycle. A mated female can lay up to 100 fertile eggs 24h after fertilization by the male sperm. There are three larval stages which take altogether abouts 4 days. During larval growth is when most cell types get differentiated. The pupal stage starts after the encapsulation of the 3rd instar larva and lasts around 4 days. Finally, adult flies emerge upon eclosion of the pupal case. The mean lifespan of adult flies is of 60-90 days. Extracted from Torres et al., 2011.

1.3.1| Systemic immune response

The living organisms rely on both innate and adaptive immune components to defend themselves from constant exposure to microbes and pathogens. Although for many years researchers have focused on the importance of the adaptive response based on its ability to generate a vast repertoire of specific recognition receptors and immunological memory, some studies have proved that innate immunity is an ancient defence mechanism that has evolved along with the hosts (98,99). The use of

Drosophila melanogaster as a model has provided a huge insight into the mechanisms of action of the innate immunity, as insects rely solely on this type of response thus avoiding the variability that adaptive mechanisms imply. Also due to the high homology of the genes that determine it with humans; approximately 75% of human disease genes have homologs in *D. melanogaster* (100).

As in vertebrates, *Drosophila*'s immune system is also divided into humoral and cellular responses, although the mode of communication between them is not completely understood yet. However, many studies have tried to shed a little light on this. For example, septic injury or exposure to LPS triggers expression of Unpaired (Upd) 3 in the hemocytes, which in turn activates JAK/STAT signalling in the fat body, leading to upregulation of immune genes (101) or that the loss of hemocytes induces the upregulation of the Toll pathway and the downregulation of the immune deficiency (Imd) pathway (102), and also results in a weakened immune response at short-term and a decrease in long-term survival after infections (103,104).

1.3.1.1| Cellular immune response

Drosophila has a primitive open blood circulation system with blood cells freely circulating in the hemolymph or associated with diverse tissues. These cells, which are collectively called hemocytes, can be differentiated into three cell types (plasmatocytes, lamellocytes and crystal cells) with different morphology and immunological functions (105,106) (Figure 9). In addition to their obvious role in immune defence against pathogens, *Drosophila* hemocytes also have crucial functions during embryogenesis and their study has expanded the knowledge about cell migration mechanisms during development and the regulation of chemotaxis during inflammation (107).

Phagocytosis represents a fundamental process of the innate immune response and in the maintenance of tissue homeostasis. In *Drosophila* this process is mainly performed by plasmatocytes, which represent up to 90% of the total circulating hemocytes (108) and are defined as homologs of vertebrates monocytes, especially macrophages. As in vertebrates, various receptors involved in phagocytosis have been described in *Drosophila*, such as Eater for Gram-positive bacteria, peptidoglycan recognition protein (PGRP)-LC for Gram-negative bacteria or Peste for mycobacteria (109–111). In addition, PGRP-SC1 and various thioester proteins (TEPs) have been described to also act as an opsonin, and therefore, contribute to bacterial phagocytosis (112,113). Interestingly, a CD36-related receptor, named Croquemort, was described to be essential for phagocytosis of apoptotic cells (114) and also to be required for proper phagosome maturation and thus an effective clearance of bacterial and fungal infections. Flies defective for this receptor entered

a state of chronic immune activation with increased levels of Upd3 production, which induced intestinal stem cell proliferation and lead to loss of gut homeostasis and shorten lifespan (115). Comparative studies of host defence genes have also revealed host factors involved in preventing intracellular growth of pathogens (116). Some examples are Rab7, CG8743 and ESCRT machinery, in which manipulation of only one of them results in intracellular growth of the otherwise non-pathogen *Mycolicibacterium smegmatis* (117) or the lysosomal enzyme beta-hexosaminidase which specifically constrains intracellular growth of *Mycobacterium marinum* (118).

Melanisation is another predominant immune response in insects based on the production and release of melanin around intruding microorganisms (119). This response is involved in wound healing, phagocytosis, hemolymph coagulation and antimicrobial peptides (AMPs) expression (120,121), and is mediated by the crystal cells that account for 5% of circulating hemocytes. These cells store inactive pro-phenol oxidase (PPO) that under stimuli is cleaved into active phenol oxidase (PO), which is the main enzyme in melanin biosynthesis (122). This process is tightly regulated to prevent the excessive formation of intermediates that are toxic to the host. Several studies also highlighted the role of PGRP-LC and PGRP-LE in melanisation induction (123,124).

Lastly, another cellular response found in *Drosophila* is the encapsulation of foreign bodies that are too large to be phagocytosed (125). This process happens in three stages: first, hemocytes recognize the non-self-structure; second, the number of circulating hemocytes increases, lamellocytes differentiate from plasmatocytes and they attach to the foreign structure and each other and create a multi-layered capsule; finally, the lysis of crystal cells triggers the melanisation of the capsule and the encapsulated pathogens are killed by asphyxiation or by the toxic compounds released by the capsule (126–129).

1.3.1.2| Humoral immune response

The main mode of action of the humoral response in *D. melanogaster* is the induction of AMPs. These are small (<40 amino acids), amphipathic molecules that are encoded in the genome of the host, have a broad spectrum of antimicrobial and antiviral activity and are produced by almost all living organisms, from bacteria, to plants and animals, both vertebrates and invertebrates (130). Up to 7 AMPs have been described in *Drosophila melanogaster*. In addition to having conserved sequences, the promoter regions of these molecules contain binding sites similar to the ones found in mammals for NFκB/Rel proteins and mutations in these regions are shown to reduce the immune-dependant expression of these genes (131,132).

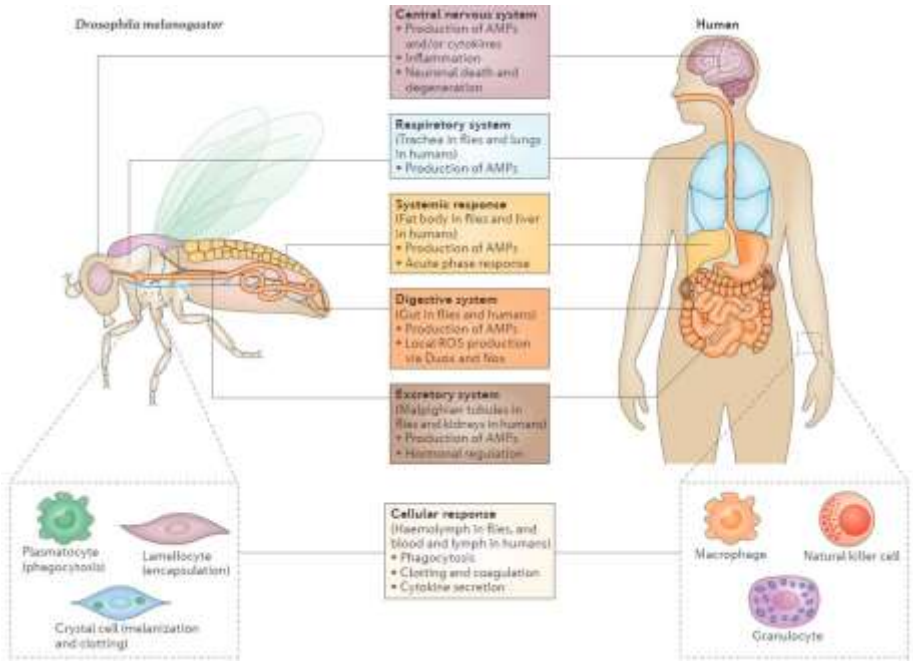


Figure 9| Innate immunity in Drosophila melanogaster compared to humans. The organ systems in Drosophila are analogous to those in vertebrates. The gut absorbs nutrients, the fat body stores and sense them, as the mammalian liver and adipose tissue, and the Malpighian tubules carry out the same function as the kidneys. The flies' circulatory system is open; the heart is essential for the circulation of nutrients and immune cells, but oxygen is provided by an independent tracheal system. Similar to mammals, flies have different types of circulating immune cells known as hemocytes as a whole. The fat body is also the main source of AMPs after a systemic infection, which are released to the hemolymph to induce a systemic response, while barrier epithelial are also capable to induce production of AMPs locally. The D. melanogaster central nervous system coordinates both organism physiology and immunity through the secretion of hormones. Extracted from Buchon et al. 2014.

After a systemic infection, AMPs are released into the hemolymph where they persist for several days and can protect the flies against a second exposure to the pathogen (133). This response is mediated by the fat body, which is the equivalent of the liver in mammals and represents the main immune-responsive organ in the fly (129). Barrier epithelial cells are also able to secrete AMPs and reactive oxygen species (ROS) in response to a localized infection (134). Three main signalling pathways have been described to play a role in the regulation of immune genes induced after infection. The Toll- and the Imd- pathways regulate the majority of immune genes, including the production of AMPs, while the JAK/STAT contribute to the regulation of other infection-induced genes (135,136).

1.3.1.2.1| Toll pathway

The Toll signalling pathway was first related to *Drosophila* embryonic development, due to the key role of its downstream protein, dorsal, in the correct development of the dorsoventral pattering of the early embryo (137). It was not until 1995 that its role in the immune defence against microbes in the fruit fly was introduced (138).

This pathway is the homolog of the mammal Toll/Interleukine-1 receptor (TIR) signalling pathway and controls the systemic AMP production by the fat body cells in response to Gram-positive bacteria and fungi (139). However, unlike in mammals, the Toll receptor does not directly interact with the microbial components, but with the cleaved form of the cytokine-like molecule Spätzle. The spätzle-processing enzyme (SPE) is responsible for this cleavage and is regulated by the detection of the lysine-type peptidoglycan (PGN), characteristic of Gram-positive bacteria, by the circulating PGRP-SA and -SD, and the Gram-negative binding protein (GNBP) 1 (140–142). The β-1-3-glucans of the fungal cell wall and the microbial-secreted virulence factors also activate SPE when recognised by GNBP3 and Persephone, respectively (143,144). Recently, the serine protease ModSP has been identified as the mediator molecule that connects PGRP-SA and GNBP3 to SPE (145). In addition, the Toll pathway has been described to be essential in the defence against acid-fast bacteria (146) and to play a role in viral infections (147). Up to 9 Toll proteins have been described in *D. melanogaster*, including Toll-7, which recognizes viral glycoproteins directly (148), and Toll-8, which down-regulated NF-κB signalling in the respiratory epithelial cells (149).

Once Spätzle binds to the extracellular part of the Toll receptor, induces the receptor's internal region dimerization and the recruitment of the intracellular adaptors *Drosophila* myeloid differentiation factor 88 (dMyD88, homolog of MyD88), the serine-threonine kinase Tube, and Pelle (homolog of IL-1R associated kinase) (150,151). This cascade culminates with the phosphorylation and subsequent degradation of Cactus (inhibitor of kappa B, IκB), which under no stimuli is attached to the Rel-family proteins (NFκB) Dorsal-related immunity factor (Dif) and Dorsal preventing their translocation to the nucleus where they would act as transcription factors for Toll-regulated genes (131,152–154). To prevent an excessive immune response, this pathway is highly regulated through the expression of the Toll-dependant Rel-family inhibitors Cactus and wnt inhibitor of Dorsal (WntD), creating a negative loop (155,156). Also, the protease inhibitor Serpin (Spn) 1 regulates the GNBP3-dependant activation of SPE (157). The pathway is illustrated in Figure 10.

Recently, the *Drosophila* model has been used to study the importance of post-translational modifications on the regulation of Toll signalling that can imply changes

in the protein localization, activity and/or the ability to bind its target. Some examples of this regulation are the Ubc9-mediated sumoylation of Dorsal (158) and the interaction of β-arrestin Kurtz (Krz) with the SUMO protease Ulp1. Mutants for either Krz or Ulp1 exhibit a pro-inflammatory phenotype with high levels of lamellocytes, accumulation of Dif and Dorsal, and increased expression of *Drosomycin* (Droso) (159).

1.3.1.2.2| IMD pathway

This pathway is the homolog of the tumour necrosis factor receptor 1 (TNFR) signalling pathway in mammals and controls the expression of AMPs both at a systemic level in the fat body cells and at a local level in response to natural infections in intestinal and tracheal epithelial cells (139). This pathway is mainly activated when monomeric or polymeric meso-diaminopimelic (DAP)-type PGN of Gram-negative and certain Gram-positive bacteria (e.g. *Bacillus subtilis*) binds to the transmembrane receptor PGRP-LC (160,161). Also, as the mammalian TNFR pathways play a major role in the antiviral response, the fruit fly model has served to gain knowledge on the role of the Imd pathway against viral infections (162,163).

The activation of PGRP-LC induces the recruitment of the intracellular adaptor molecule Imd (164), the Fas-associated death domain protein (dFadd) and the caspase-8 homolog, Dredd (165). Dredd is activated by ubiquitination by the *Drosophila* inhibitor of apoptosis-2 (dIAP2) (166,167). Activated Dredd cleaves Imd removing the N-terminal fragment and creating a new binding site for dIAP2 which ubiquitinates and activates Imd (168). This leads to the recruitment of the transforming-growing-factor-β activating kinase (dTAK1) and its activation by the TAK-1 binding protein 2 (TAB2). The activated dTAK1 phosphorylates the IκB-kinase complex (169), which in turn phosphorylates the NF-κB-like protein Relish (170). As in mammalian Rel proteins, an inhibitory domain is contained in the C-terminus of Relish, which hides the nuclear localization signalling in the N-terminal and inhibits the protein dimerization by the Rel homology domain (171). Thus, the activation of Relish also requires the cleavage of the C-terminal domain (Rel-49), and the dimerization of the N-terminal domain (Rel-68), which migrates into the nucleus and induce the expression of the Imd pathway-related genes, such as *diptericin* (Dpt) and *cecropin* (Cec). This cleavage is likely produced by Dredd (171,172). Another homology with the mammalian TNFR signalling pathway is that the Imd pathway also bifurcates into the Janus Kinase (JNK) pathway at the TAK1 level (173). The Imd pathway is illustrated in Figure 10.

In addition to the transmembrane receptor PGRP-LC, it has been described as a role for PGRP-LE in the activation of the Imd pathway. This receptor can be found in two forms (174,175). The short form of this protein is secreted and binds PGN in the haemolymph and brings them to PGRP-LC. It can also induce a pro-phenoloxidase cascade together with PGRP-LC. The long-form is the only intracellular PGRP described and recognizes monomeric PGN fragments known as tracheal cytotoxins (TCT) that have managed to get into the cell. In addition, the intracellular form can interact directly with Imd and activate the Imd pathway independently of PGRP-LC as well as induce autophagy. Ectopic expression of PGRP-LE in the fat body cells is sufficient to activate the AMP expression in absence of infection (123,124,174,175). Also, a role for PGRP-LA being a positive regulator of the Imd pathway in barrier epithelia has been described (176).

As happens with Toll, the Imd pathway is also highly regulated mainly in three ways. First, some secreted PGRPs (-SC1, -SC2, -SB1) have been described to have an amidase activity and digest PGN, converting them into non-immunostimulatory fragments. PGRP-LB also has amidase activity and is upregulated by the pathway itself creating a negative loop and is the major regulator of this pathway in the gut (177). In addition, the transmembrane receptor PGRP-LF does not bind PGN, but it does bind to PGRP-LC creating a non-signalling complex that competes for PGN recognition (178). In addition, several proteins block the signalling cascade at different levels. Caspar and Dnr1 block the cleavage of Relish by Dredd (179,180), Pirk/Rudra/PIMS disrupt the Imd-PGRP-LC association (181–183) and Caudal prevents Relish from binding to the promoters of the Imd target genes (184).

1.3.1.2.3| JAK/STAT pathway

The JAK/STAT signalling pathway was first described in mammals and was shown to control several biological processes including tissue homeostasis and humoral immunity in response to stress conditions, such as infection or cellular damage (185). Further studies in mammalian models revealed a canonical pathway based on a transmembrane cytokine receptor that dimerizes and recruits two tyrosine kinases, JAKs, that phosphorylate each other. This allows the cytosolic STATs to bind to their associated receptors. JAK-mediated phosphorylation of STATs results in the dimerization of these molecules and their translocation to the nucleus where they act as transcription factors (185). In mammals, up to 4 *JAK* and 7 *STAT* genes have been described as well as many cytokines and growth factors have been shown to activate the pathway (139). As in mammals, this pathway regulates a wide range of biological processes in the fruit fly, such as hematopoiesis, stem cell self-renewal, long-term memory formation, circadian behaviour, as well as, immune response (101,186–191). This pathway has also been reported to interact with many others involved in

development such as Notch and Hedgehog pathways (192). However, the first evidence for its role as an immune modulator in invertebrates was shown in the mosquito *Anopheles*, with the observation that a STAT protein accumulated in the nucleus after infection (193).

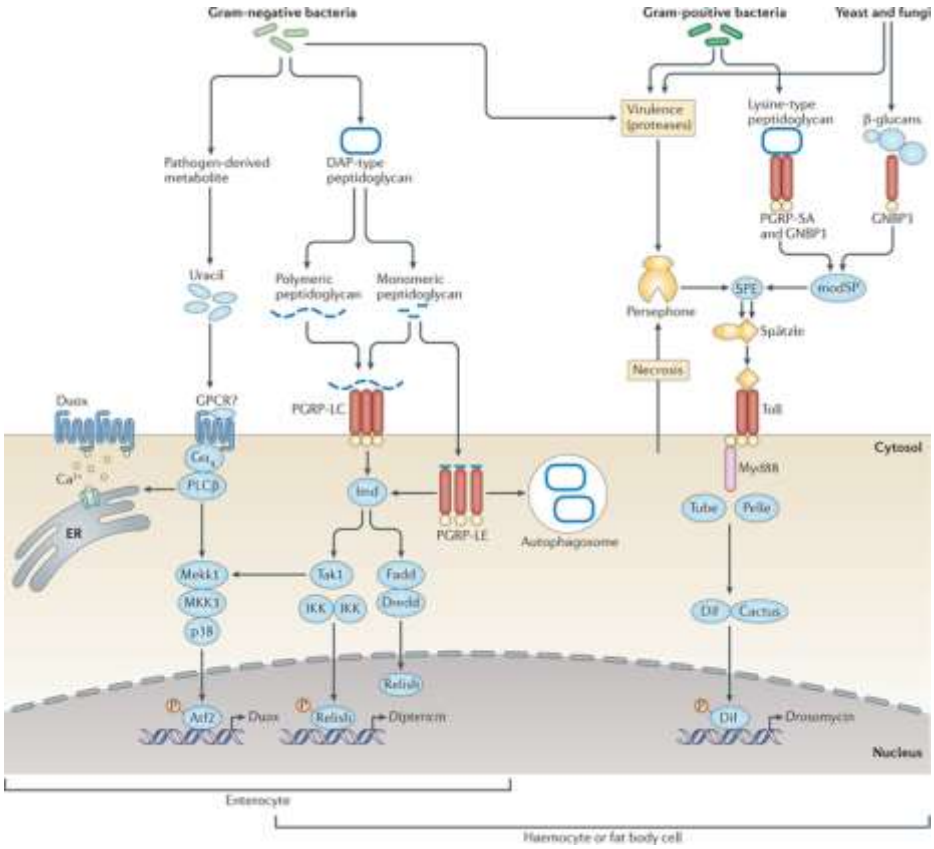


Figure 10| Humoral immune response in Drosophila melanogaster. Both Imd and Toll pathways control the systemic production of AMPs when activated in the fat body in response to the detection of microbial cell wall components. The Imd pathway is also activated in epithelial barriers and controls local production of AMPs and ROS. PGRP-LC and PGRP-LE bind to DAP-type peptidoglycans and activate Imd signaling. Imd binds to FADD, and then the caspase Dredd is recruited. Dredd cleaves Imd, which is then activated by ubiquitination, and connects to TAK1. TAK1 is responsible for activating the IKK complex, which phosphorylates the NF-κB-like transcription factor Relish. Dredd is also required for the cleavage of Relish precursor. Nuclear translocation of Relish activates the expression of AMP genes. The Toll pathway is activated when the processed form of Spätzle binds the receptor. This process triggers the dimerization of the TIR domains and promotes binding of the adaptor proteins Myd88 and Tube, which in turn recruits the protein kinase Pelle. Once Pelle is autophosphorylated, it triggers the phosphorylation and destruction of the inhibitor Cactus, which allow nuclear translocation of the transcription factors Dif or Dorsal, depending on the context. Extracted from Buchon et al., 2014.

The *Drosophila* model has been highly used for the study of this pathway as it provides a less complex yet complete version of the pathway only based on one transmembrane receptor (Domeless/Dome) (194), one JAK (Hopscotch/Hop)(195) and one STAT (STAT92E/Marelle) (196,197). Also, only three cytokine-like molecules have been described as ligands for this pathway, called unpaired, which share similarities with mammals' leptins (198). Although all three molecules are induced locally in response to tissue damage Upd is associated with the extracellular matrix and induces a strong response (199,200), Upd2 is secreted and induces the longest response (200,201). Upd3 is also secreted and expressed in haemocytes of adult flies in response to bacterial infections and is required for the activation of the pathway in the fat body cells (101). Both Upd2 and Upd3 are also induced in response to viral infections (202).
Although the antimicrobial production in *Drosophila* is mainly regulated by the Toll and the Imd pathways, the JAK/STAT pathway also contributes to the humoral response by inducing the expression of several additional immune proteins in the fat body after the Upd3 production by the haemocytes in response to infections or other stresses, such as Thioester-containing protein (Tep) and Turandot (Tot) protein families. The Tep family have a high similarity with proteins of the complement C3/alpha2-macroglobulin (α2M) superfamily that is expressed at basal levels in the fat body cells and larval haemocytes but are highly induced upon immune challenge (203). The expression of *totA* is induced by the Upd3-dependent activation of JAK/STAT, although it also requires Relish induction (101).

As this pathway is involved in many biological processes, it needs to be highly controlled and many regulators are as conserved as the pathway itself. First, the eye transformer (ET) non-signalling protein (resembles mammalian gp130), which share structural similarity with the receptor Dome but has a shorter cytoplasmatic tail and cannot bind Stat92E, seems to play a role in inhibiting the signalling cascade, although the precise mechanism is not completely known yet (204,205). Also, three suppressors of cytokine signalling (SOCS) proteins have been described so far, although only SOCS36E is responsible for the main negative feedback loop regulator of the pathway (206). As in mammals, SOCS36E has two main domains: a central SH2 domain that possibly interferes in the Dome receptor phosphorylation via Hop, and a C-terminal SOCS box that interacts with ubiquitinating enzymes and regulate the lysosomal degradation of Dome (207,208). The *Drosophila* homologs of the Ras-like guanine nucleotide-binding-protein 3 (RanBP3) and RanBP10 control the STAT92E translocation to the nucleus (209) and the protein inhibitor of activated STATS (PIAS) seems to act as the mammalian one by blocking the binding of phosphorylated STATS to DNA (210,211). The JAK/STAT pathway is illustrated in Figure 11.

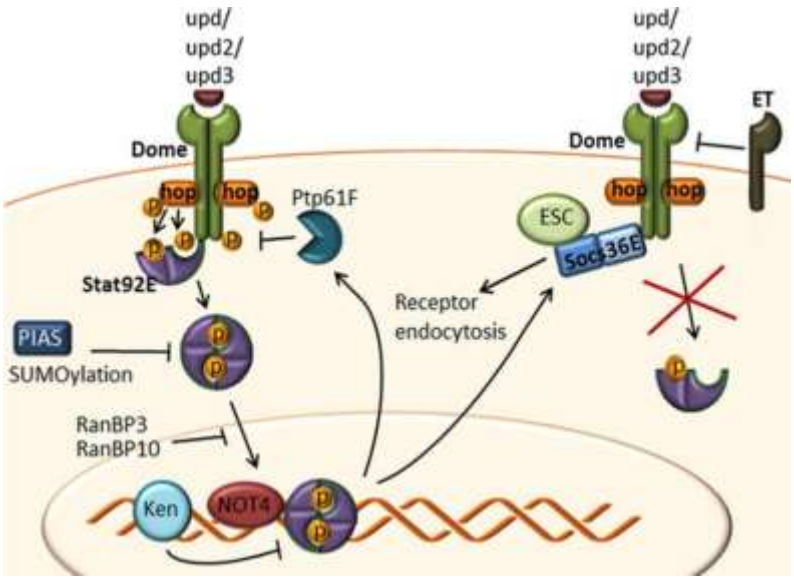


Figure 11| The JAK/STAT pathway in *Drosophila melanogaster*. The cytokine receptor, Domeless (Dome) is activated by binding Upaired (Upd) cytokines. This causes the JAK kinase Hopscoth (Hop) to phosphorylate itself and the tail of Dome. The transcription factor Stat92E binds the receptor and is phosphorylated by Hop. This promotes Stat92E to dissociate from the receptor, dimerize and translocate to the nucleus, where it induces the transcription of thioester-containing protein genes (Teps) and Turandot (Tot) stress genes. Ptp61F, Socs36E and ET act as inhibitors of the pathway at different levels. Extracted from Rämet and Myllymäki, 2014.

1.3.2| Epithelial immune response

It is important for all living organisms to have an inducible immune response in the epithelial and mucosal barriers as these are the first line of defence to prevent the entrance of potentially pathogenic microbes. In *Drosophila*, the mechanisms by which a septic infection causes a systemic response controlled by the fat body have been well-characterised, even though these types of infections are rare. However, although the so-called natural infections take place constantly, the epithelial immune response remains poorly characterised. It is known that this local response is mainly regulated by the Imd-pathway (212) and is shaped against different microbes via the JAK/STAT and the JNK signalling pathways (213). In 2013, a microarray analysis performed by Lemaitre et al. showed that the induction of Imd-dependant genes varies substantially among tissues with only very few "universal genes" being expressed in the fat body, the gut, and the trachea (176). This set of genes includes mainly AMPs and pathway components.

1.3.2.1| The respiratory epithelia

The respiratory tract is similar among all animals, being made of a complex network of branched tubules. In *Drosophila* this structure is the simplest epithelial organ, comprising only one type of epithelial cells organized in a multi-branched monolayer that surrounds the lumen, where the gases are transported (214). Several pattern recognition receptors are expressed in the tracheal epithelial cells, such as 4 Toll-receptors (*toll*, *18 wheeler*, *toll-7* and *toll-8*), GNBP 1 and 3, and several members of the PGRP gene family (with the only exception of -SB1 and -SB2) (215). Genes encoding for several enzymes responsible for oxidative stress response are also constitutively expressed in the tracheal cells (215) as well as the gene encoding for Transferrin1, which in humans has been associated with inhibition of bacterial growth in the lungs (216).

The natural infection of the respiratory tract in *Drosophila* induces the expression of genes from the Imd-, the JNK-, and the JAK/STAT pathways (215). It also induces the Imd-dependant reactivation of a group of developmental genes that are not activated in the fat body to promote the renewal of the tissue damaged (213). The production of AMPs seems to be induced only by the Imd-pathway, as the key components of the Toll-pathway, *pelle* and *tube*, are not expressed in tracheal epithelial cells (215). Thus, the expression of the typically Toll-dependant AMP Drosomycin is difficult to explain, even though this situation has been also reported in other epithelial barriers (134). The increased expression of the apoptosis-inducer Autophagy-related (Atg)1 has been also reported after infection, however not dying cells have been observed potentially due to an effective counteract of these cells through the production of Thor and forkhead box, sub-group O (Foxo) (213). Since these epithelial cells are constantly exposed to potentially pathogenic microbes, the immune response must be quick but also highly regulated. Two serpins, Spn28D and Spn77Ba, prevent the phenol oxidase activity (217,218) and Tollo downregulates the Imd-dependant expression of AMPs, although the mechanism reminds unknown (149).

1.3.2.2| The intestinal epithelia

As in vertebrates, *Drosophila* intestinal system needs to adapt to metabolism and nutritional changes, ensure epithelial integrity and control both transient pathogens and commensal microorganisms. *Drosophila* and mammalian intestines are similar both in structure and function, but *Drosophila* has a more simple microbiota composed of only 2 to 30 bacterial species (219), mainly *Acetobacter* and *Lactobacillus*, although Proteobacteria and some species previously defined as pathogenic, such as *Providencia*, *Serratia*, *Erwinia* and *Enterobacteriaceae*, may also be present (220). Although germ-free flies fed with a rich food source are viable, the

gut microbiota is essential for development and gut homeostasis under limiting conditions. For example, generation of acid acetic by *Acetobacter pomorum* and alteration of amino acid levels by *Lactobacillus plantarum* accelerate larval development through activation of the insulin pathway (221–223). A recent study also showed that the presence of localized necrosis in adult flies lead to a systemic immune response mediated by commensal gut microbiota, since removing the microbiome attenuated the hyperactivation of the Imd pathway in necrotic flies (224). In addition, both gut microbiota and transient pathogenic bacteria stimulate intestinal turnover and intestinal stem cells (ISCs) proliferation through the production of Upd3 by gut epithelium cells during steady-state conditions or in response to tissue damage, respectively (225–229). A similar response is induced in mammalian lungs, with the production of IL-6 in response to injury (230). However, in the *Drosophila* gut, the innate immune response is mainly based on the production of AMPs and ROS (231).

ROS are induced by two enzymes: dual oxidase (Duox) is induced through pathogen-derived uracil and peptidoglycan (232) and NADPH oxidase (Nox) is induced by microbiota-derivate lactate (233). In the gut, Duox-derived ROS are mainly involved in immune response and repair tissue damage, while Nox-derived ROS regulate epithelial renewal (233). However, excessive production of ROS is ultimately toxic to the host and induces epithelial cell death and early ageing, thus Duox expression is tightly regulated through the p38 mitogen-activated protein kinase–activating transcriptional factor 2 (p38 MAPK – Atf2) pathway (234). The uracil secreted by some pathogenic bacteria activates the phospholipase Cβ (PLCβ), which in turn activates the p38MAPK-Atf2 pathway. The PLCβ pathway also promotes the release of Ca^{2+} from the endoplasmic reticulum to bind and activate Duox (235). Peptidoglycan also induces the expression of Duox through the activation of the Imd pathway at the TAK1 level (235). In response to commensals, the levels of both uracil and peptidoglycan are lower and the concentration of cytosolic Ca^{2+} is reduced, thus Duox activity is kept minimal (234).

On the other hand, the production of AMPs is crucial to control ROS-resistant bacteria (236). As in mammals, the induction of AMPs is compartmentalized (237), with the Toll pathway being non-functional in the midgut and the expression of Imd-activating receptors and inhibitors also varying throughout the gut (184,238). For example, PGRP-LE is the main receptor expressed in the middle and posterior midgut, which might prioritize an immune response against intracellular pathogens but also immune tolerance to microbiota through upregulation of pathway inhibitors Pirk and PGRP-LB. Loss of PGRP-LE-mediated detection of bacteria in the gut results in systemic immune activation that can be rescued by overexpressing PGRP-LB (239). In addition, the JAK/STAT pathway regulates the expression of three Drosomycin-type AMPs only in the midgut (240,241). As with ROS, a weak immune response leads to dysbiosis and

a dysregulated ISCs activation, but an excessive induction of the Imd pathway is also ultimately detrimental for the host. Thus, several negative regulators are expressed in the gut: enzymatic PGRPs, such as PGRP-SC or PGRP-LB; and inhibitory proteins as Pirk, PGRP-LF, SkpA and Caudal (242). Flies lacking PGRP-LB or Caudal were shown to have shortened lifespans which can be rescued by the elimination of commensal bacteria, thus showing the importance of a balanced immune response (184,243).

But not only infections can affect gut homeostasis. With age, microbial load and diversity increase in *Drosophila*, as well as the production of AMPs and ROS (228,244,245), which leads to tissue damage, increased dysbiosis and uncontrolled ISCs proliferation and differentiation. These age-related changes correlate with epithelial barrier failure and the induction of the systemic immune response (246,247). The mechanisms underlying these age-related mechanisms are not fully understood yet, however, it has been observed that there is a chronic expression of the transcription factor Foxo that reduces the expression of PGRP-SC2, leading to hyperactivation of the immune response (248,249). Similar age-related changes are observed in humans (250). The intestinal immune response of *Drosophila* is represented in Figure 12.

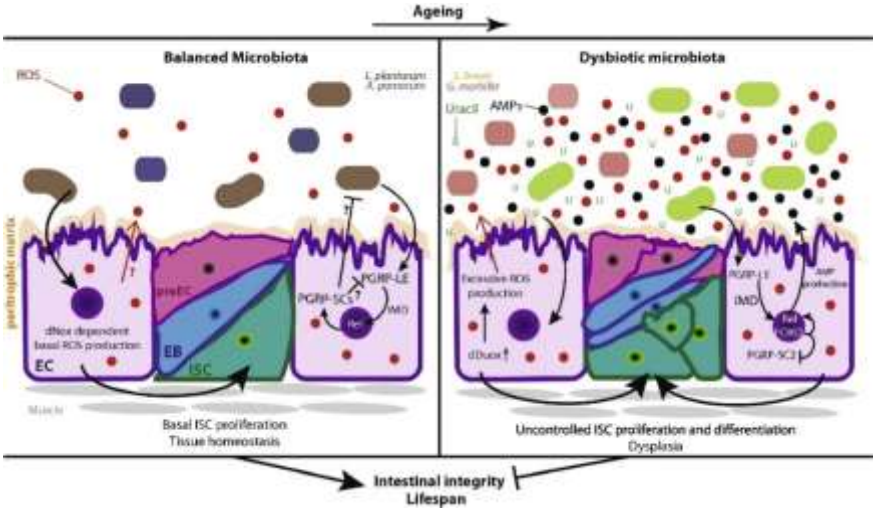


Figure 12| Drosophila melanogaster intestinal immune response. In young and healthy adult flies (left) the microbiota promotes basal Nox-dependent production of ROS, which stimulates ISCs proliferation, and also promotes a basal Imd induction through PGRP-LE and negative regulators. In ageing gut or after infection (right) Duox-dependent ROS production and expression of dFoxo are activated, and production of PGRP-SCs is reduced. This leads to dysbiosis, uncontrolled ISCs proliferation and reduced lifespan. Extracted from Erkosar and Leulier, 2014.

1.3.3| Systemic control of the immune system

It is well known that hormones and metabolic homeostasis, as well as circadian cycles, have central roles in the regulation of systemic physiology, including immunity, in both insects and mammals (251–253). To build an immune response is a high energy-demanding process and that energy has to be obtained from other physiological processes, such as reproduction and growth. Thus, a trade-off exists between immunity and host fitness.

1.3.3.1| Metabolic regulation

The link between metabolic homeostasis and immunity has been the centre of many studies in the past years. Insulin resistance and cachexia represent the most common comorbidities that come together with inflammatory processes, including infections (254,255), but an energy surplus due to high-fat diets or obesity also impairs the immune system and induces chronic inflammation (253). However, the complexity of the immune system in vertebrates difficult the complete understanding of this relationship (256). And here is where insects take a predominant role.

The insulin/insulin-like signalling (IIS) pathway is key for metabolic regulation in insects. In *Drosophila*, the fat body acts stores fat in form of triglycerides and releases factors that affect insulin production in response to nutrient sensing (257,258). The insulin-like peptides (dILPs) are mostly produced by the neurosecretory cells (also called insulin-producer cells, IPCs) in the brain and bind a single insulin-receptor (InR) (259,260). Activation of this receptor induces the phosphorylation and activation of AKT, which in turn phosphorylates the transcription factor dFoxo preventing its nuclear translocation. AKT also regulates Target of rapamycin (Tor), which inhibits autophagy (261). The reduction of activated AKT induces the dFoxo-dependent expression of genes involved in the loss of energy stores and AMPs production (262,263). The transcription factor myocyte enhancer factor (Mef) 2 is responsible for the shift in energy usage in the fat body. Under normal conditions, Mef2 is phosphorylated, potentially under AKT signalling, and promotes the expression of anabolic enzymes. After an immune stimulus phosphorylation levels decrease and Mef2 induces the transcription of immune genes, including Eiger (TNF-α homolog, and necessary for Imd activation in the fat body) (264). The activation of both immune signalling pathways in the fat body results in loss of energy storage and suppression of host growth: the Toll pathway seems to suppress dILPs signalling in the fat body (265,266) and Eiger binds the receptor of IPCs and reduce the production of dILPs in the brain (267).

Another link between metabolism and immunity is the metabolic switch that activated phagocytic cells undergo towards aerobic glycolysis, which is a highly energy-demanding process, induced by the hypoxia-inducible factor (Hif)1α (268). Recently, Bajgar et al., hypothesized about a "selfish immune system theory" (Figure 13), supported by several studies, based on the fact that activated phagocytes release signalling molecules (selfish immune factors, SIFs) that regulate host energy to steal resources from other non-immune tissues to induce an efficient acute immune response (269–272). As insulin signalling is the major energy regulator in insects, and the cytokine-induced insulin resistance increases the number of available energy-rich compounds, these authors assume that inhibiting insulin signalling during infection is the main target of this strategy. They also propose the insulin/IGF antagonist Imaginal morphogenesis protein late 2 (Impl2) and Upd3 as potential SIFs in *Drosophila*.

Impl2 has been identified as a cancer-derived cachectic factor in flies, which induces the mobilization of nutrients towards the tumour for its growth via insulin resistance (273). It has been described that the expression of HIF1α in plasmatocytes not only induces the metabolic switch but also the expression of Impl2 (274). This, added to the fact that Impl2 binds to dILPs and inhibits the IIS pathway (275), makes of this molecule a great candidate. On the other hand, Upd3 released by plasmatocytes induces a dFoxo-dependent transcription that leads to lipid-stores mobilization in the fat body and reduces glucose consumption by muscles through the activation of JAK/STAT (276,277) (278–280). However, the link between plasmatocytes metabolic switch and Upd3 production has not been completely studied yet. This process might be overall beneficial to fight extracellular pathogens, but it may be also used for intracellular pathogens to favour their own growth, as described for mycobacteria (281).

1.3.3.2| Hormonal regulation

As in mammals, in insects also exists a sexual dimorphism affecting immunity at basal conditions, after a pathogenic challenge, and even upon ageing (282,283). However, the mechanisms underlying these differences between males and females remain understudied, as few works include both sexes or do not stratify their results by sex. These few studies, however, revealed that these differences might be mediated by different immune players depending on the pathogen and the route of infection. For example, males appeared to be more resistant to systemic infections by *Providentia* species, and *Enterococcus faecalis*, while females seemed to be more resistant to *Pseudomonas aeruginosa*, *S. aureus* and *Serratia* infections (284–286). Recently, a role for the Toll pathway in mediating sex dimorphisms has arisen with the finding that the loss of Toll-7 reduces resistance to *P. aeruginosa* and *E. faecalis* in males but not in females (287).

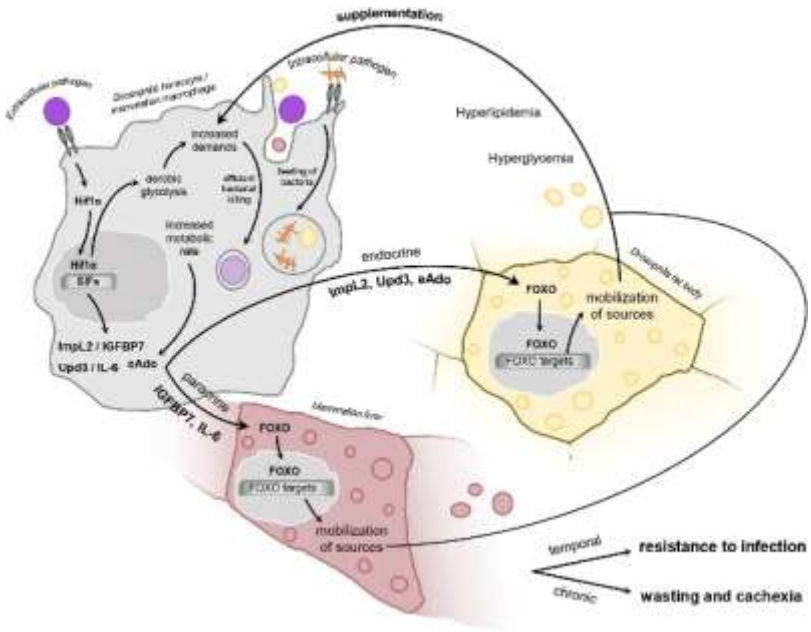


Figure 13| Schematic representation of the "selfish immune system theory". In infection-activated phagocytic cells, Hif1α induces a metabolic switch and the production of selfish immune factors. These released molecules affect systemic metabolism via insulin resistance and induce Foxo-dependent mobilization of sources. This results in increased numbers of circulating energy-rich molecules that are then used by energy-demanding phagocytes. Extracted from Bajgar et al., 2021.

But it is not only sext that affects immunity, but also the reproductive status. In *Drosophila*, mated females showed decreased survival, higher pathogen loads and reduced AMPs production after pathogenic infections (288–290), although mating does not affect the clearance of non-pathogenic *E.coli* (291,292). On the other hand, increased resistance to infections correlates with a reduction in egg viability (293), but female flies unable to generate eggs do not have reduced immunity after mating (290) and females fed with yeast *ad libitum* improved both fecundity and resistance to infections proving that reproduction and immunity also compete for energy resources(294). In the same lines, males exposed to a higher number of females also showed increased susceptibility to bacterial infections (295).

In mammals, these sex differences in immune response have been linked to steroid hormones, and interactions between these types of hormones and the immune system in *Drosophila* have also been described (296,297), which together with the presence of conserved immune pathways makes *Drosophila* a powerful model to study sex dimorphism in host-pathogen interactions. The steroid hormone Ecdysone

is the main regulator of the insect life cycle (298). In adult flies, Ecdysone is produced in the ovaries after mating, thus showing higher levels in females than in males (299–301). Its signalling through the receptor complex formed by the Ecdysone receptor (EcR) and Ultrapiracle (Usp) is required for the proper expression of the pathogen-sensing receptor PGRP-LC and, thus, the production of Imd-dependent AMPs (302) and the cellular immunity (297,303). At the same time, Ecdysone levels are regulated by stress signals and is has been related to age-related immune changes, as its depletion increases *Drosophila* lifespan (304,305). On the other hand, the Juvenile hormone (JH) promotes the expression of the proteins responsible for the egg production in the fat body and induces immunosuppression after mating in females (296,306,307). In *Drosophila*, JH is synthesized when the protein Sex Peptide (Acp70A) is transferred to females through the male's seminal fluid (308). It was shown that females mating males lacking this protein were as resistant to bacterial infections as virgin females, while females mating wild-type males showed lower resistance (290). Thus, supporting the idea that mating increases the synthesis of JH and suppresses immunity. In addition, a decrease in the IIS pathway leads to lower levels of JH, reduction of fecundity and increased levels of Ecdysone, leading to higher immune response, while increased levels of IIS and JH promoted oogenesis and inhibits immunity.

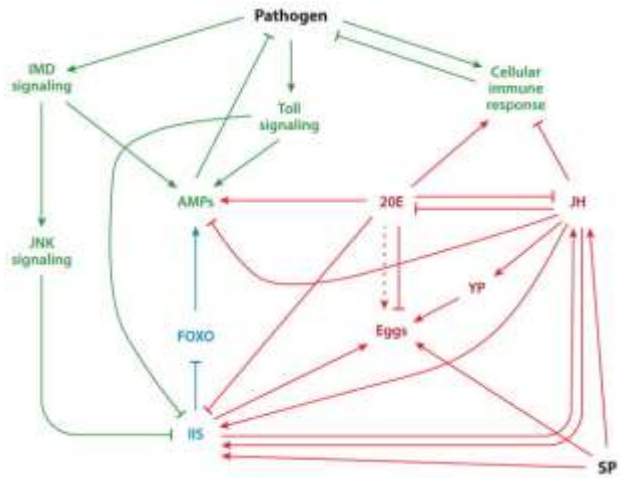


Figure 14| Schematic representation of the interactions between reproduction and immunity. The color code is as followed: reproductive pathways are in red, immune pathways are in green, and metabolic pathways are in blue. The activation of the Imd pathway inhibits insulin signaling via the JNK pathway and produces AMPs. The production of Ecdysone (20E) inhibits the IIS pathways and favors immunity, while the production of JH after mating favors eggs production and inhibits immunity. Extracted from Schwenke et al., 2015.

1.3.4| *Drosophila melanogaster* as a model for mycobacterial infections

Mycobacterium marinum is the most common pathogenic mycobacteria used for infections in non-mammal animal models because, due to its optimal growth range (25-30ºC) (309), it is a natural pathogen of ectotherms such as fish and frogs causing them a granulomatous infection that highly resembles TB in humans (310–312), although it can also cause superficial granulomatous infections in warm-blooded hosts including humans (313). In addition, this species is closely related to *Mycobacterium tuberculosis* (*Mtb*), grows faster and requires fewer biosafety measures although it is able to secret ESAT-6, which is the major virulence factor in Mtb to avoid phagolysosome fusion, generate NETs and induce cording.

Although many studies have been performed with zebrafish, *Caenorhabditis elegans* and even in mice (314–316), very few studies have been done in the *D. melanogaster* model. Dionne et al., showed that even very low initial doses of this mycobacterium were able to kill flies and that it was able to replicate inside the *Drosophila* hemocytes at early stages of the infection by preventing acidification of the phagosome using the same mechanism seen previously in mice and resembling what *Mtb* causes in vertebrates (317,318). However, as the infection evolves and bacteria start growing both inside and outside of the host cells, there is severe widespread tissue damage, but no granuloma formation is observed (317). They also compared the infection by *M. marinum* with the infection with the non-pathogenic species *M. smegmatis*. This study showed that neither of the mycobacteria triggered the production of AMPs at the early stages of the infection and that the susceptibility of mutant flies lacking both humoral response pathways was not increased (317). However, infection with *Mycobacterium abscessus*, a pathogen responsible for a broad-spectrum of infections in immunocompromised patients, was the first mycobacteria proved to trigger a severe humoral response in *Drosophila*, mainly the Toll pathway (146).

On the other hand, Dionne *et al.* proved that *M. marinum* causes a progressive loss in energy storage inducing a cachexia-like process that eventually, accompanied by widespread tissue damage, is responsible for killing the flies. This wasting process is mediated by the disruption of the insulin signalling pathway (ISS), which causes the degradation of activated AKT, thus allowing FOXO to enter the nucleus and induce the production of AMPs, that probably is the reason why at late stages of the disease some AMP expression is observed. However, the mechanism by how this happens is unclear, as the InR-AKT signalling is not affected because the injection of even low doses of insulin restore this pathway and flies are less susceptible to the infection (262). So, the authors hypothesized that either there is an active degradation of activated AKT or there are no circulating dILPs available to bind to the InR receptor.

In another study Péan, Dionne *et al.* showed that hemocytes-derived production of Upd3 induces activation of the JAK/STAT signalling pathways back to hemocytes and reduces the expression of *atg2*. This leads to the formation of larger and irregular lipid droplets (LD), which favours the replication and survival of *M. marinum*, although the protective mechanism remains unknown (Figure 15). This process is similar to the observed in human macrophages in response to IL-6 sensing (281).

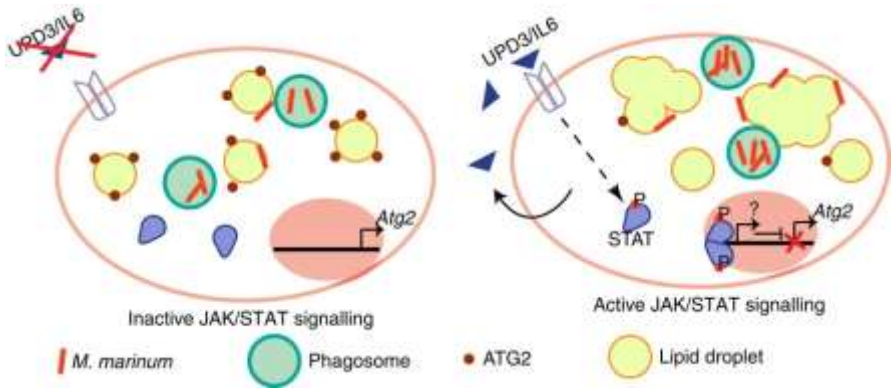


Figure 15| Model of the role of JAK/STAT signaling in M. marinum infections. After infection, hemocytes release Upd3, which activates the JAK/STAT pathway back in hemocytes. Downstream this pathway, Atg2 expression is inhibited and LD became larger and irregular, favoring the bacterial growth. Extracted from Péan et al., 2017.

1.4| Trained immunity: the innate immune memory

1.4.1| Trained immunity in vertebrates

For so long, the dogma in immunology was that the innate immune response was rapid and non-specific, while the adaptive immune response was slower, but antigen-specific and led to long-term immunological memory. However, it is unlikely that a crucial trait like immune memory is restricted to the adaptive arm of the immune response when more than 95% of species do not rely on this immune system. Thus, many studies have provided pieces of evidence that some vaccines and infections protect against secondary exposure in a specific or unspecific way (319,320). For example, vaccination with bacille Calmette-Guerin (BCG) was probed to confer protection against a range of infectious diseases (321–323) as well as induce antitumoral effects (324–326). Altogether, the non-specificity of this protection, the role of innate immune cells like monocytes and macrophages and the evidence that plants and invertebrates, which only rely on innate immunity, also showed greater

protection against reinfections revealed that immunological adaptation may also occur on the innate immunity (320,327–330).

The term “trained immunity” was established to define the long-term reprogramming of innate immune cells after a stimulus that leads to a stronger and faster response to a second challenge with the same or different stimulus (320). This reprogramming is mainly due to epigenetic changes rather than gene recombination. Several studies have shown epigenetic-related chromatin modifications in monocytes, macrophages and DCs that affected the inflammatory profile of these cells in response to secondary challenges (331–333). Intriguingly, epithelial stem cells were also reported to have inflammatory memory behaviour, showing faster mobilization and higher induction of interferon-stimulated genes during secondary challenges (329,334–336). More recent studies have also shown that stimuli like vaccination with BGC and exposure to β-glucan may also reprogram hematopoietic stem cells in the bone marrow, thus explaining the long-term protection that would not be explained otherwise, as mature myeloid cells have an average half-life of 5 to 7 days (337–340). In addition, the possibility of generational transmission of the trained immunity has also been explored (341,342).

The mechanisms of this reprogramming are not completely understood yet, though some evidence supports the existence of multiple regulators. One layer of regulation is mediated by the modification of the chromatin organization. In quiescent myeloid cells, immune genes are organized into topologically associated domains (TADs) and are found in a repressed configuration to prevent its transcription. After a first challenge, the chromatin unfolds to allow transcription of immune genes and immune gene-priming long non-coding RNAs (ILPs) interact with these TADs and promote the histone-modifying complex to “mark” these regions, preventing the chromatin to fully condensate again and allowing a faster and robust activation of these genes in response to secondary challenges (343–345). However, how these “marks” are maintained through DNA replication and cell cycle remain unknown (346). New studies have also suggested changes in DNA methylation patterns and changes in cellular metabolism as a mediator of trained immunity. For example, individuals that exhibit higher protection against Mtb after BGC vaccination showed lower methylation levels among the promoter regions of immune genes (347,348). Also, exposure to β-glucan and BCG vaccination modify monocytes metabolism toward aerobic glycolysis via the AKT-mTOR-HIF1α pathway, which modulated the activity of chromatin-modifying enzymes (349–352).

1.4.2| Other innate adaptive programs

It is obvious that trained immunity provides a great advantage in host defence, however, it may also be detrimental in the context of chronic inflammatory diseases. Also, the dose and the duration of the first stimulus determine whether adaptation of innate immune cells will enhance or reduce the immune response to a second challenge. Thus, based on the functional status in which these cells after before the second challenge, different adaptive programs can be defined. The change of an immature cell into its mature counterpart is defined as cell differentiation and often comes together with morphological changes (Figure 16a) (353). When the first stimulus changes the state of the innate immune cells and the active gene expression does not return to basal levels before the second challenge it is named "priming", and often the response to the second stimulus is synergistic with the first one (Figure 16b). In contrast, in trained immunity the gene expressions levels return to basal levels when the first stimulus is removed, but epigenetic changes persist favouring a faster and higher immune response in subsequent infections (Figure 16c). Finally, tolerance is the opposite of trained immunity, where after activation by a first stimulus and returning to basal levels the immune response is reduced in subsequent challenges (Figure 16d) (354).

1.4.3| Innate immune adaptation in *Drosophila melanogaster*

Few studies have been performed in *Drosophila melanogaster* addressing this topic. Pham *et al.*, found that the first exposure to a non-lethal dose of *Streptococcus pneumoniae* conferred protection against a second exposure to lethal doses of the same pathogen. This response was found to last the rest of the fly's life, to be specific and to be mediated by the Toll pathway together with phagocytes, but not the Imd pathway or AMPs. They also showed that heat-killed bacteria, which are known to be potent immune activators, did not confer protection to subsequent infections (355). Similar results were found when priming flies with the less virulent strain of *Pseudomonas aeruginosa* CF5, however, these studies revealed that both Toll and Imd pathways were necessary for the priming of the immune response against subsequent infections with the more virulent strain PA14 and that heat-killed CF5 did confer protection although this was shorter (356,357). Recent studies have gone deeper into the mechanisms of these innate immune adaptations. Chakrabarti and Visweswariah defined the ROS production and accumulation in hemocytes after an injury as the key regulators for the induction of the Toll pathway, which in turn confers protection to subsequent infections with *Enterococcus faecalis* (358), while Bozler *et al.* showed that increased maternal PGRP-LB expression levels after exposure to parasitic wasp correlated with more rapid induction of lamellocytes and, thus, a more successful immune response to the parasite in the offspring (359).

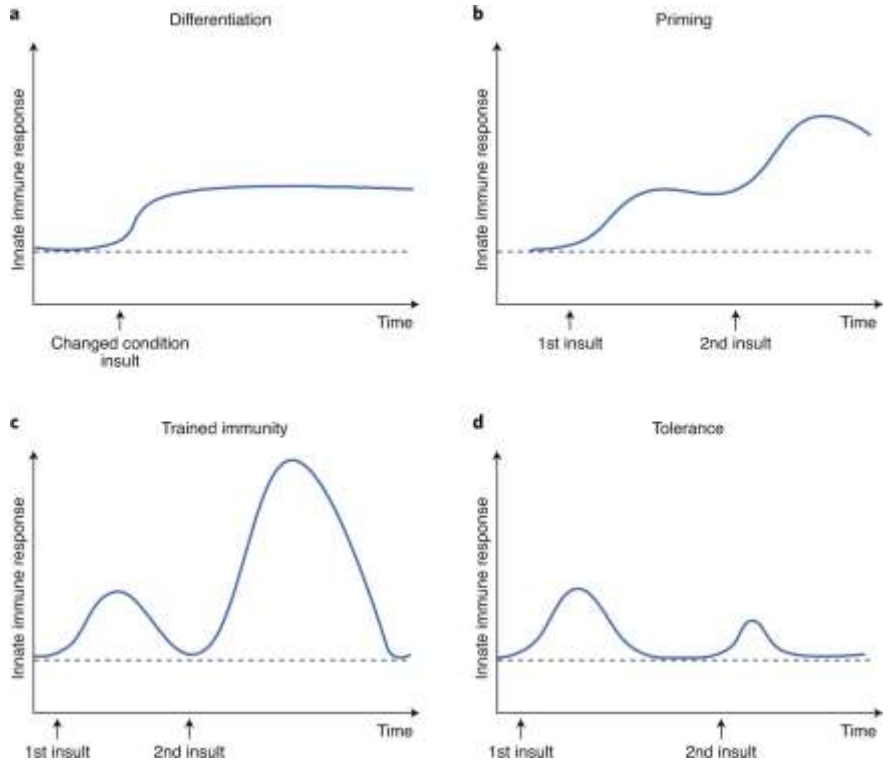


Figure 16| Schematic representation of the behaviour of innate immune response during different adaptive programs. (a) Cell differentiation is defined as the change of an immature cell into its mature counterpart and is a long-term change. (b) Priming is characterised by an activation of gene expression in innate immune cells that is sustained over time and does not return to basal levels before the second challenge. Often the response to the second stimulus is synergistic with the first one. (c) In trained immunity the gene expressions levels return to basal levels when the first stimulus is removed, but epigenetic changes persist favouring a faster and higher immune response in subsequent infections. (d) Finally, tolerance is the opposite to trained immunity, where after activation by a first stimulus and returning to basal levels the immune response is reduced in subsequent challenges. Extracted from Divangahi et

2| Hypothesis and Objectives

So far, the role of the Tregs ins the tolerance to TB acquired by the oral administration of hkMm has been proven. However, as mentioned in section XXX, the innate immune response also plays a major role in oral tolerance. So, our aim is to study this.

Previous work at the unit showed that the administration of low doses of heat-killed *Mycolicibacterium manresensis* (hkMm) induces oral tolerance to Mtb in a murine model, delaying the progression of the infection toward active TB, through the production of antigen-specific memory Tregs. These studies also showed the safety of the treatment in humans. However, all these studies focused on the adaptive immune response, although the innate immune response also plays a major role in the defence against Mtb and the progression towards active TB, as tolerogenic DCs generated by oral tolerance could induce Tregs at local and systemic levels. Thus, our unit aimed to study the role of the innate immune response in the acquisition of oral tolerance due to the administration of hkMm.

We hypothesised that *Drosophila melanogaster* could be a suitable animal model to study the role of the innate immune response in the induction of oral tolerance to mycobacteria by the administration of heat-killed *M. manresensis* and that this tolerance could not be restricted only to mycobacteria.

In order to demonstrate the hypothesis, 3 objectives were defined:

1| To characterise the innate immune response induced by mycobacterial infections within the *Drosophila melanogaster* model, as well as to assess the importance of sex and reproductive status of the host in the tolerance to mycobacterial infections.

2| To test the protective effect given by oral administration of heat-killed *M. manresensis* against subsequent infections with different pathogens.

3| To evaluate the impact of *M. marinum* infection and heat-killed *M. manresensis* oral administration in the resistance/tolerance response of *D. melanogaster* after coevolution for several generations.

3| Materials and Methods

3.1| Drosophila melanogaster husbandry

Oregon-R-C wildtype flies were obtained from the Bloomington Drosophila Stock Centre (BDSC, Indiana University) and they belong to stock number #5.

3.1.1| Media preparation

The Bloomington standard cornmeal formulation containing yellow cornmeal, corn syrup solids, inactive nutritional yeast, agar and soy flour was used to fed flies. A pre-mixed dry version is available at Genesee Scientific. One bag containing 176g of dry medium was poured into 1L of water and heated until boiling point. The mix was constantly stirred until homogenization and autoclaved. Once sterile, 2ml of propionic acid were added to prevent yeast contamination and 50ml of media was poured into polypropylene bottles (57 x 103mm) for stock maintenance and 10ml into polystyrene tubs (28.5 x 95mm) for experimental procedures. Tubs with fresh media were left open inside the sterile vertical flow hood to solidify and stored at room temperature for less than a month.

3.1.2| Stock maintenance and synchronization

In all cases flies were kept at 25ºC with constant light:dark cycles of 12 hours each and a humidity of 70%. Stocks were checked periodically to ensure absence of contamination and/or mites and flies were transferred onto new bottles with fresh medium every 3-4 weeks.

For experimental procedures was crucial that all flies were same age to prevent aging bias. To do this, 30 males and 30 females were placed onto new bottles for 24h to allow females to lay eggs. After 24h, adults were removed and the empty bottles were kept at 25ºC, where new flies emerged after 10 days, thus ensuring that all flies were born on the exact same day. Males and females can be easily differentiated when anaesthetized with carbon dioxide (CO_2) and viewed under a light microscope. Males are smaller in size, possess dark structures on their forelegs called “sex comb” and a dark pigmented area on the dorsal posterior part of the abdomen (360) (Figure 17).

3.1.3| Virgin collection

Female flies do not mate in the first 8 hours post-eclosion from the pupae at 25ºC (361). Thus, 10 days after synchronization all adults that might have born were cleared and the bottle was checked every 2-3 hours to collect all the flies that have emerged from the pupae over that period of time and checked for the characteristic

appearance of recent born flies (362) (Figure 17). Virgin flies are placed in new tubs and after 2-3 days those tubs with females are checked for larvae, since virgin females can lay eggs but they will be sterile.

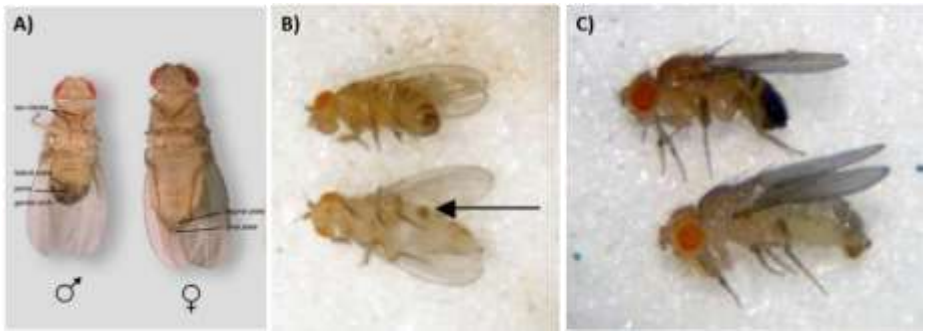


Figure 17| Pictures of differences among males and females. (A) Picture of the ventral section of flies. Males (left) are smaller, have dark and rounded genitalia and the sex comb on the forelegs, while females (right) are bigger and have light, pointed genitalia. **(B and C)** Pictures of differences between mature (top) and virgin (bottom) flies. Virgin females **(B)** and males **(C)** are much larger than older flies and do not have the dark coloration of mature flies. In addition, in the early hours after eclosion, a dark spot on the abdomen is visible in females (the meconium, remains of their last ingest before pupation). Extracted from Tauber Lab and Berg Lab reports, respectively.

3.2| Bacterial strains

3.2.1| Mycobacterial strains

Three mycobacterial species were used in this project. *Mycobacterium marinum* E11 strain resistant to kanamycin (a kind gift from Wilbert Bitter, Vrije Universiteit Amsterdam) (363), *M. manresensis* with intrinsic resistance to doxycycline and *M. smegmatis* with intrinsic resistance to ampicillin. Ten microliters of each mycobacteria were inoculated in 5ml of 7H9 liquid media complemented with each corresponding antibiotic and placed at 30ºC and constant agitation (170 revolutions per minute, rpm) until an optical density at 600nm (OD_{600nm}) of 1.5 was achieved. The cultures were then centrifuge for 5min at 4000g, resuspended in 5ml of phosphate buffered saline (PBS) with 0.2% Tween 80 and centrifuge again for 5min at 500g to remove clumps. Supernatants were transferred to a new tub, centrifuged for 5min at 4000g and resuspended in 1ml of 7H9 with 15% glycerol. The cultures were then split into 50µl aliquots and frozen at -80ºC. Each stock was tittered after frozen at least overnight. One aliquot of each mycobacteria was defrosted, centrifuged 5min at 5000g and resuspended in 50µl of PBS. Serial dilutions were plated by spotting 5µl into 7H10 plates complemented with the indicated antibiotic. Plates were incubated at 30ºC for

10 days for *M. marinum* and *M. manresensis* and 3 days for *M. smegmatis* and colony forming units (CFU) were counted.

3.2.1.1| Mycobacterial Growth Curve

The three mycobacteria were inoculated in 7H10 liquid media complemented with ADC but no antibiotics, at a final OD_{600nm} of 0.05. These cultures were kept at 25ºC and constant agitation of 170rpm for 10 days. The OD_{600nm} was measured for each bacterial culture twice a day. Three independent replicates were performed.

3.2.2| *Salmonella typhimurium* and *Staphylococcus aureus*

Salmonella typhimurium resistant to ampicillin and *Staphylococcus aureus* resistant to penicillin were obtained from clinical isolates at the Hospital Universitari Germans Trias i Pujol. Ten microliters of each bacteria were inoculated in 5ml of Luria-Bertani (LB) broth complemented with the corresponding antibiotic and incubated at 37ºC with constant agitation (170 rpm) overnight. The grown cultures were then centrifuged 5min at 5000g, resuspended in 1ml of LB with 15% glycerol, split into 100µl aliquots and frozen at -80ºC. Each stock was tittered after frozen at least overnight. One aliquot of each bacteria was defrosted, centrifuged 5min at 5000g and resuspended in 100µl of PBS. Serial dilutions were plated by spotting 5µl into LB plates complemented with the indicated antibiotic. Plates were incubated at 37ºC overnight and CFUs were counted.

3.3| *D. melanogaster* infections

3.3.1| Systemic infection

Infections were performed in 3 to 5-days-old flies always in the morning, as *Drosophila* immunity is influenced by circadian rhythm. Bacterial strains aliquots were defrosted, centrifuged and resuspended in PBS, prior to dilute them to the proper infection concentration. For systemic infections, flies were anesthetised using CO_2 and injected in the anterior abdomen on the ventrolateral surface with 13.8nl of either PBS for wounding control or the corresponding bacterial solution, using a Nanoject II (Drummond). Flies were never exposed to CO_2 for more than 15 minutes. Infected flies were kept in groups of 15 males and 15 females, unless otherwise stated, at 25ºC. Survival of the control group was checked daily until stabilized, usually 3 to 4 days after inoculation. Flies from all groups that died within these days were eliminated from the experiments and surviving flies were transferred into new tubs every 3 to 5 days.

3.3.2| Oral infection and treatment

Oral infections were also performed with 3 to 5-days-old flies. Bacterial solutions were prepared as for systemic infections and 100µl of the corresponding dilution were added on the surface of the media. Tubs were kept at 30ºC to allow the media to absorb the bacterial solution. Once dried enough, flies were transferred to these tubs and left at 25ºC for 24h to allow them to ingest the media with bacteria. After that time, flies were transferred to new sterile tubs and kept at 25ºC. Survival was checked every day and surviving flies were transferred into new tubs every 3 to 5 days. For the oral administration of heat-killed *M. manresensis* the same procedure was used, but mycobacterial aliquots were heated at 95ºC for 30min prior to dilute them to the proper concentration and add it to the media.

3.3.3| Bacillary load

At the indicated time points for each experimental procedure, singles flies were washed once with 70% ethanol, rinsed with PBS and smashed into 200µl of cold PBS. Serial dilutions were made and 5µl of each dilution were plated in 7H10 plates for mycobacteria and LB plates for *S. typhimurium* and *S. aureus*. In all cases, the corresponding antibiotics were added to the plates to prevent the growth of the microbiota of the flies, as well as the antifungal Amphotericin B. Mycobacteria plates were kept at 30ºC for 3 or 10 days depending on the species and the rest of bacteria were kept overnight at 37ºC. Viable bacteria were determined by counting colony forming units (CFU) after incubation time.

3.4| Gene expression analysis

3.4.1| RNA extraction, cDNA synthesis and real-time PCR analysis

Total RNA was extracted from single flies using the MasterPure™ Complete DNA and RNA Purification Kit (Lucigen), converted to cDNA with the PrimeScript RT Master Mix (Takara), and subjected to Real-Time PCR. Conventional PCRs were done to check amplification temperatures and to validate each set of primers (Table 1). Real-Time PCR was carried out in 10µl reaction solution containing 5 µl of KAPA SYBR® FAST Mix (Sigma), 2µl cDNA (diluted 1:3), 0.1µl of each specific primer (20µM stock) and 2.8µl of water. PCR conditions were 95ºC for 5min followed by 40 cycles of 95ºC for 10s and 60ºC or 62ºC for 20s. the specificity of each pair of primers was checked by melting curve analysis (95ºC for 5s, 65ºC for 1min and a continuous raise in temperature to 97ºC at 2.5ºC/s ramp rate followed by 97ºC for 30s). To check reproducibility, each assay was performed with technical triplicates for each biological sample. The relative transcripts levels of target genes were calculated using the $2^{-\Delta\Delta CT}$

method (364) with r*pl32* used as the reference gene for normalization of target gene abundance.

3.4.2| RNA-Seq Data Analysis

Total RNA was extracted from a pool of 5 flies using the MasterPure™ Complete DNA and RNA Purification Kit (Lucigen). To determine the total RNA quality and quantity was used Qubit® RNA HS Assay (Life Technologies) and RNA 6000 Nano Assay on a Bioanalyzer 2100 (Agilent).

The RNASeq libraries were prepared following the TruSeq Stranded mRNA Library Prep protocol. Briefly, total RNA (500ng) was enriched for the polyA mRNA fraction and fragmented by divalent metal cations at high temperature. In order to achieve the directionality, the second strand cDNA synthesis was performed in the presence of dUTP. The blunt-ended double stranded cDNA was 3´adenylated and Illumina platform compatible adaptors with unique dual indexes and unique molecular identifiers (Integrated DNA Technologies) were ligated. The ligation product was enriched with 15 PCR cycles and the final library was validated on an Agilent 2100 Bioanalyzer with the DNA 7500 assay (Agilent).

The libraries were sequenced on NovaSeq 6000 (Illumina) with a read length of 2x51bp following the manufacturer's protocol for dual indexing. Image analysis, base calling and quality scoring of the run were processed using the manufacturer's software Real Time Analysis (RTA v3.4.4) and followed by generation of FASTQ sequence files.

Reads were mapped against the *Drosophila melanogaster* reference genome (BDGP6.32) with STAR 2.7.8a (365) using ENCODE parameters. Gene quantifications (release 104) were performed with RSEM 1.3.0 (366) using default options. Only genes with >1cpm in >= 20 samples were kept for the analysis. Differential expression analysis was performed with the R package limma (version 3.5.0) using the *voom* transformation (367). Differentially expressed genes (DEGs) with absolute $\log_2$ fold change >1 and a P value <0.05 corrected for multiple testing using the Benjamini-Hochberg method were considered significant. Functional enrichment analysis was performed using the Gene Set Enrichment analysis (GSEA) software (version 4.1.0) and the gene matrix provided by Cheng *et al.* (368). As indicated for RNASeq data, the pre-ranked analysis was used and data was ranked by decreasing value of thet-statistics (all genes included). Modules with enrichment scores with nominal P values <0.05 and False Discovery Rate (FDR) <25% were considered significant and plotted. Volcano and GSEA plots were graphed using the ggplot2 package in R (version 3.3.5). For heatmaps representations, significant DEGs that belong to the considered enriched modules were selected, as well as genes

extremely differentially expressed which function was considered relevant, and graphed with the R package pheatmap (version 1.0.12)

3.5| Statistical Analysis

Data were graphed and analyzed using GraphPad Prism (version 9.0.0). Survival curves were analyzed using Log-rank (Mantel-Cox) test. CFU counts and gene expression data were checked for normality with Shapiro-Wilk normality test and analyzed using one-way ANOVA for multiple comparisons, t-test for single comparisons with normally distributed groups and Mann-Whitney test for non-normally distributed groups. The Principal component analysis (PCA) was performed with Factoextra package in R (version 4.0.1). Linear regressions on tolerance curves were analyzed using ANCOVA test. Significant differences are as follow: *p≤0.05; **p≤0.01; ***p≤0.001 and ****p≤0.0001 (same for #).

Table 1| Oligonucleotide sequences used for Real-Time PCR

Gene	Primer forward (5'-3')	Primer reverse (5'-3')
Rpl32	ACAGGCCCAAGATCGTGAAG	TCGACAATCTCCTTGCGCTT
Diptericin	GGCTTATCCGATGCCCGACG	TCTGTAGGTGTAGGTGCTTCC
Drosomycin	CCAAGCTCCGTGAGAACCTT	CAGGTCTCGTTGTCCCAGAC
Upd3	GCAAGAAACGCCAAAGGA	CTTGTCCGCATTGGTGGT
Impl2	GCCGATACCTTCGTGTATCC	TTTCCGTCGTCAATCCAATAG
Ecdysone receptor	CAACAGCTCGGACTCAATATTCTT	GTTCTCCTCCTGGGTAATCTGAA

4| Study 1

Deciphering the innate immune response against mycobacterial infections in *Drosophila melanogaster*

This first study aimed to characterize the innate immune response of *D. melanogaster* against mycobacterial infections and was divided into three sub-studies. The first sub-study addresses the suitability of *Drosophila melanogaster* as a model to study the interactions between mycobacteria and the host by establishing the best infection method taking into consideration the route of infection and the inoculation dose. The second sub-study evaluates the host response to different pathogenic and non-pathogenic mycobacterial species and, last, the third sub-study investigates the role of both sex and the reproductive status of the host have on mycobacterial infections.

4.1| Assessing *D. melanogaster* as a model for mycobacterial infections

4.1.1| *M. marinum* kills *D. melanogaster* in a dose-dependent manner systemically

To establish a robust and reproducible protocol for the infection with *Mycobacterium marinum*, 3 to 5 days old flies were infected systemically with increasing doses of the pathogen. Their survival was checked daily, and the bacillary load was measured at 2, 5 and 7 days post-infection. For each inoculation dose, a total of 60 males were used for survival and 9 for CFU counting at each time-point, divided into three independent experiments (20 males for survival and 3 for CFU counting at each time-point, respectively). Only males were used in these experiments to mimic the conditions used by Dionne et al., 2003 which was the only reference existing in the literature.

The systemic infection was proved to be a robust method as no significant differences were found in the three independent replicates, neither in the bacillary load nor in the survival (Figure 18). All the doses tested killed flies in a dose-dependent manner, while flies that received sterile PBS showed no significant mortality other than the expected by ageing and wounding. Significant differences were observed among all conditions, except between doses 10^4 and 10^5.

Bacillary load at 2 days post-infection correlated with the initial inoculation dose, showing that systemic injection was a reliable and controllable infection model. As stated previously by Dionne et al., *M. marinum* was capable to replicate within the flies as the bacillary load increased exponentially over time (Figure 18).

4.1.2| *M. marinum* is not capable to infect *D. melanogaster* naturally

The natural infection of *D. melanogaster* with *M. marinum* was also assessed. Both male and female flies (20 each for 3 independent replicates) were exposed to the

presence of the pathogen in the feeding media for 24h. Survival was checked every day and the bacillary load was assessed at 2, 5 and 7 days post-infection. Results showed no statistically significant decrease in survival compared to control flies, and the bacillary load was reduced drastically to the point that no CFU were counted at 5 days post-infection (Figure 18).

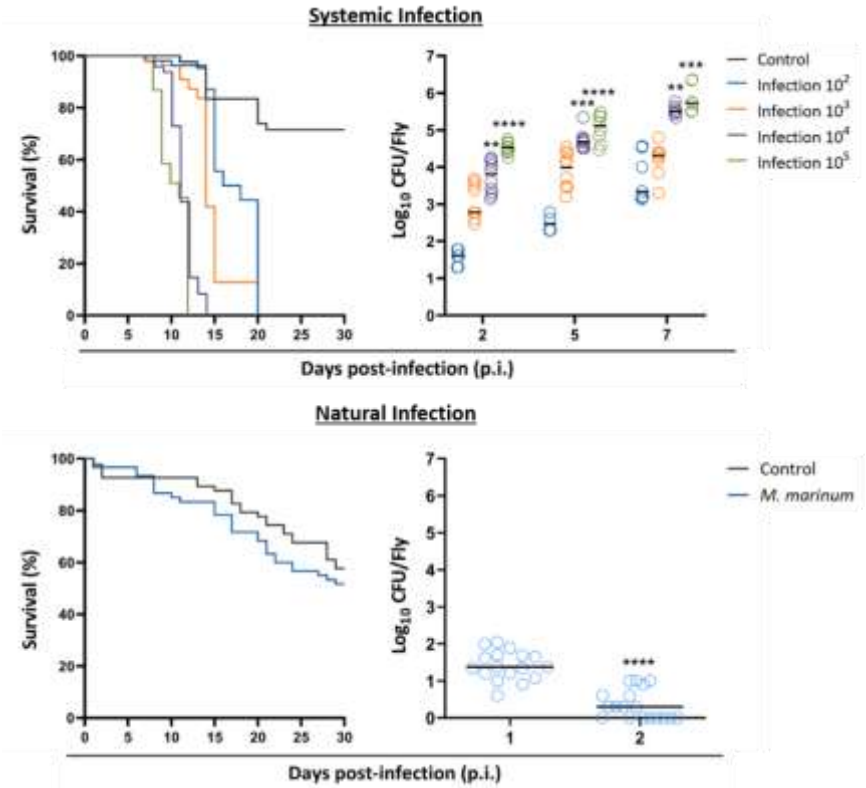


Figure 18| Effect of systemic and natural *M. marinum* infections on *D. melanogaster* survival and bacillary load. Systemic infection. Survival (left) was observed daily and statistically significant differences were observed among all curves ($p<0.0001$; Log-rank test) except for comparison between infections 10^4 and 10^5. Bacillary load (right) is expressed as Log_{10} CFU/Fly. Each circle represents an individual and lines are medians. Data as tested for normality and statistically significant differences were observed between dose 10^2 and 10^4-10^5 at all time-points (**p≤0.01, ***p≤0.001, ****p≤0.0001; Kruskal-Wallis test). **Natural infection**. Survival (left) was checked daily and no statistically significant differences were observed (Log-rank test). The bacillary load (right) was significantly reduced 2 days p.i. (****p≤0.0001, Mann-Whitney t-test).

4.2| Evaluating the innate immune response of *D. melanogaster* to mycobacterial infections

The response of *D. melanogaster* against three mycobacterial species was evaluated by infecting male and female flies with an initial infectious dose of 500 CFU of each microorganism: *Mycobacterium marinum*, as a pathogenic model species;

Mycolicibacterium smegmatis as a non-pathogenic species; and *Mycolicibacterium manresensis* (belonging to the *M. fortuitum* complex), which was proved non-pathogenic and immune beneficial in mice, but was never studied in the fly model.

4.2.1| All mycobacterial species are able to grow at 25ºC *in vitro*

The three mycobacterial species selected for this study had different optimal growing temperatures. *M. marinum* is a natural pathogen for ectotherms and therefore has an optimal growth temperature between 25ºC to 35ºC, while *M. smegmatis* is considered an environmental organism and has a wide growth temperature range, although in laboratory conditions is typically grown at 37ºC. Finally, *M. manresensis* optimal growing temperature in laboratory conditions is 30ºC, although can perfectly grow at 37ºC. Thus, to establish an infection protocol for the *D. melanogaster* model it was crucial that all of them were able to grow at 25ºC. Three independent growth curves were performed as stated in section 2.1.1.

The growth curves showed that all three species were able to grow at 25ºC in vitro (Figure 19). The three species showed significantly different growing kinetics (****p≤0.0001; Extra sum-of-squares F test). On one hand, *M. smegmatis* is considered a fast-growing *Mycolicibacterium* and therefore entered the log phase almost immediately compared to the other species. On the other hand, both *M. marinum* and *M. manresensis* showed a more progressive growth with a longer lag phase previous to the logarithmic growth. However, *M. manresensis* and *M. smegmatis* reached the stationary phase at a much lower concentration compared with *M. marinum*.

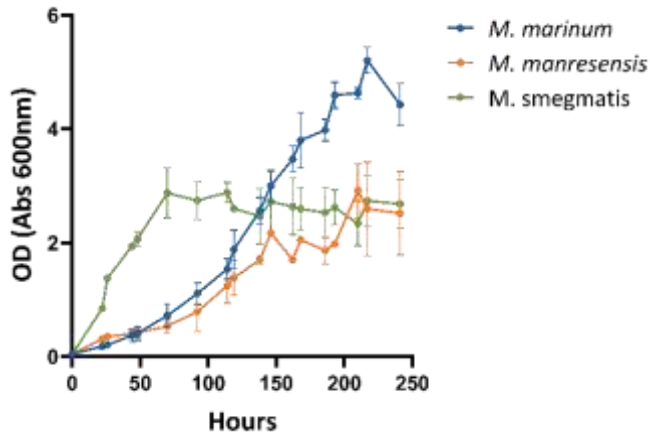


Figure 19| Mycobacterial growth curve in 7H10 medium. Cells were grown at 25ºC in constant agitation (170rpm) for 10 days and the growth was determined by measuring OD at 600nm twice a day. The values are the mean and the standard deviation of three independent incubations. The three growth curves showed statistically significant differences (****p≤0.0001; Extra sum-of-squares F test).

4.2.2| *Drosophila*'s death is species specific and not generic for all mycobacteria

The virulence of the three mycobacterial species was evaluated by infecting male and female flies with an initial infectious dose of 500 CFU of each microorganism. For each group, a total of 90 males and 90 females were used for survival divided into three independent experiments (30 males and 30 females respectively). Males and females were kept together during the infection in a ratio of 15:15 each and survival was checked daily.

Flies infected with *M. marinum* showed the same death pattern that the one observed in the previous sub-study where most of the flies infected with an initial dose of 10^2 CFU died around 20 days post-infection, while *M. smegmatis* did not show a statistically significant decrease on the survival compared to flies injected with PBS. Surprisingly, *M. manresensis* killed flies more rapidly than *M. marinum* even thought this species is considered an environmental, non-pathogenic for mammals. In addition, no statistically significant differences on survival were observed between males and females in *M. marinum* and *M. smegmatis* infections, but females died significantly earlier than males when infected with *M. manresensis* (*p<0.05, Log-rank test) (Figure 20).

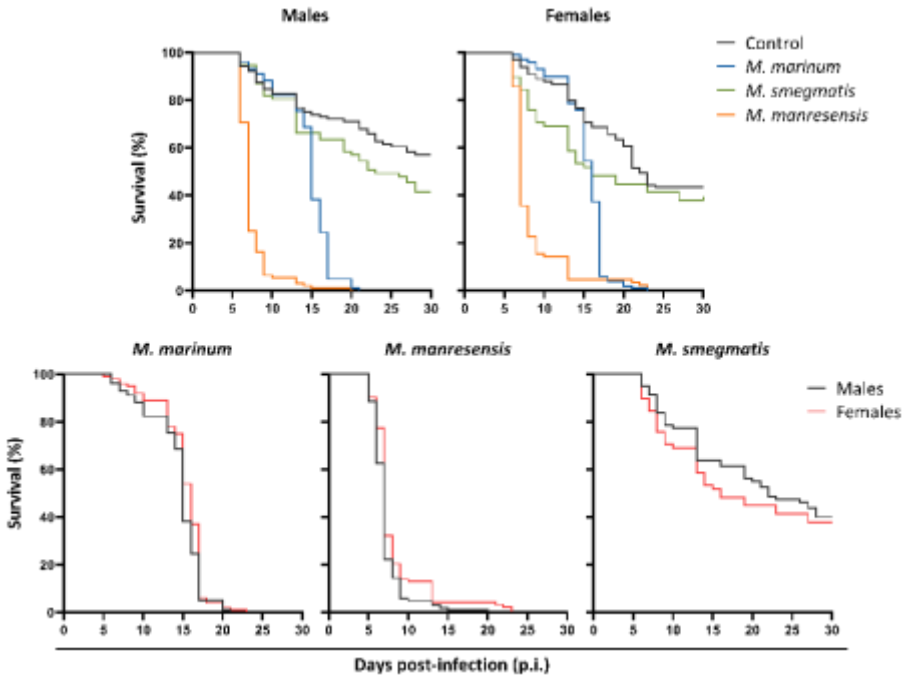


Figure 20| Effect of systemic mycobacterial infections on *D. melanogaster* survival. Statistically significant differences were observed between *M. marinum* and *M. manresensis* infections compared to PBS in both males and females (p<0.0001, Log-rank test) and between males and females in *M. manresensis* infection (p=0.0115, Log-Rank test).

4.2.3| *M. manresensis* replicates faster within *D. melanogaster*

The progression of the bacillary load during the infection for each mycobacterial species was also evaluated. For this, from the previously explained experiment additional 60 males and 60 females were infected for each group and 15 of each were used for CFU counting at different time-points (5 males and 5 females from each biological replicate). Results are presented in Figure 21

As observed in the sub-study 1, *M. marinum* progressively replicated within flies, while *M. smegmatis* seemed to be able to survive within the host for some time, as the bacillary load did not increase and the infection was eventually cleared. On the other hand, *M. manresensis* showed the fastest replication rate within the flies, which was not observed in the growth in vitro. In fact, the bacillary load of *M. manresensis* reached the highest levels of all mycobacteria tested by day 5 p.i. and it reached the plateau by day 7. All flies died previous to the measurement at 10 days post-infection.

When comparing the bacillary load between males and females, only *M. marinum* infection showed significantly increased bacillary load in females, although survival rates were the same for both sexes (Figure 20). On the other hand, after *M. manresensis* infection females survived longer than males, even when the bacillary load was the same. In both sexes, the bacillary load of *M. smegmatis* was cleared by day 10.

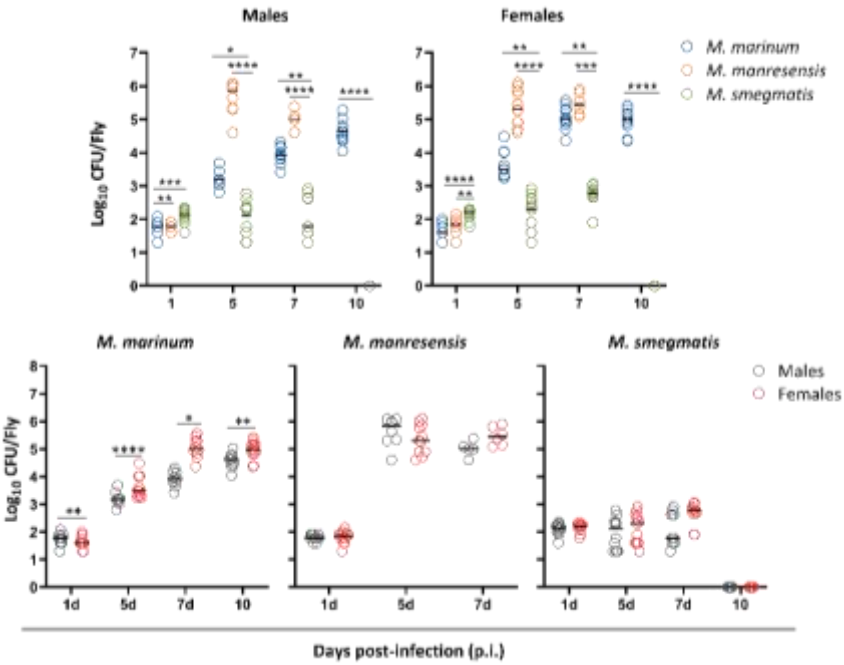


Figure 21| Bacillary load of mycobacterial infections in *D. melanogaster*. Bacillary load is expressed as Log_{10} CFU/Fly. Each circle represents an individual fly and the lines are medians. Data was testes for normality and statistically significant differences were represented as follow: *p≤0.05, **p≤0.01, ***p≤0.001, ****p≤0.0001 (Kruskal-Wallis and Mann-Whitney test).

4.2.4| The innate immune response of *D. melanogaster* against mycobacteria is species-specific

The innate immune response induced by the three different mycobacterial species was also characterized. To do so, the relative expression of selected genes from each immune pathway between infected and uninfected flies were analysed by Real-Time PCR: *diptericin* (Imd pathway), *drosomycin* (Toll pathway), *upd3* (JAK/STAT pathway) and *impl2* (Insulin pathway). For each group, 9 males and 9 females from the previous explained experimental procedure were analysed at different time-points: at the beginning of the infection (early; 24 hours), at an intermediate time point (mid; 5 days), and at a late stage of the infection (late; 10 days for *M. marinum* and *M. smegmatis* infections, and 7 days for *M. manresensis*). Results are shown in Figure 22.

Infection with *M. marinum* did not trigger immediate production of any of the genes neither in males nor in females. However, at 5 days post-infection males showed a peak in the production of Diptericin and Upd3, which was not sustained over the course of the infection. The rest of the genes were not induced at any time point. Females, on the other hand, showed increased production of Diptericin also at day 5 but it was sustained throughout the rest of the infection and late production of Drosomycin. As males, they also showed a punctual production of Upd3 at the same time-point and also an early increase on Impl2.

Opposite to data obtained by Dionne *et al.*, the infection with *M. smegmatis* did show increased production of both AMPs since the beginning of the infection in both males and females, as well as, increased production of Upd3 and Impl2 from day 5. Both in males and females, and for all the genes, the response was sustained over the whole curse of the infection, with the exception of Impl2 in females.

Finally, *M. manresensis* was not only the more virulent species causing lower host survival and higher bacillary load, but it was also the one that triggered a higher immune response. Both males and females showed a statistically significant increased expression for all of the genes at 5 days post-infection that was sustained over time. Again, we found the exception of Impl2, which in both sexes only a punctual peak at day 5 was observed.

The principal component analysis (PCA) of the relative expression for each infection at all time-points revealed that in both males and females, *M. manresensis* infection triggered a significant higher innate immune response compared to flies infected with *M. marinum* mainly characterized by the production of AMPs, as well as those infected with *M. smegmatis* although the response was not as intense (Figure 23).

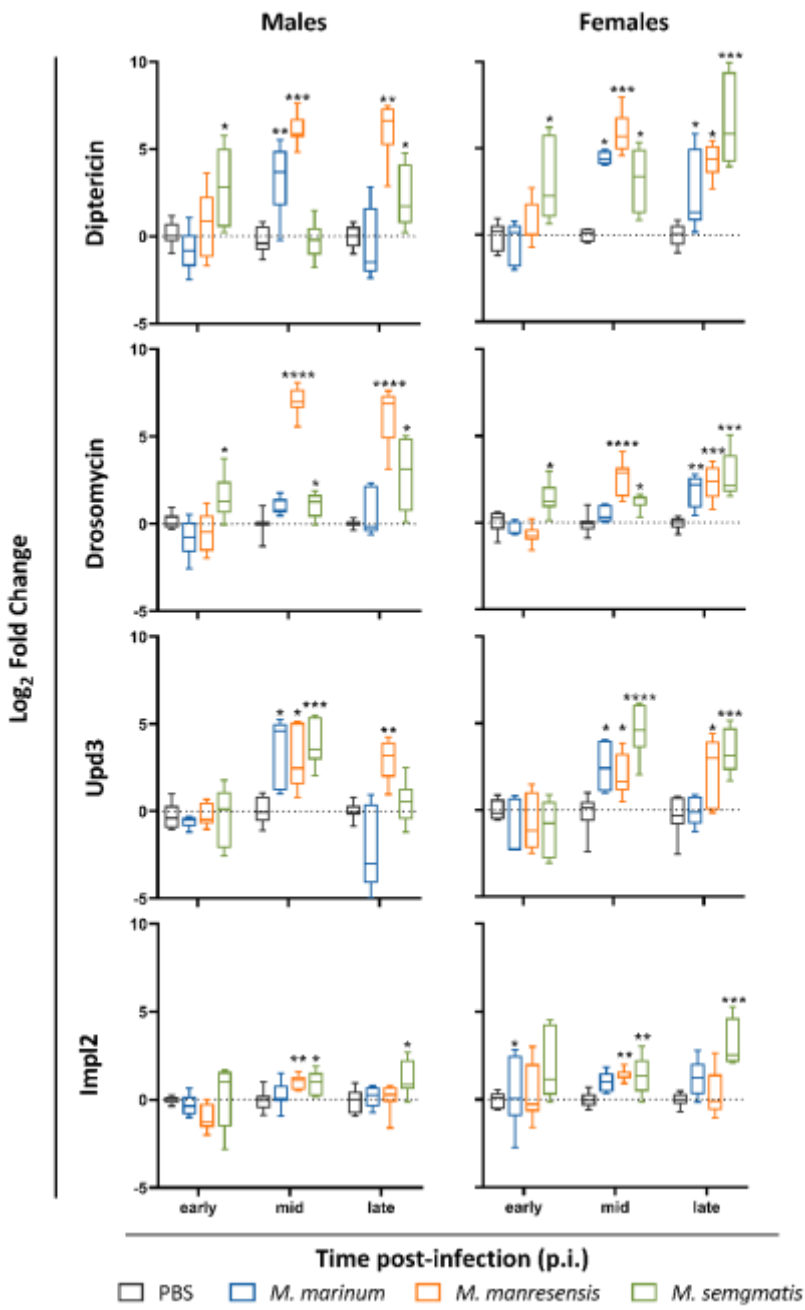


Figure 22| Effect of mycobacterial infections on the innate immune response of *D. melanogaster*. Expression of selected genes in flies infected with *M. marinum*, *M. manresensis* and *M. smegmatis*. Real-Time qPCR results were normalized with the *rpl32* gene and expressed as the log2 fold change between the PBS injected and infected groups (the dot line in each graph represents the controls' relative expression with a fold change of 1). Each infection against the control group at each time-point were analysed independently and data was tested for normality. Significant differences were represented as follow: *p≤0.05, **p≤0.01, ***p≤0.001, ****p≤0.0001 (Kruskal-Wallis test).

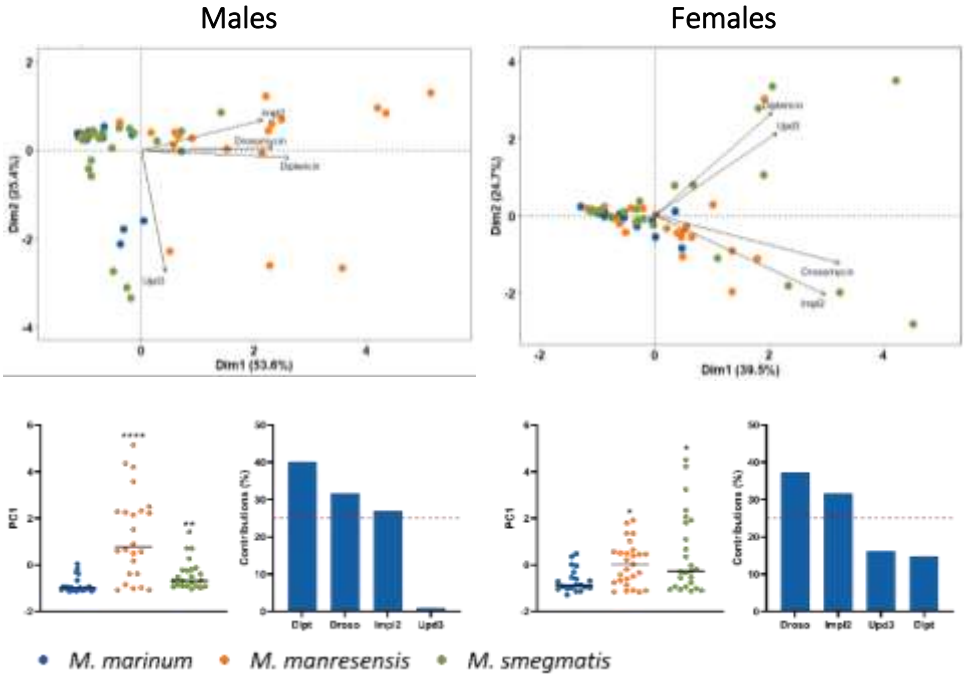


Figure 23| Heterogeneity of gene expression among mycobacterial infections of *D. melanogaster*. (Top) Principal component analysis (PCA) based on expression of selected genes in male and female flies infected with *M. marinum, M. manresensis* and *M. smegmatis* at all time-points. **(Bottom)** PC1 scores (left) and variable contribution (right), each circle represents an individual and lines are means and statistically significant differences were represented as follow: *p≤0.05, **p≤0.01, ****p≤0.0001 (Kruskal-Wallis test).

4.3| Impact of sex and reproductive status of the host in *Mycobacterium marinum* infections

For this sub-study, virgin flies were separated during the first hours after eclosion from pupae to ensure they were not sexually mature yet and the rest of flies were left together to mate. Flies were infected with increasing doses of *M. marinum* at 3 to 5 days after eclosion and mated flies were then either separated by sex or kept together in equal proportions. For the three different groups (virgins, alone and together) survival was checked daily, bacillary load was measured at the time of death and differential gene expression was obtained at 24 hours, 5 days and 10 days post-infection. A total of 90 flies for each group were used for survival and bacillary load (30 flies from each replicate), and 9 flies for each group for gene expression (30 flies from each replicate), divided into three independent experiments.

4.3.1| Tolerance and resistance to *M. marinum* depends on the reproductive status and the sex of the host

Tolerance was measured as the slope of the regression line between the inoculation dose and the days survived post-infection for each group. A slope close to 0 would mean that the increasing the inoculation dose does not reduce the survival of the individuals and, thus, that they are more tolerant to the infection. On the other hand, resistance was represented as the Y-intercept of the regression line between the inoculation dose and the bacillary load at the time of death. When slopes are equal, the lower the Y-intercept the more resistant is the group. If slopes differ significantly between groups, a flattest slope indicates increased resistance against higher bacillary loads. In both cases, slopes and Y-intercepts were analysed for statistically significant differences with the Extra sum-of-squares F test. Results are presented in Figure 24.

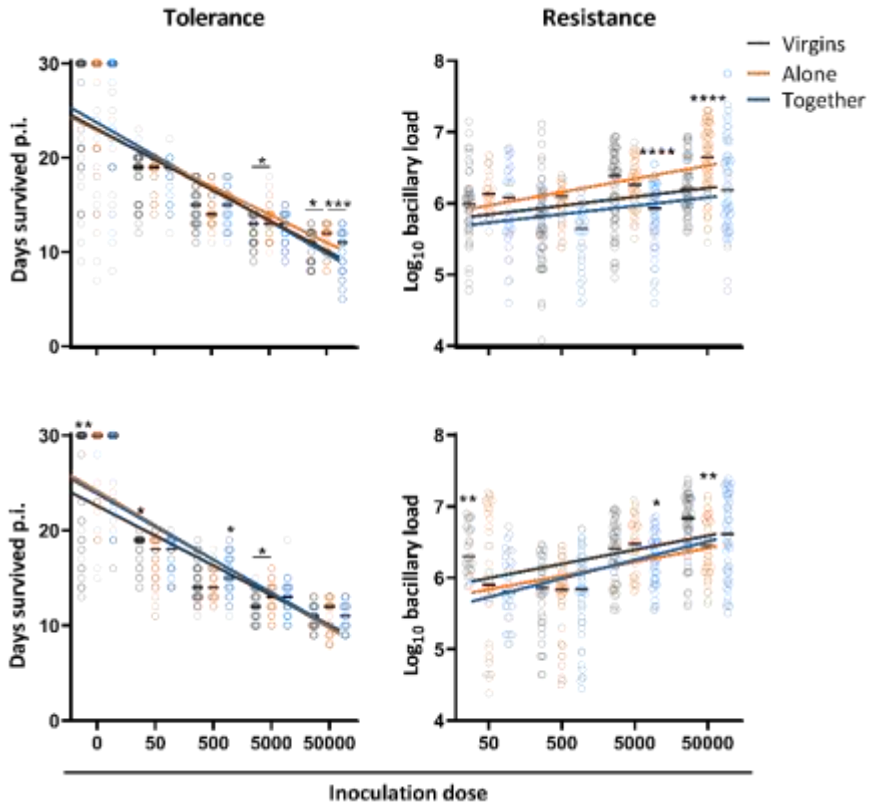


Figure 24| Tolerance and resistance curves of *D. melanogaster* against *M. marinum* depending on the reproductive status of the host. Tolerance and resistance of males (top) and females (bottom). Lines represent the regression lines fitted for each group and each circle represents an individual. Both survival and bacillary load between the groups were analysed independently for each inoculation dose and were tested for normality. Statistically significant differences were represented as follow: *p≤0.05, **p≤0.01, ***p≤0.001, ****p≤0.0001 (Kruskal-Wallis test).

Our study revealed no differences in the general vigour when male flies were not infected, neither in the overall survival at low doses of infection (50 and 500), depending on the reproductive status. At increasing initial doses (5000 and 50000), males that had mated but were kept alone after the infections showed slower mortality although no changes on the tolerance levels compared to virgin males were found. On the other hand, males that were kept in the presence of females after the infection showed lower tolerance to *M. marinum* infections (Table 2).

When looking into the resistance we found that males kept alone had higher bacillary loads independently of the initial dose and were significantly less resistant to *M. marinum*, while virgin males and males in the presence of females showed no statistical difference on the Y-intercept but in the slopes, revealing that males together with females showed higher resistance to increasing inoculation doses (5000 and 50000) (Table 3).

Taken together, this data revealed that in general virgin males were more tolerant and resistant to *M. marinum* infections. Once mated, being in the presence of females reduced their tolerance to the infection but increased their resistance to higher doses of the pathogen, while being kept alone reduced their resistance but not their tolerance.

Table 2| Tolerance to *M. marinum* infection in male *D. melanogaster*

Group	Slope	Comparison	p values
Virgins	-3.098	Alone	ns
Alone	-2.936	Together	0.0025
Together	-3.422	Virgins	0.0307

Table 3| Resistance to *M. marinum* infection in male *D. melanogaster*

Group	Slope	Y-inter	Comparison	p value slope	p value Y-inter
Virgins	0.1531	5.535	Alone	ns	<0.0001
Alone	0.2088	5.586	Together	ns	<0.0001
Together	0.1319	5.492	Virgins	ns	0.0204

In females, the general vigour when not infected was significantly lower in virgin flies, although they also showed a better survival to low inoculation doses and an overall higher tolerance when compared with both mated groups. Females that had mated but kept alone after infection showed increased survival at higher doses (5000 and 50000) while females kept together with males showed better survival at the

intermediate dose (500). However, no significant differences were observed between their general tolerance levels (Table 4)

As observed in male flies, the more tolerant group, which were the virgin females, showed lower resistance to the infection when compared with the lower tolerant groups (Table 5). Both mated females' groups showed no differences neither in the slope nor in the Y-intercept in the resistance regression lines. Thus, seems that once females have mated they become less tolerant but more resistant to *M. marinum* infections than virgin females, independently to the presence or absence of males.

Table 4| Tolerance to *M. marinum* infection in female *D. melanogaster*

Group	Slope	Comparison	p value
Virgins	-3.073	Alone	0.0026
Alone	-3.545	Together	ns
Together	-3.404	Virgins	0.0334

Table 5| Resistance to *M. marinum* infection in female *D. melanogaster*

Group	Slope	Y-inter	Comparison	p value slope	p value Y-inter
Virgins	0.2208	5.575	Alone	ns	0.0062
Alone	0.2150	5.427	Together	ns	ns
Together	0.2903	5.177	Virgins	ns	0.0038

When comparing males and females for each reproductive status independently (Figure 25), we found that virgin flies did not differ neither in general vigour when not infected nor in general tolerance to the infection, but males were significantly more resistant. Same pattern was observed in flies that were kept together in equal proportions after the infection: both males and females had same general vigour and same tolerance, but males showed up to be more resistant as the inoculation doses increased. On the other hand, when flies mated but were kept separated by sex after the infection, females had increased general vigour when not-infected and were overall less tolerant but more resistant to the infection compared to males (Table 6-7).

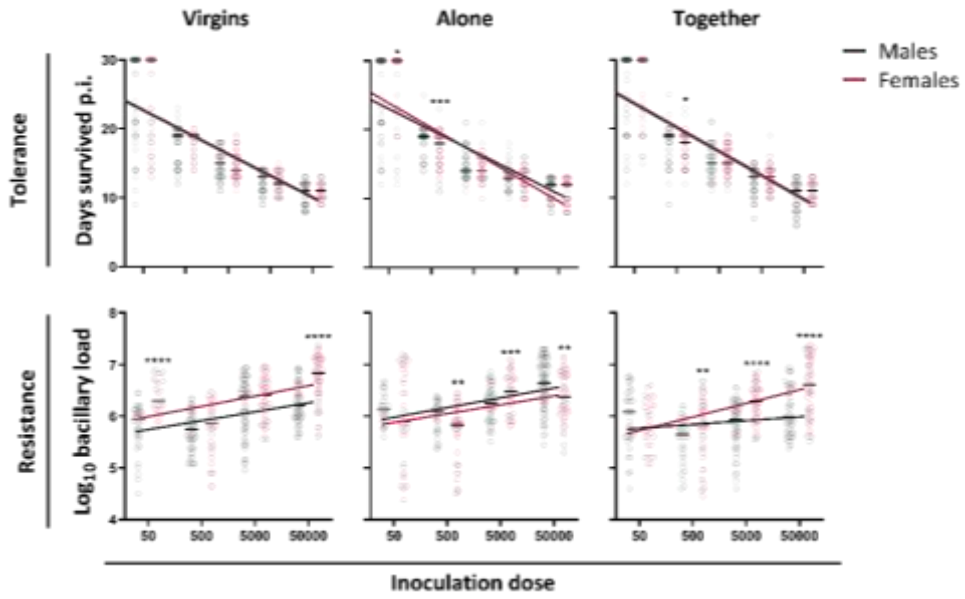


Figure 25| Tolerance and resistance curves of *D. melanogaster* against *M. marinum* depending on the sex of the host. Lines represent the regression lines fitted for each group and each circle represents an individual. Both survival and bacillary load between the groups were analysed independently for each inoculation dose and were tested for normality. Statistically significant differences were represented as follow: * p≤0.05, **p≤0.01, ***p≤0.001, ****p≤0.0001 (Welch's corrections for normally distributed data and Mann-Whitney test for not normally distributed data).

Table 6| Tolerance to *M. marinum* infection in *D. melanogaster*

Group	Slope males	Slope Females	p value
Virgins	-3.098	-3.073	ns
Alone	-2.936	-3.545	0.0021
Together	-3.422	-3.404	ns

Table 7| Resistance to *M. marinum* infection in *D. melanogaster*

Group	Slope males	Slope females	p value	Y-inter males	Y-inter females	p value
Virgins	0.1384	0.2208	ns	5.593	5.575	<0.0001
Alone	0.2088	0.1894	ns	5.586	5.522	0.0166
Together	0.1319	0.2903	0.0024	5.492	5.177	ns

4.3.2| Innate immune response against mycobacterial infections depends on the reproductive status and the sex of the host

The innate immune response induced by the three different reproductive status in both males and females was also characterized. To do so, relative expression of selected genes from sub-study 2 between infected and uninfected flies was analysed by Real-Time qPCR. For each group, 9 males and 9 females from the previous explained experimental procedure were analysed at 24 hours, 5 days and 10 days post-infection. Results are presented in Figure 26.

Virgin males showed a general late response to the infection with production of Diptericin only at 10 days post-infection and no increase on Upd3. Surprisingly, there was an early production of Drosomycin and also a pick on Impl2 at day 5, although none of these were sustained throughout the infection. Males that had mated and were kept together with females showed the opposite pattern, with early production of Diptericin, but not Drosomycin and a pick of Upd3 5 days post-infection. No changes on the expression levels of Impl2 were observed. On the contrary, males that had mated and were then kept alone showed significant increased production of both AMPs from 5 days post-infection, that in the case of Drosomycin was sustained over time. In addition, increased production of Upd3 was also observed since the beginning of the infection, as well as Impl2. The more resistant groups (males together and virgin males) coincided with the low expression levels of Drosomycin and Upd3 and the more tolerant (virgin males and males alone) showed late production of Diptericin, compared to the less tolerant (males together).

Females showed more controversial results with not as good concordance between phenotypic and gene expression results as males. The expression levels profile of virgin females and females alone were more similar, with high production of Diptericin 5 days post-infection and low production Drosomycin, Also, both groups had early increased production of Upd3. However, virgin females were more tolerant and less resistant to the infection than females alone. On the other hand, females that were kept together with males after the infection were equally tolerant and resistant than females kept alone, but they showed a more sustained production of Diptericin since the beginning of the infection and late production of Drosomycin and Upd3. None of the groups showed increased production of Impl2. In addition, virgin females and females kept alone presented even significant repression of these gene expression at the later stage of the infection.

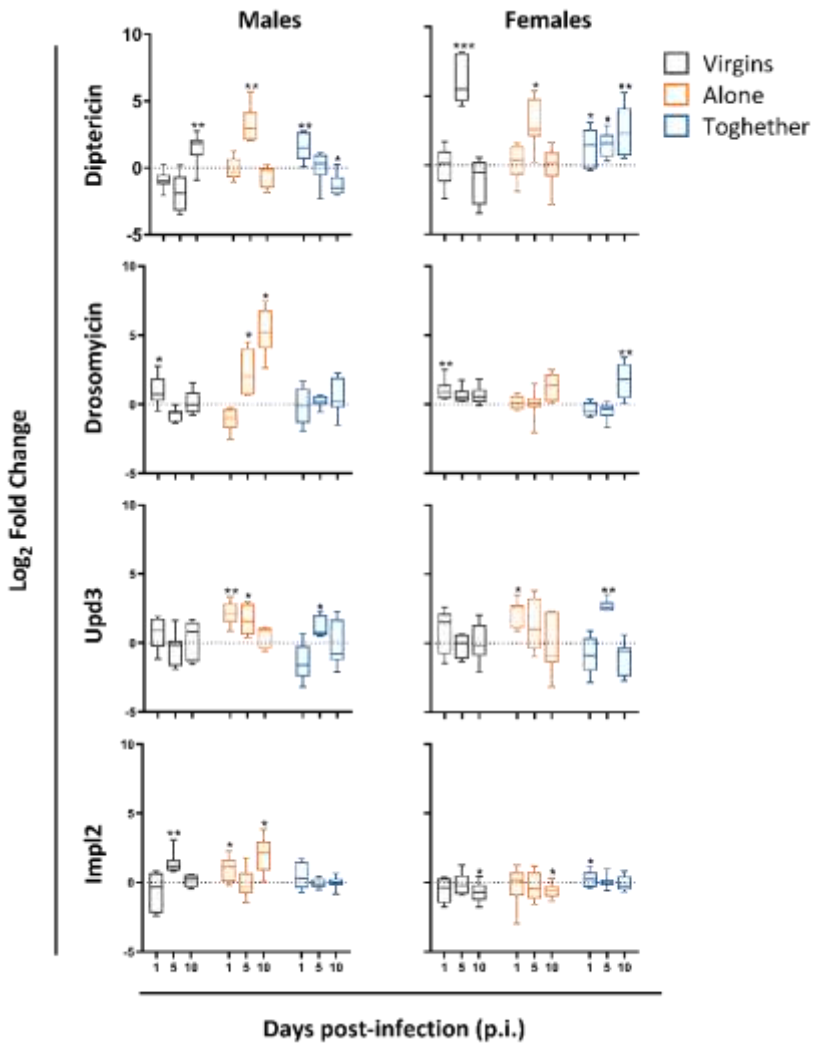


Figure 26| Effect of *M. marinum* infection on the innate immune response of *D. melanogaster* based on reproductive status. Expression of genes of interest in male and females flies infected with *M. marinum* over time. Real-Time qPCR results were calculated with the $2^{-\Delta\Delta CT}$ method using the *rpl32* gene for normalization and expressed as the $\log_2$ fold change between uninfected and infected flies. Each group at each time-point was compared to its relative control independently (the dot line in each graph represents the controls' relative expression with a fold change of 1). Data was analysed for normality and significant differences were represented as follow: *p≤0.05, **p≤0.01 (Welch's corrections for normally distributed data and Mann-Whitney test for not normally distributed data).

When comparing males versus females for each reproductive status independently, data revealed that when virgins, females showed higher production of Diptericin and same levels of Drosomycin, and the same happened with flies that were kept together. On the other hand, when males and females were kept separated they showed same expression levels for Diptericin but males showed significantly more Drosomycin than

females. Overall, females showed higher production of Upd3 and lower production of Impl2 in all reproductive statues when compared to males (Figure 27).

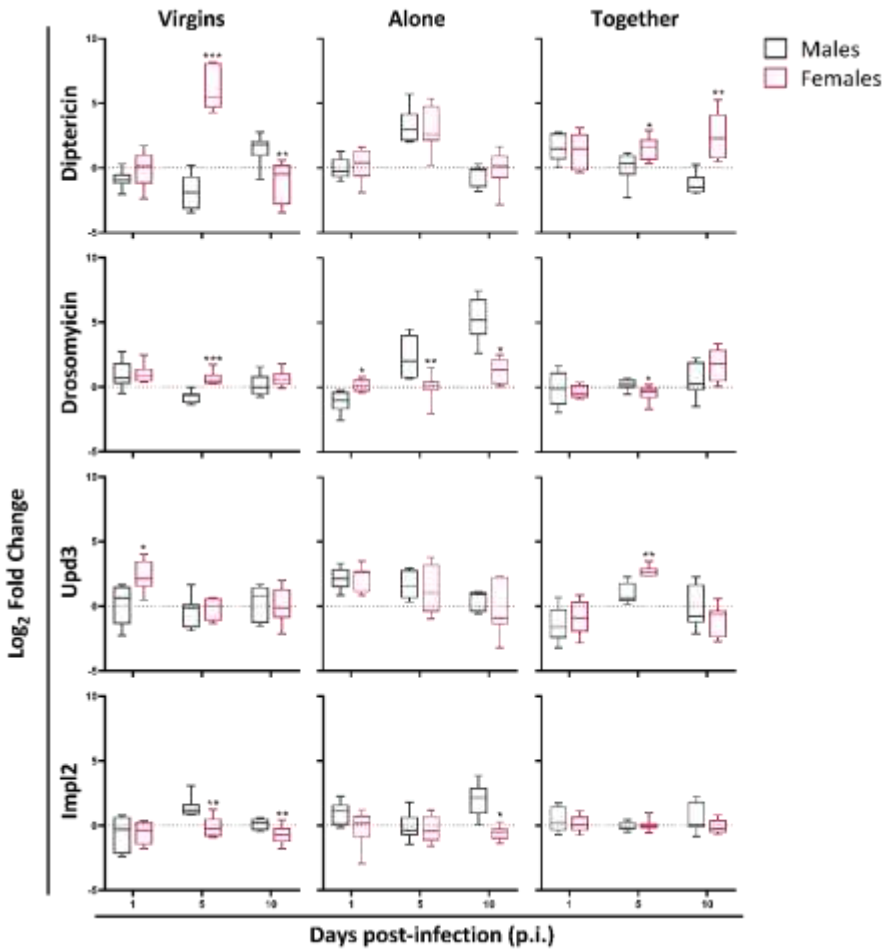


Figure 27| Effect of *M. marinum* infection on the innate immune response of *D. melanogaster* based on sex. Expression of genes of interest in male and females flies infected with *M. marinum* over time. Real-Time qPCR results were calculated with the $2^{-\Delta\Delta CT}$ method using the *rpl32* gene for normalization and expressed as the $\log_2$ fold change between uninfected and infected flies. Each time-point was compared independently (the dot line in each graph represents the controls' relative expression with a fold change of 1). Data was analysed for normality and significant differences were represented as follow: *p≤0.05, **p≤0.01 (Welch's corrections for normally distributed data and Mann-Whitney test for not normally distributed

We also evaluated the expression levels of the ecdysone receptor (EcR) due to the tight relationship that Ecdysone has with immunity and reproduction. In this case, we measured the relative expression of this gene after the infection, but also the expression levels when injected with PBS to evaluate the differences on the basal expression depending on the reproductive status (Figure 28).

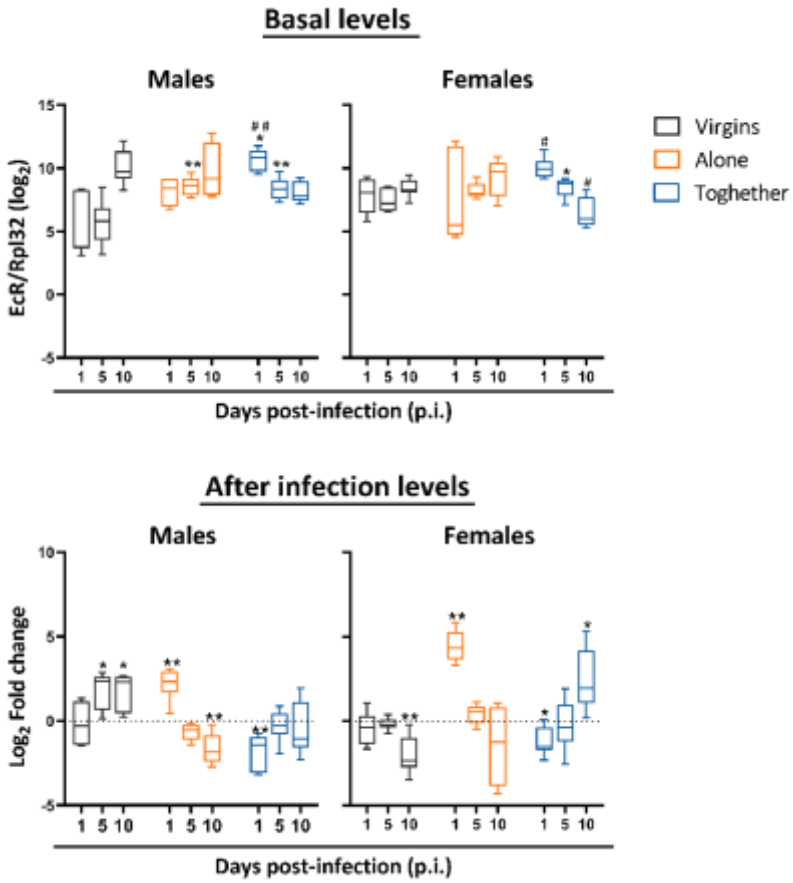


Figure 28| Expression of EcR in *D. melanogaster*. Basal expression of the gene in uninfected flies. Expression levels were calculated using the $2^{-\Delta CT}$ method with the *rpl32* gene for normalization (all values were multiplied by 10^4 for more visual results). Groups were compared independently for each time-point (* indicates differences with virgins and # represent differences between alone and together). Data was analysed for normality and significant differences were represented as follow: *p≤0.05, **p≤0.01, ***p≤0.001 (Kruskal-Wallis test). **After the *infection,*** the relative expression of the gene was measured in infected flies. Relative expression levels were calculated with the $2^{-\Delta\Delta CT}$ method. Each group at each time-point was compared to its relative uninfected control independently (the dot line represents the controls' relative expression with a fold change of 1). Data was analysed for normality and significant differences were represented as follow: *p≤0.05, **p≤0.01 (Welch's corrections for normally distributed data and Mann-Whitney test for not normally distributed data).

Virgin males showed increasing production of EcR over the course of the infection and decreasing levels in the case of males kept alone. Males that were kept together did not show any increased induction of this gene. However, when observing the basal expression levels when flies were not infected, we observed significant differences among groups. Virgin males and males kept alone showed increasing basal levels of EcR over time, although virgin males had lower values initially, while males kept together with females presented decreasing values of EcR at basal levels.

Females that mated showed similar pattern are their males' counterpart. Females kept alone showed increasing basal levels of EcR and decreasing induction when infected, while females kept together with males showed increasing production of EcR over the course of the infection, but decreasing basal levels. However, virgin females showed no changes neither in the basal levels nor in the induction of EcR after the infection.

When comparing males and females for each reproductive status we observed that mated females also showed increased production of EcR at early stages of the infection when were kept alone and at late stages when were kept together with males, but lower production when virgins. On the other hand, females showed significant lower basal levels of EcR compared to males when they were virgins or separated from males, but had the same basal levels as males when they were kept together (Figure 29).

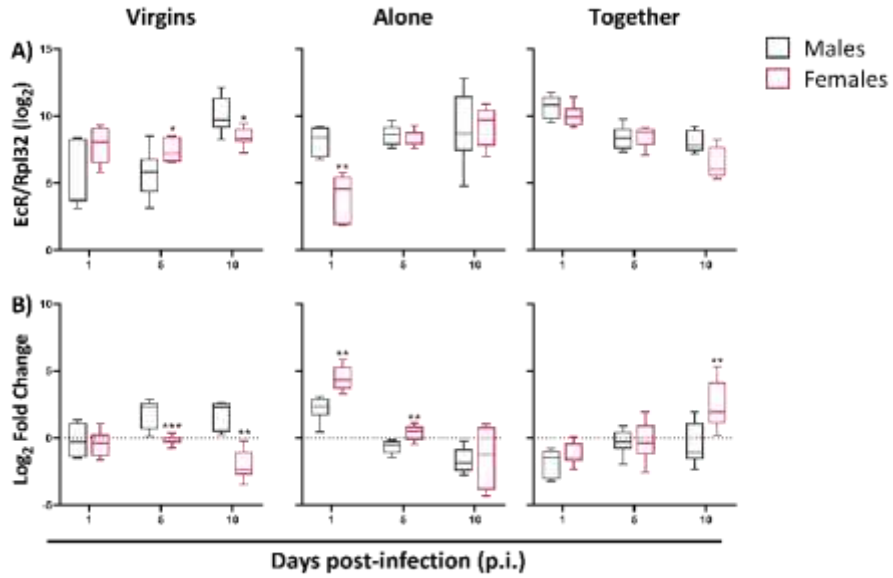


Figure 29| Expression of EcR in *D. melanogaster*. (A) Basal expression of the gene in uninfected flies. Expression levels were calculated using the $2^{-\Delta CT}$ method as before (all values were multiplied by 10^4 for more visual results). Each time-point was compared independently. Data was analysed for normality and significant differences were represented as follow: *p≤0.05, **p≤0.01, ***p≤0.001 (Welch's corrections for normally distributed data and Mann-Whitney test for not normally distributed data) **(B) Relative gene expression** of the gene in infected flies. Relative expression levels were calculated as before. Each time-point was compared independently (the dot line represents the controls relative expression with a fold change of 1). Data was analysed for normality and significant differences were represented as follow: *p≤0.05, **p≤0.01 (Welch's corrections for normally distributed data and Mann-Whitney test for not normally distributed data).

The Principal component analysis (PCA) performed with the relative expression levels of infected flies including all time-points for each condition revealed that males that were kept alone after the infection and virgin females were significantly different at gene expression level from the rest of their respective groups. In the case of males, this difference was mainly driven by the differential expression of Impl2, while in females the main differences were driven by EcR and Drosomycin (Figure 30).

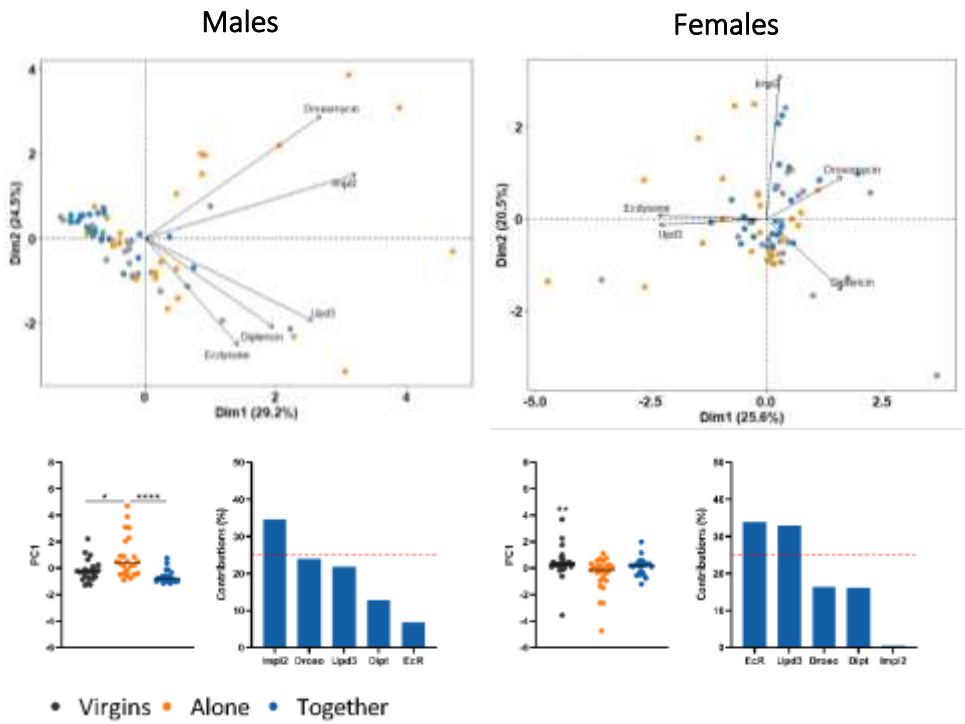


Figure 30| Heterogeneity of gene expression among flies with different reproductive status. (Top) Principal component analysis (PCA) based on expression of genes of interest in males and females infected with *M. marinum* at all time-points. **(Bottom)** PC1 scores (left) and variable contribution (right), each circle represents an individual and lines are means and statistically significant differences were represented as follow: *p≤0.05, **p≤0.01, ****p≤0.0001 (Kruskal-Wallis test).

4.4| Discussion

This first study aimed to further characterise mycobacterial infections in the *Drosophila melanogaster* model to demonstrate its suitability for the study of the role of the innate immune response in TB infection. The data provided herein suggest that, indeed, *Drosophila melanogaster* is a suitable model for the study of mycobacterial infections. The establishment of our infection protocol revealed that systemic infection with *Mycobacterium marinum* kills flies in a dose-dependent manner and the analysis

of the three independent biological replicates also revealed that this infection method is robust and consistent among experiments and that inoculation doses can be highly controlled, thus, allowing the selection of the optimal initial infectious dose for each further procedure.

As TB is an airborne disease, we aimed to establish a "natural infection", as has been described for other pathogens as a feasible route of entry into the *D. melanogaster* host (369,370). However, our data reveal that *M. marinum* is not able to survive inside flies' intestinal tract long enough (bacillary load is reduced significantly within 2 days) and that the infection cannot be established. Previous studies demonstrate that this mycobacterium is an obligate intracellular pathogen that requires replication within *Drosophila*'s hemocytes for at least 2 days before being found replicating extracellularly (317). In the flies, hemocytes mainly localize close to the midgut on normal conditions and after infection, they attach to the visceral muscle transiently surrounding the gut, but they have never been described in the lumen (371). Thus, the lack of hemocytes in the gut of flies could be one reason why *M. marinum* is not able to establish a proper natural infection in this host model.

Once the infection protocol was properly established, we aimed to define the specificity of the innate immune response of *Drosophila* to several mycobacterial species. The infections with *M. marinum* and *M. smegmatis* were previously described, however, very few studies have been published about this host-pathogen system. Dionne *et al.*, characterised *M. marinum* as a pathogenic species that could replicate within *Drosophila*'s hemocytes and *M. smegmatis* as a non-virulent species, although bacillary load was not recorded (317). Thus, our results with *M. marinum* and *M. smegmatis* were in line with these previously published findings on survival levels. Surprisingly, the environmental species *M. manresensis*, which we hypothesised should be non-virulent as none of the characteristic virulence factors of pathogenic mycobacteria (i.e. ESAT-6) have been described in this species, proved to be more virulent for the flies.

Our data also reveals that unlike the growth pattern observed in vitro, where *M. smegmatis* grows rapidly followed by *M. marinum* and *M. manresensis*, the opposite happens inside the host. Here we demonstrate that *M. smegmatis* seems to be able to survive within the host throughout the infection but not to replicate, and is finally cleared. Interestingly, *M. manresensis* grows the fastest inside flies and we hypothesised that this ability to rapidly replicate within the host should be a key factor in determining the virulence of the mycobacterial species, as *M. manresensis* grows faster than *M. marinum* and kills flies sooner.

We also assessed the innate immune response triggered by these mycobacteria. To do so, we selected some genes of interest from the main immune pathways. We

chose Diptericin as the main downstream gene of the Imd pathway and Drosomycin for the Toll pathway. Our data shows that early activation of the innate immune system, as described for many other pathogens, only happens for the non-pathogenic mycobacteria. Flies infected with *M. smegmatis* showed immediate activation of both immune pathways and production of Diptericin and Drosomycin throughout the whole infection. This data does not correlate with the previous finding by Dionne *et al.*, but it explains the lack of bacillary load increase observed previously. This data also suggests that virulent mycobacteria somehow inhibit the innate immune response of the host at the beginning of the infection.

If we take a look at *M. marinum* infection, our results differ from the data published previously for males. This data generates some controversy, as it has been described that mycobacteria are recognized by the Toll signalling pathway in *Drosophila*, however, here we show that males only induce expression of the Imd-related AMP, Diptericin, 5 days after the infection coinciding with the extracellular presence of the bacilli. On the contrary, in females, we show increased production of both AMPs also when the bacilli already replicate extracellularly. On the other hand, *M. manresensis* triggers a high production of AMPs from day 5 that is sustained throughout the infection, although this does not translate into the reduced bacillary load as happens with *M. smegmatis*. We hypothesise that this cannot be explained by an intracellular localization of the bacteria but by the rapid replication of the bacilli that might cope with the innate immune response.

We also assessed the expression levels of the cytokine-like molecule Upd3, as is the homolog of the proinflammatory cytokine IL-6 in humans. Upd3 is known to be secreted by hemocytes after an infection or detection of tissue damage and to be the ligand for the activation of the JAK/STAT pathway, which regulates immune genes other than AMPs, mainly related to tissue repair and homeostasis and also favours metabolic switch in the fat body to favour hemocytes activity. Our results reveal that all mycobacteria tested here trigger the production of this molecule at least 5 days after the infection, although only for *M. manresensis* this induction is sustained over time.

Finally, we also analysed expression levels of Impl2. *M. marinum* has been described to kill flies by inducing a cachexic state in which the host loses energy storage (262) via the reduction of AKT signalling and induction of Foxo-dependent transcription. However, the signalling between the insulin receptor and AKT is not broken, as injection of insulin restores the signalling (262). Impl2 is known as a cancer-derived cachexic factor, that induces mobilization of energy towards tumours and also induces insulin resistance by binding to circulating dILPs, but also induce this mobilization towards activated hemocytes. The induction of Impl2 is controversial, as a temporal activation leads to resistance but chronic activation of Impl2 leads to

wasting and cachexia. (273,274,325). We observed that all species, whether virulent or not or intracellular or extracellular, had a punctual increased expression of Impl2 during the infection. For the extracellular species, the production of Impl2 together with Upd3 by hemocytes could lead to higher bactericidal activity within the immune cells. However, once the pathogen is found replicating extracellularly, the induction of this metabolic switch is inefficient and ultimately detrimental for the host.

Repeatedly, we have observed significant differences between males and females throughout the study, from increased bacillary load in females infected with *M. marinum*, to increased survival in *M. manresensis* infection or differences in gene expression levels. Thus, we decided to evaluate the effect that sex and also reproduction have in the response to mycobacterial infections.

Typically, laboratory studies have revealed a significant "cost of mating" to *Drosophila* females in the form of reduced longevity. However, here we present that virgin females show a significant decrease in general vigour compared to mated females when uninfected, which is not observed in virgin males. This phenomenon has only been described previously for wild-caught flies (372). Previous studies described that females reduce their resistance to some infection after mating, while males exposed to females are more susceptible (290,295,308). However, data presented herein suggest that males will only prioritise the infection clearance when reproduction is assured, whereas when females are not present, they tend to prioritise fitness. In contrast, females seem to devote more effort to the clearance of the infection once they have mated than when they are virgins. Still, in general, males tend to have a higher ability to control infection compared to females, except for those that are alone, which in that case increase their fitness over infection. In addition, our data reveal that, in flies, tolerance and resistance to *M. marinum* infections are in inverse proportion.

We also assessed the innate immune response triggered by the different sexes at each reproductive status and the hormonal levels of each group before and after the infection.

Table 8| Hypothesis on the effect of the expression of the following genes on tolerant or resistant phenotype.

	Tolerance	Resistance	Hypothesis
Diptericin	-	?	Increases in immune futile expense
Drosomycin	+	+	Reduces hemocytes activation and LD
Upd3	-	+	Low levels reduce LD / High levels increase hemocytes activation
Impl2	-	+	Increases hemocytes activation
bEcR	-	?	Stimulates Imd pathway activation

Our data shows that a certain EcR levels are required for all the flies after the infection, as when flies have higher basal levels the infection does not induce and even represses its production. On the other hand, flies with lower basal levels of EcR trigger its expression after the infection. Previous studies showed that ecdysone induces the expression of the Imd-related receptor PGRP-LC and, thus, modulates the Imd pathway (302). Our data supports this finding, as those flies with higher basal levels of EcR (males alone and together and females together) show higher expression levels of Diptericin when infected.

If we try to connect the innate immune response with the phenotype observed (Table 9), our data correlate with previous studies that linked Upd3-deficient flies with over-expression of Atg2, reduction of lipid droplet accumulation within cells and reduced bacillary loads (281). Thus, making flies more resistance to *M. marinum* infections, as reflected in the phenotype of males and females kept together.

On the other hand, we also see that production of the Toll-dependant AMP, Drosomycin, links with results found in the murine model that the activation of Toll-like receptors leads to impaired effective killing of intracellular bacteria in macrophages and increased bacillary load in the lung upon Mtb infections (373). Thus, explaining the tolerant phenotype observed in virgin flies. Thus, suggesting a role for the Toll pathway in regulating tolerance to *M. marinum* infections, maybe by reducing oxidative stress in cells. However, recent studies have described a Toll-dependant metabolic switch that directs fatty acids from neutral cellular storage toward phospholipid biosynthesis (374), thus increasing resistance to infection as observed for virgin males.

When females are kept alone, although they show high production of Upd3 and thus should have higher bacillary loads, the bactericidal activity of hemocytes is not reduced by the Toll pathway and is also triggers by the Upd3 itself. These results suggest a dual role of Upd3 in the immune response against *M. marinum* and that the bactericidal activity is enough to reduce the bacillary load. On the other hand, males that have mated but are kept alone, have more Toll pathway activation, thus reducing the oxidative stress at expenses of higher bacillary loads. These links with their tolerant phenotype.

Altogether, this data suggests a role for the Toll pathway in determining the hemocytes activity against *M. marinum* infections and regulating tolerance levels. Previous studies had related the Imd pathway in the control of resistance to infections (375) as well as the Toll pathway had been described as key in determining resistance to infection in males, but not in females (287). However, any of these studies were performed in mycobacterial infections.

In conclusion, this first study demonstrates that *Drosophila melanogaster* is a suitable model for the study of mycobacterial infections, with an easy and controlled infection protocol with robust outcomes. We have also demonstrated that the innate immune response induced is non-species-specific, but that the ability to replicate within the host has a key role in the virulence of these pathogens. We have also shown that this host is a suitable model to study the sex dimorphisms in infectious disease, revealing antagonism for tolerance and resistance in *M. marinum* infections and also that this sexual dimorphism is highly related, not only to the sex of the host but also to its reproductive status.

Table 9| Summary table of the results of sexual dimorphism study

Males	**Gene expression**					**Phenotype**	
	Diptericin	**Drosomycin**	**Upd3**	**Impl2**	**bEcR**	**Tolerance**	**Resistance**
Virgins	Late	Early	None	Late	Low	+	+
Alone	Late	High late	Early	Early	Mid	+	-
Together	Early	None	Late	None	High	-	++
Females	**Gene expression**					**Phenotype**	
	Diptericin	**Drosomycin**	**Upd3**	**Impl2**	**EcR**	**Tolerance**	**Resistance**
Virgins	Late	Early	None	None	Low	+	-
Alone	Late	None	Early	None	Low	-	+
Together	Early	Late	Late	Early	High	-	+
M vs. F	**Gene expression**					**Phenotype**	
	Diptericin	**Drosomycin**	**Upd3**	**Impl2**	**bEcR**	**Tolerance**	**Resistance**
Virgins	F late	F	F earl.	M late	F	=	M
Alone	=	M late	=	M late	M	M	F
Together	F late	=	F late	=	=	=	M

5| Study 2

Assessing the protective effect of heat killed *M. manresensis* against subsequent infections

5.1| Experimental design

For the study of the effect that oral administration of heat-killed *Mycolicibacterium manresensis* (hkMm) could have on the induction of innate immune memory, male and female flies were orally administrated the treatment for 24h or 48h (with addition of new treatment at 24h) and were fed with normal medium for 72h prior to the second challenge. The experimental design for this study is outlined in Figure 31, and can be divided in two parts.

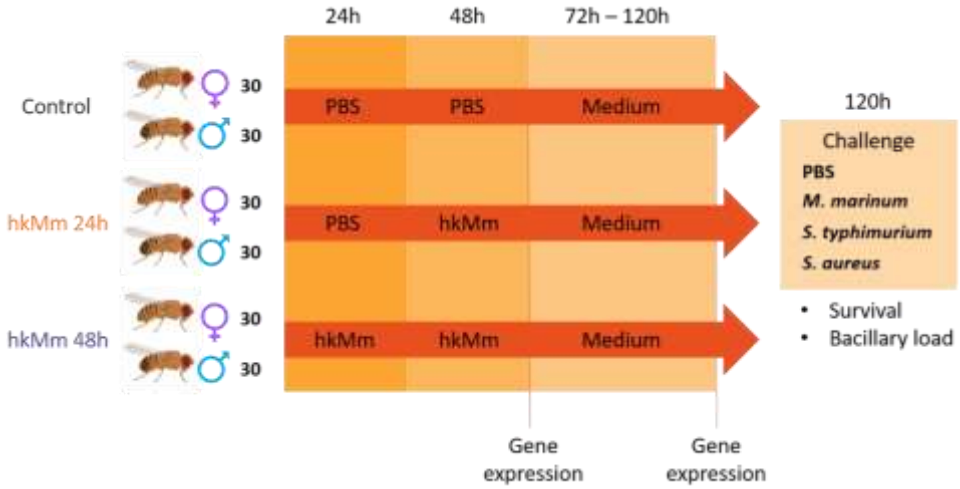


Figure 31| Experimental design for Study 2

First of all, we evaluated the innate immune response induced by the oral administration of 10^5 CFU of hkMm for 24h or 48h by Real-Time qPCR. For each group, 9 males and 9 females from 3 independent experiments (3 males and 3 females from each replicate) were analysed at the end of the treatment and 72h after the treatment (120h from the beginning of the experiment), prior to exposure flies to the subsequent infections.

On the other hand, we studied the protective effect of the hkMm treatment against subsequent infections. For a specific infection, 500CFU of *M. marinum* were injected systemically in treated and control flies. For unspecific infections, we injected flies systemically with the Gram-positive *Staphylococcus aureus* (376). Diverse inoculation doses were tested initially based on previous studies and finally a dose of 20CFU was selected for optimal survival rates. As a Gram-negative, we selected *Salmonella typhimurium* at an initial dose of 10^4 CFU, based on the study by Brandt *et al.* (377). Subsequent injection with PBS was used as control. For this, 360 males and 360 females were used for each treatment (PBS, 24h and 48h) and were then separated into the 4 subsequent infections (90 males and 90 females from each

treatment group for each infection). Data came from three independent biological replicates.

5.2| Results

5.2.1| Orally administrated hkMm induces the innate immune response of *D. melanogaster* in the absence of subsequent challenges

Treatment with 10^5CFU oh hk *M. manresensis* was administrated orally to flies for 24 or 48 hours, and were fed with normal media for 3 days prior to infect them with different bacterial pathogens. The expression levels of selected genes of interest were evaluated both at the end of the treatment and before subsequent infections in order to evaluate the innate immune response triggered by the treatment and the length of this effect. Results are presented in Figure 32.

Immediately after the treatment, no increased expression was observed for any evaluated gene in both sexes. Surprisingly, 72h after the treatment ended we observed an increase in the innate immune response of males in a dose-dependent manner. On the other hand, this induction was subtler in females and only significant for those treated for 48h.

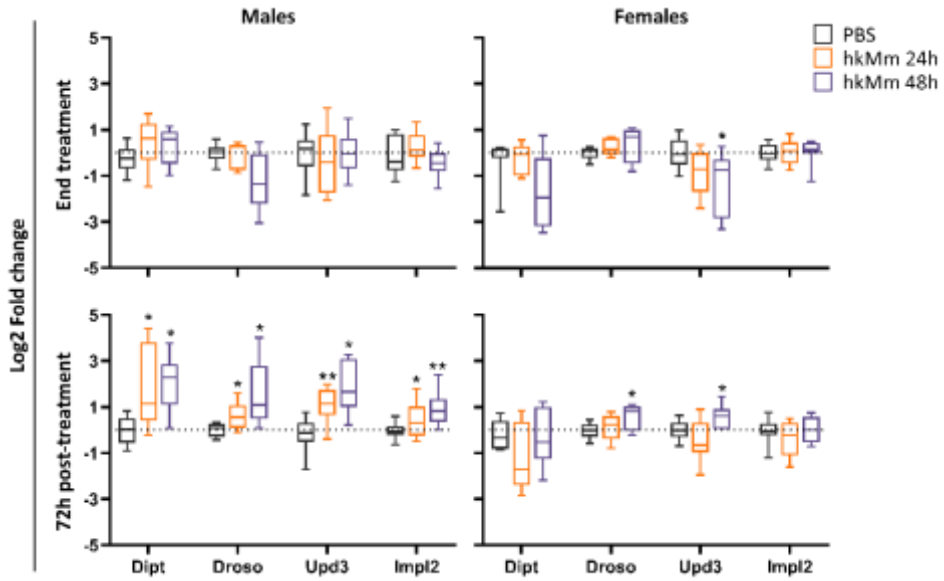


Figure 32| Effect of oral administration of 10^5 hkMm on the innate immune response of D. melanogaster. Flies were treated for 24h and 48h and expression levels of genes of interest were assessed at the end of the treatment and 72h after. Real-Time qPCR results were normalized using *rpl32* and expressed as the $\log_2$ fold change between treated and non-treated flies (PBS). The dot line represents the average expression of the untreated flies with fold change of 1. Each time point was analysed independently. Data was analysed for normality and significant differences were represented as follow: *p≤0.05, **p≤0.01 (Kruskal-Wallis test).

5.2.2| Oral administrated hkMm induces Nox-dependent production of ROS

An important part of the intestinal immune response in *D. melanogaster* is the production of ROS. Thus, we also evaluated the effect that the oral treatment with hk bacilli may have in the expression levels of the two NADPH oxidase enzymes, which are responsible for the production of ROS in flies: Duox and Nox. Results are presented in Figure 33.

None of the treatment regimens induced production of any of the NADPH oxidase enzymes immediately after the treatments ended neither in males nor in females. However, the both treatment regimens induced the production of Nox 3 days after the treatment ended, and prior to any other subsequent infections, in both males and females.

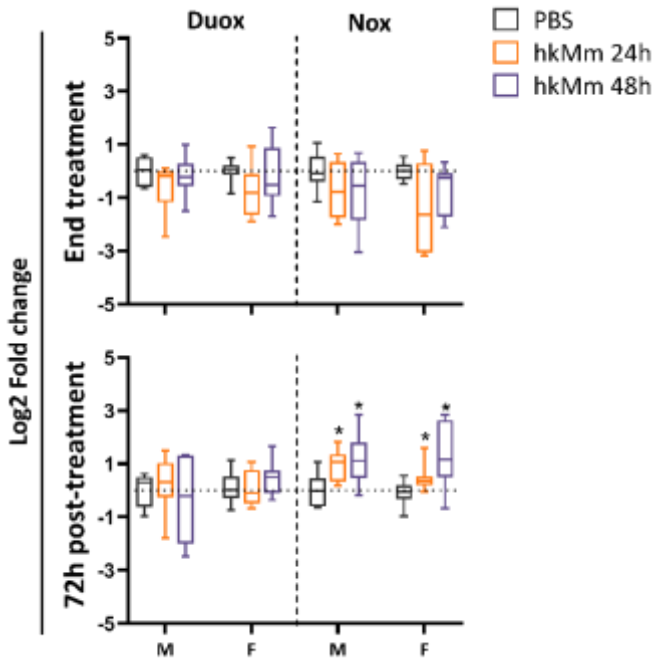


Figure 33| Effect of oral administration of 10^5 hkMm in ROS production in *D. melanogaster*. Flies were treated for 24h and 48h and expression levels of genes of interest were assessed at the end of the treatment and 72h later. Real-Time qPCR results were normalized using *rpl32* and expressed as the $\log_2$ fold change between treated and non-treated flies (PBS). The dot line represents the average expression of the controls with a $\log_2$FC of 0. Each time point was analysed independently. Data was analysed for normality and significant differences were represented as follow: *p≤0.05 (Kruskal-Wallis test).

5.2.3| Oral administration of heat-killed *M. manresensis* (hkMm) effect on *D. melanogaster* response to subsequent infection

First, we evaluated whether the oral administration of hkMm would give specific protection to *D. melanogaster* against subsequent infections with other mycobacteria or not. To do so, flies were infected systemically with 500CFU of *M. marinum* 72h after the end of the treatment. Survival was checked daily until all flies died and the bacillary load of each individual was measured at the time of death.

No significant differences were observed on survival times neither in males nor in females due to any of the treatment regimens. However, both treatment regimens significantly reduced the bacillary load in both sexes (Figure 34).

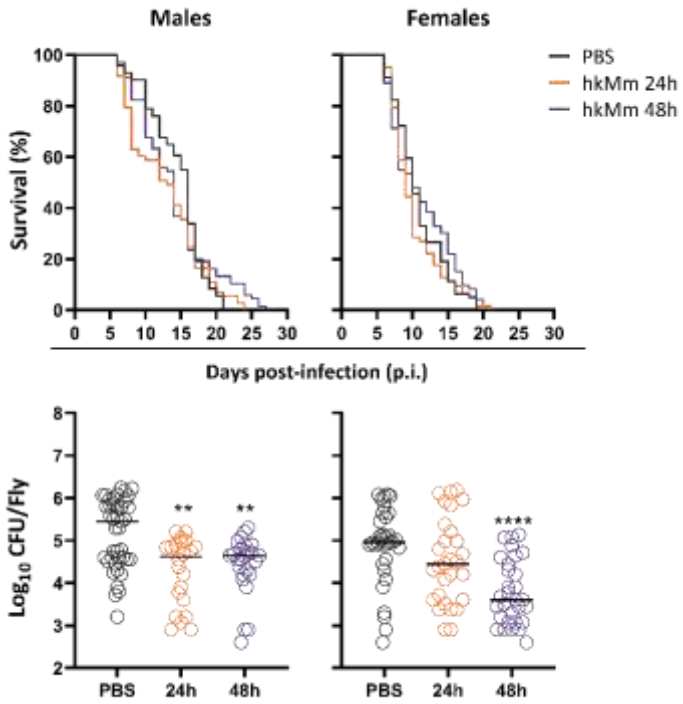


Figure 34| Effect of oral treatment with 10^5 CFU of hkMm on *D. melanogaster* subsequently infected with *M. marinum*. Survival was observed daily for 30 days and no statistically significant differences were observed among groups (Log-rank test). Bacillary load was measured at the time of death and is expressed as Log_{10} CFU/Fly. Each circle represents an individual fly and lines are medians. Statistically significant differences were represented as follow: *p≤0.05, **p≤0.01, ****p≤0.0001 (Kruskal-Wallis test).

To evaluate the capacity of hkMm to induce unspecific protection to subsequent infections in *D. melanogaster*, flies were systemically infected with either the Gram-positive *S. aureus* or the Gram-negative *S. typhimurium*. Results are presented in Figure 35.

Flies infected systemically with *S. aureus* 72h after the end of the oral treatment with hkMm did not show significant differences on survival times compared to untreated flies, however, both males and females showed increased bacillary load at the time of death when were previously treated for 48h, but only males showed the same increase when treated only for 24h. On the other hand, flies that were infected systemically with *S. typhimurium* showed different effect depending on sex. Males showed a significant reduction on the lifespan after the infection but also a significant reduction on the bacillary load, while females presented significantly higher survival rates and increased bacillary loads at the time of death.

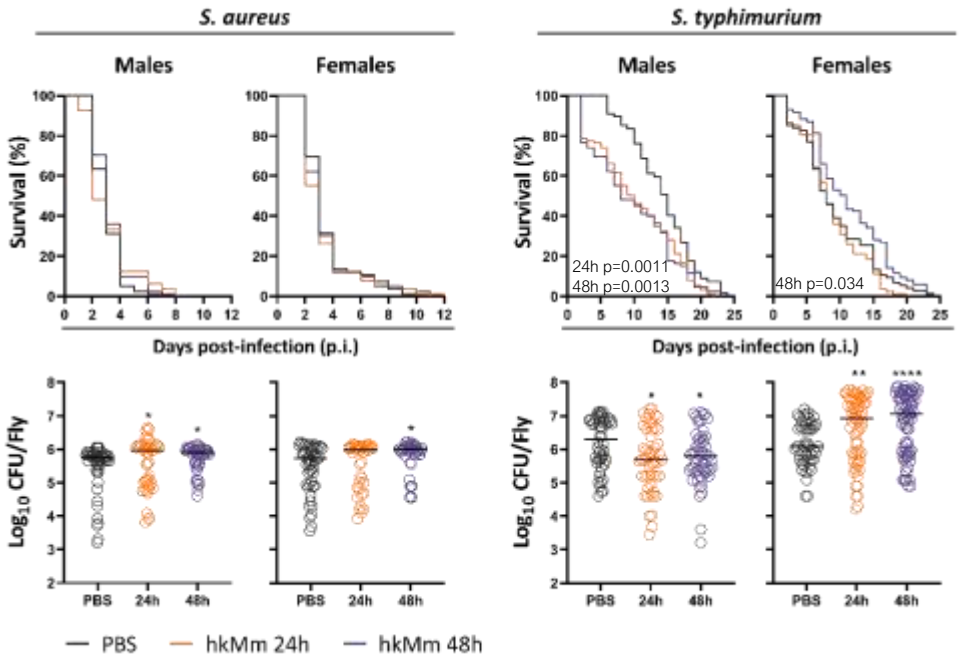


Figure 35| Effect of oral treatment with 10^5 CFU of hkMm on *D. melanogaster* subsequently infected with *S. aureus* and *S. typhimurium*. Survival was observed until all flies died. No statistically significant differences were observed for flies infected with *S. aureus*. Males infected with *S. typhimurium* showed significant reduction in the lifespan compared to untreated flies when treated for 24h (p=0.0011, Log-rank test) and 48h (p=0.0013, Log-rank test). Females infected with *S. typhimurium* showed significant increased lifespan only when treated with hkMm for 48h (p=0.034, Log-rank test). Bacillary load was measured at the time of death and is expressed as Log_{10} CFU/Fly. Each circle represents an individual fly and lines are medians. Statistically significant differences were represented as follow: *p≤0.05, **p≤0.01, ****p≤0.0001 (Kruskal-Wallis test).

5.3| Discussion

The data provided herein suggest, for the first time, that oral administration of a low dose treatment based on heat-killed *M. manresensis* could induce protection against subsequent infections based only on the innate immune response, as previous studies performed in mice focused only on the adaptive arm of the immunity (91). We also probe for the first time that a mycobacteria can prime flies, as only heat-killed *M. marinum* has been tested before but it was not able to induce protection against a subsequent infection with the same mycobacterium (355).

Previous studies showed that the immune pathways activated, the duration and the specificity of the protection triggered in primed flies is dependent on the microbe used for the priming. For example, priming flies with sublethal doses of *Streptococcus pneumoniae* induces a specific, long lasting and Toll- and hemocytes-dependent protection against subsequent infection of lethal doses of the same bacteria, but not against other pathogens (355). On the other hand, flies primed with heat-killed *Pseudomonas aeruginosa* conferred shorter protection than priming with live, low-in-virulence bacteria and also in a species-specific manner. In both cases the Imd and the Toll pathways were implicated in the protection (357).

Our characterization of the effect that the oral treatment with hkMm has on *D. melanogaster* reveals a delayed activation of the innate immune response in treated flies 72h after the end of the treatment. This response could be categorized as priming based on the classification that Divangahi *et al.* published recently (354) because the immune response is still activated prior to the second challenge. However, the duration of the immune activation and the response triggered by these subsequent infections should be assessed to ensure this statement.

Data also shows a dose dependency on the effect of the treatment based on the higher activation of the immune response in flies treated for 48h compared to those treated only for 24h. In addition, we show that the sex of the host is also a key factor for the priming outcome and that a sexual dimorphism exists at least with the hkMm priming, as females seem to need higher doses or longer treatment times to be able to induce the same innate immune response as males. This reduced protection in females has been also observed with the symbiont *Wolbachia*-mediated protection against oral bacterial infections(378).

As expected, we observe a significant increase in the expression of the Imd-dependent AMP Diptericin, although only in males. This pathway has been widely described to be responsible for the humoral response in the gut of *Drosophila* (184). However, in both males and females we observe also increased expression of the Toll-dependent AMP Drosomycin, although this pathway has been described to be non-functional in the gut (238). Feasible explanations for this could be the FoxO-

dependent expression of AMPs in intestinal epithelial cells (379) or the gut-to-fat body communication induced by the expression of ROS in the gut (380).

Surprisingly, our data also shows that the hkMm given orally triggers the Nox-dependent production of ROS, also 72h after the end of the treatment, but no increase expression of Duox is observed in any sex and treatment regimen. Typically, the expression of Nox has been related with the commensal bacteria and to induce tissue homeostasis, while Duox has been linked to invading pathogens and activation of the immune response (232,233). Thus, this induction of Nox together with the increased expression of Upd3, which has also been linked with intestinal epithelia renewal and homeostasis (225,227–229,381), suggest that the oral administration of hkMm induces a homeostatic response in the gut of *D. melanogaster*. In addition, the increased expression of Impl2 might indicate a metabolic reprogramming in the innate immune cells (272,382).

Finally, in this study we probe that oral administration of hkMm gives unspecific protection against subsequent infections with a different mycobacterium, a Gram-positive, and a Gram-negative and that protections against each bacterium correlates with the innate immune response triggered by the treatment. Thus, it has been described that the Toll pathway was in charge of the humoral response against acid-fast bacteria (146), although *M. marinum* does not activate it (317). However, our results show that those flies that have increased expression of Drosomycin prior to the infection present lower bacillary loads of *M. marinum* at the moment of death, although survival is not affected.

Those same flies with activated Toll pathway showed higher tolerance levels to subsequent infection with the Gram-positive *S. aureus*, with same survival rated but higher bacillary loads at the moment of death. A previous study by Nehme *et al*. showed that the response against Gram-positive bacteria is mainly mediated by the cellular immune response and that increasing the expression of Toll-related AMPs did not induce any protection against *S. aureus* infection (383). Thus, our hypothesis is that treated flies might increase the tolerance to *S. aureus* infections through Upd3-mediated reduction of tissue damage.

More particular is the case of flies infected with the Gram-negative bacteria, *S. typhimurium*. Here, we observe a sexual dimorphism in the protective response induced by the treatment. While in males both regimens increase the resistance to the infection at expenses of the lifespan, in females both regimens increase the tolerance to the infection. The increased resistance in males correlates with the increased expression of the Imd-related AMP, Diptericin, while females only show increased expression of Nox in common in both treatment regimens.

Overall, our data confirms our hypothesis that the oral administration of hkMm modulates the innate response of the host and brings protection against subsequent infections with a broad spectrum of pathogens.

6| Study 3

Evaluating the impact of coevolution with mycobacteria in tolerance/resistance of *D. melanogaster*

6.1| Experimental design

In this study we aimed to evaluate the impact that *M. marinum* infection and oral administration of hkMm would have in tolerance/resistance of *D. melanogaster* after coevolution for several generations. To do so, flies were divided into 4 groups: flies injected with PBS, flies orally treated with hkMm and injected with PBS, flies infected with 500CFU of *M. marinum*, and flies that combined infection and treatment (Figure 36A). This study will be divided into two differentiated experiments, although results for generation 0 are shared.

In the first experiment we aimed to assess the host's response. For each group, 12 vials with 15 males and 15 females each were used, from which 2 were daily checked for survival. The oral treatment was added into the media and the infection was performed with a fresh vial of our frozen stock of *M. marinum*. Flies were transferred into new vials every 5 days and, based on the results of the previous study that suggested that females might need higher doses or longer treatment times, here we administrated the treatment continuously, adding new doses with every change of vials. After 12 days, flies from the 10 non-survival vials were transferred into big tubs in a ratio of 30 males and 30 females for synchronization. Adult flies were discarded after 24h and tubs were kept at 25ºC for 10 days until the eclosion of the new generation. When descendants were 3 to 5 days old, the procedure was repeated until generation 10 was reached (Figure 36B). At generations 0, 5 and 10 each group was infected with increasing doses of *M. marinum* from the frozen stock to perform the tolerance/resistance test and to also obtain samples for transcriptomics (Figure 36C).

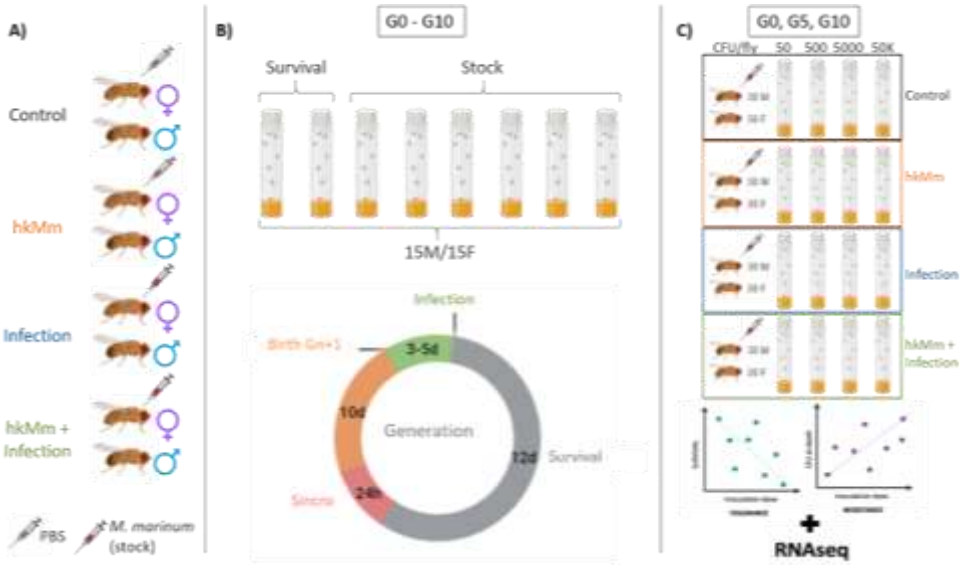


Figure 36| Experimental design of the experiment 1 from study 3

In the second experiment we aimed to assessed the coevolution of both the host and the mycobacteria in the conditions explained above. Thus, the same procedure as for experiment 1 was followed with the exception of the infected groups. For those groups, after adults were placed for synchronization and prior to discard them, 10 males and 10 females from each group were used for recovering the *M. marinum*. The next generation of each group was then infected with the corresponding recovered *M. marinum* (we also distinguished between males and females). The general procedure for the 10 generations is represented in Figure 37.

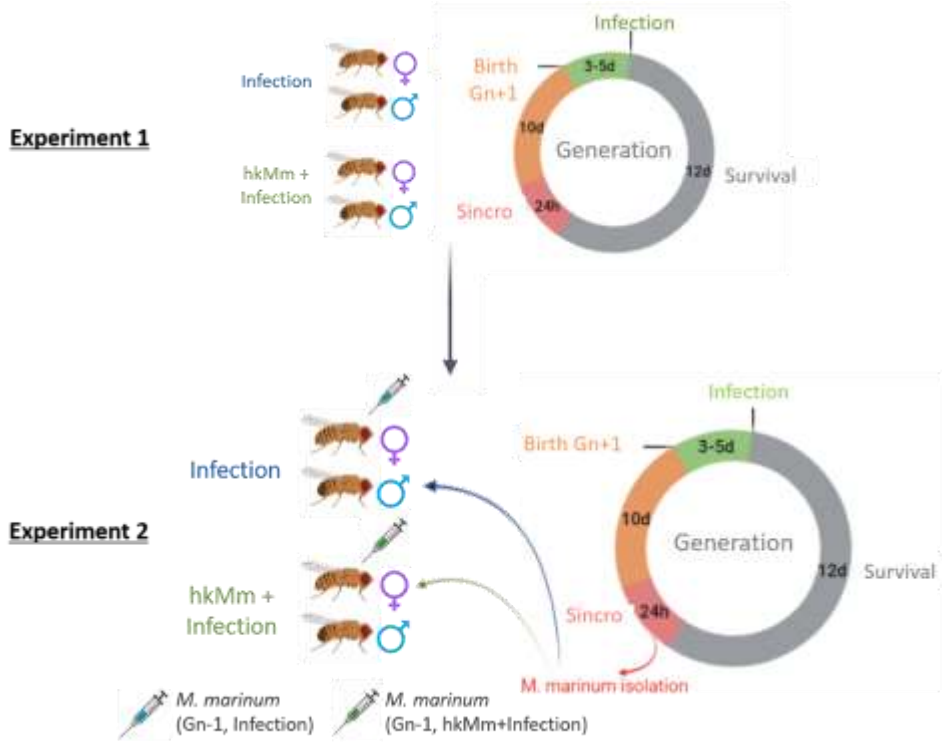


Figure 37| Experimental design for the general procedure of experiment 2 of study 3.

In the second experiment we also assessed changes on the tolerance/resistance of the host at generation 5 and 10, as in the experiment 1. However, here we used the *M. marinum* strains recovered from the infected groups for the tolerance/resistance test, in order to determine if there were changes in the virulence of *M. marinum* after coevolution with the host. Thus, the non-infected groups (Control and hkMm) were infected with *M. marinum* strains isolated from the both infected groups (Figure 38A), while infected groups (Infection and hkMm+Infection) were tested for tolerance/resistance using a fresh frozen stock (Figure 38B) and also its own isolate (Figure 38C). Samples for transcriptomics were also obtained at these generations.

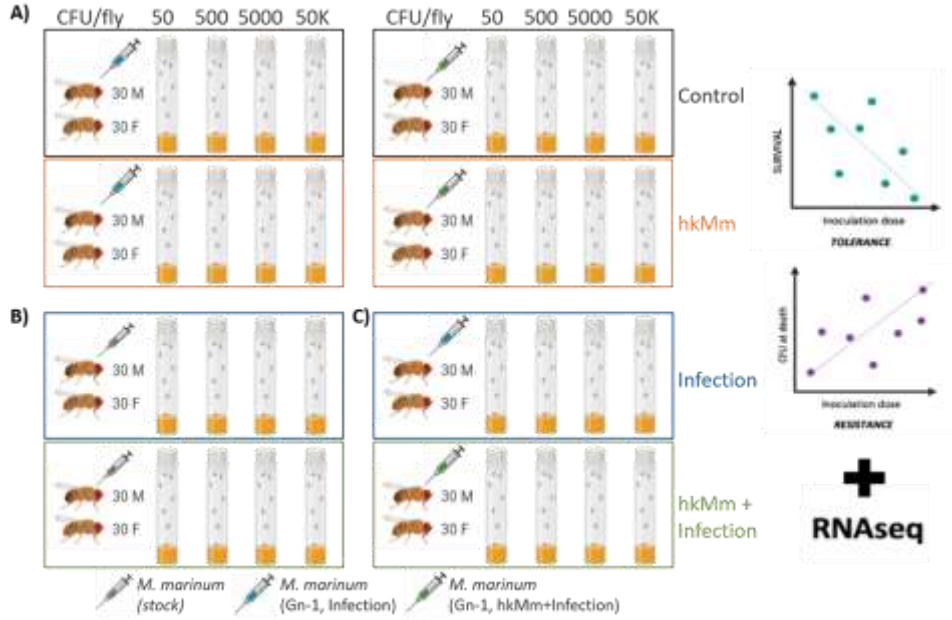


Figure 38| Experimental design for the tolerance/resistance test of experiment 2 of study 3

6.2| Results

6.2.1| Evaluation of the tolerance and resistance against *M. marinum* infections

6.2.1.1| Oral treatment with hkMm induces resistance but not tolerance to *M. marinum* infection on *D. melanogaster*

First, we assessed the immediate impact that the oral treatment with 10^5 hkMm had on *D. melanogaster*. At that point, only the control and the treated groups were tested, as Infection and hkMm+Infection groups were essentially the same as the above when no evolution had happened yet. Results are presented in Figure 39.

Male and female flies were infected with increasing doses of *M. marinum* and were given or not the treatment with 10^5 hk bacilli. As mentioned before, the treatment was renewed when flies were transferred to new vials every 5 days. In line with results obtained in study 2, no significant differences were observed in the tolerance levels of neither of the sexes (Table 10), but in both cases treated flies showed a significant increased resistance levels to the infection with *M. marinum* (Table 11).

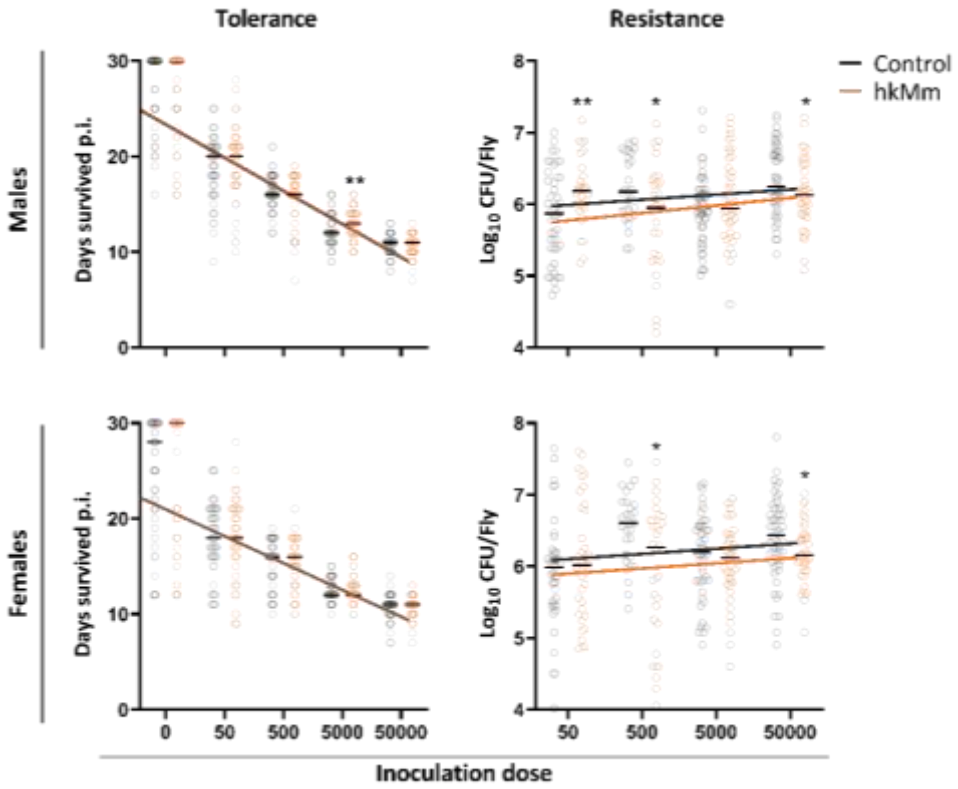


Figure 39| Effect of hkMm treatment on tolerance and resistance of *D. melanogaster* to *M. marinum* infections. Lines represent the regression lines fitted for each group and each circle represents an individual fly. Both survival and bacillary load between groups were analysed independently for each inoculation dose and data was tested for normality. Statistically significant differences were represented as follow: *≤0.05, **p≤0.01 (Mann-Whitney test).

Table 10| Tolerance to *M. marinum* infection in treated *D. melanogaster*

Group	Slope control	Slope hkMm	p values
Males	-3.354	-3.356	ns
Females	-2.984	-2.857	ns

Table 11| Resistance to *M. marinum* infection in treated *D. melanogaster*

Group	Slope control	Slope hkMm	p value	Y-inter control	Y-inter hkMm	p value
Males	0.079	0.116	ns	5.840	5.551	0.014
Females	0.074	0.093	ns	5.969	5.702	0.009

6.2.1.2| A transgenerational adaptation occurs in *D. melanogaster* exposed to mycobacteria

Flies from experiment 1 were tested for tolerance and resistance to subsequent infection with increasing doses of *M. marinum* at generations 5 and 10. To do so, 30 males and 30 females from each group in each generation were used and no biological replicates could be performed due to the complexity of the experimental procedure. For tolerance, survival of the flies was daily checked and the bacillary load was measured at the time of death for resistance. Results were plotted as previously (Figure 40).

Opposite to what we observed in generation 0, the oral treatment with hkMm lead to increased tolerance to subsequent infection with increasing doses of *M. marinum* in both male and female flies after 5 generations. However, only in females this protection is sustained until generation 10, while in males the effect of the treatment seems to dilute over the generations (Table 12).

The constant exposure to a systemic infection of *M. marinum*, as well as the combination of both the oral treatment and the infection, also induced an increase in the levels of tolerance to subsequent infections that last throughout all generations in both male and female flies (Table 12). In addition, flies that have been exposed to the infection seem to reduce their general vigour as generations pass, although they survived better to increasing initial doses of the pathogen.

Surprisingly, and also contrary to what we observed in generation 0, the oral treatment did not increase the levels of resistance of either male or female flies to the *M. marinum* infection. Flies that had been exposed to the systemic infection also showed no increase in resistance to subsequent infections in any of the generations (Table 13).

On the other hand, the combination of the two stimuli over the course of the experiment did favour an increase in resistance to subsequent infection with *M. marinum* in generation 5, but only in males. This effect was not maintained in the 10th generation, possibly due to the reduced effect of the oral treatment that we observed in the tolerance test (Table 13).

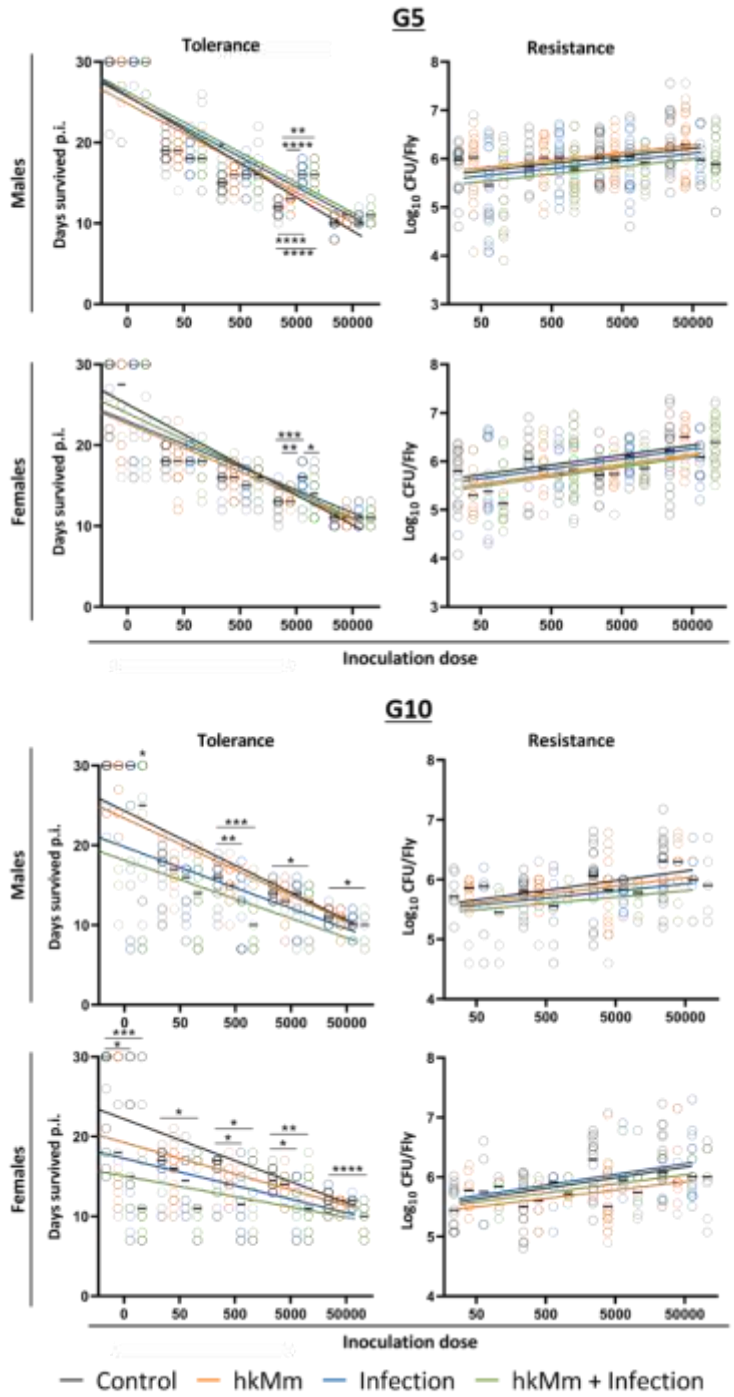


Figure 40| Effect of treatment and/or infection for 5 and 10 generations on the tolerance and resistance of *D. melanogaster* to *M. marinum* infections. Lines represent the regression lines fitted for each group and each circle represents an individual fly. Both survival and bacillary load between groups were analysed independently for each inoculation dose and data was tested for normality. Statistically significant differences were represented as follow: *p≤0.05, **p≤0.01, ***p≤0.001, **** p≤0.0001 (Kruskal-Wallis test).

Table 12| Tolerance to *M. marinum* infection in the 5th and 10th generation of *D. melanogaster*

	Group	Slope	Comparison	Slope	p value
5th generation	Control Males	-4.123	hkMm	-3.618	0.0028
			Infection	-3.671	0.0101
			hkMm + Inf.	-3.681	0.0175
	Control Females	-3.673	hkMm	-2.916	0.0007
			Infection	-2.791	<0.0001
			hkMm + Inf.	-3.191	0.0280
10th generation	Control Males	-3.299	hkMm	-3.370	ns
			Infection	-2.528	0.0324
			hkMm + Inf.	-2.395	0.0361
	Control Females	-2.580	hkMm	-1.947	0.0441
			Infection	-1.676	0.0105
			hkMm + Inf.	-1.275	0.0005

Table 13| Resistance to *M. marinum* infection in the 5th and 10th generation of *D. melanogaster*

	Group	Slope	Y-inter	Comparison	Slope	Y-inter	p value slope	p value Y-inter.
5th generation	Control Males	0.158	5.515	hkMm	0.145	5.542	ns	ns
				Infection	0.196	5.183	ns	ns
				hkMm + Inf.	0.188	5.148	ns	0.008
	Control Females	0.141	5.592	hkMm	0.229	5.412	ns	ns
				Infection	0.147	5.594	ns	ns
				hkMm + Inf.	0.259	5.382	ns	ns
10th generation	Control Males	0.144	5.448	hkMm	0.152	5.355	ns	ns
				Infection	0.100	5.484	ns	ns
				hkMm + Inf.	0.182	5.127	ns	ns
	Control Females	0.179	5.356	hkMm	0.293	4.955	ns	ns
				Infection	0.179	5.328	ns	ns
				hkMm + Inf.	0.313	4.928	ns	ns

6.2.1.3| *D. melanogaster* and *M. marinum* both coevolve after coexisting for generations

Once we have tested the ability of the host to evolutionarily adapt to mycobacterial infections, we decided to also assess the ability of the mycobacterium to adapt to the host as well as the co-evolution between the two. To test whether the pathogen increases or decreases its virulence when coevolving with the host we proposed two approximations.

First, we evaluated the tolerance and resistance levels to the infection with the adapted mycobacteria of those hosts that have not been exposed to the pathogen and we compared it with the levels of tolerance and resistance to the infection with the mycobacteria from our frozen stock from the experiment 1.

Results showed that both groups presented increase tolerance to both mycobacteria isolates compared to the frozen stock, independently of the sex, at generation 5 (Table 14). However, none of them presented significant changes on the resistance levels, with the exception of the females from the control group, which showed reduced resistance to both adapted mycobacteria (Figure 41and Table 15).

Surprisingly, and contrary to the continuity we observed in the tolerance levels from experiment 1, here things changed when we infected the 10^{th} generation of those groups with the mycobacteria isolated from the infected groups and we compared it with the infection with the frozen stock from the first experiment as well (Figure 42). At this time point, males from the control group showed no changes in the tolerance levels to the different infections, while females showed lower tolerance to the both coevolved mycobacteria (Table 15). On the contrary, both males and females that have been exposed to the oral treatment presented an increased tolerance to the mycobacteria that has coevolved with flies that had been also treated with hkMm (Table 16).

While at generation 5, none of the hosts showed increased resistance to the coevolved mycobacteria, at this time point all of them revealed a significant reduction in the bacillary loads of the *M. marinum* that coevolved with the also treated hosts. In this case, we found the exception again in females but from the treated group, which did not show significant increased resistance to any of the mycobacteria (Table 17).

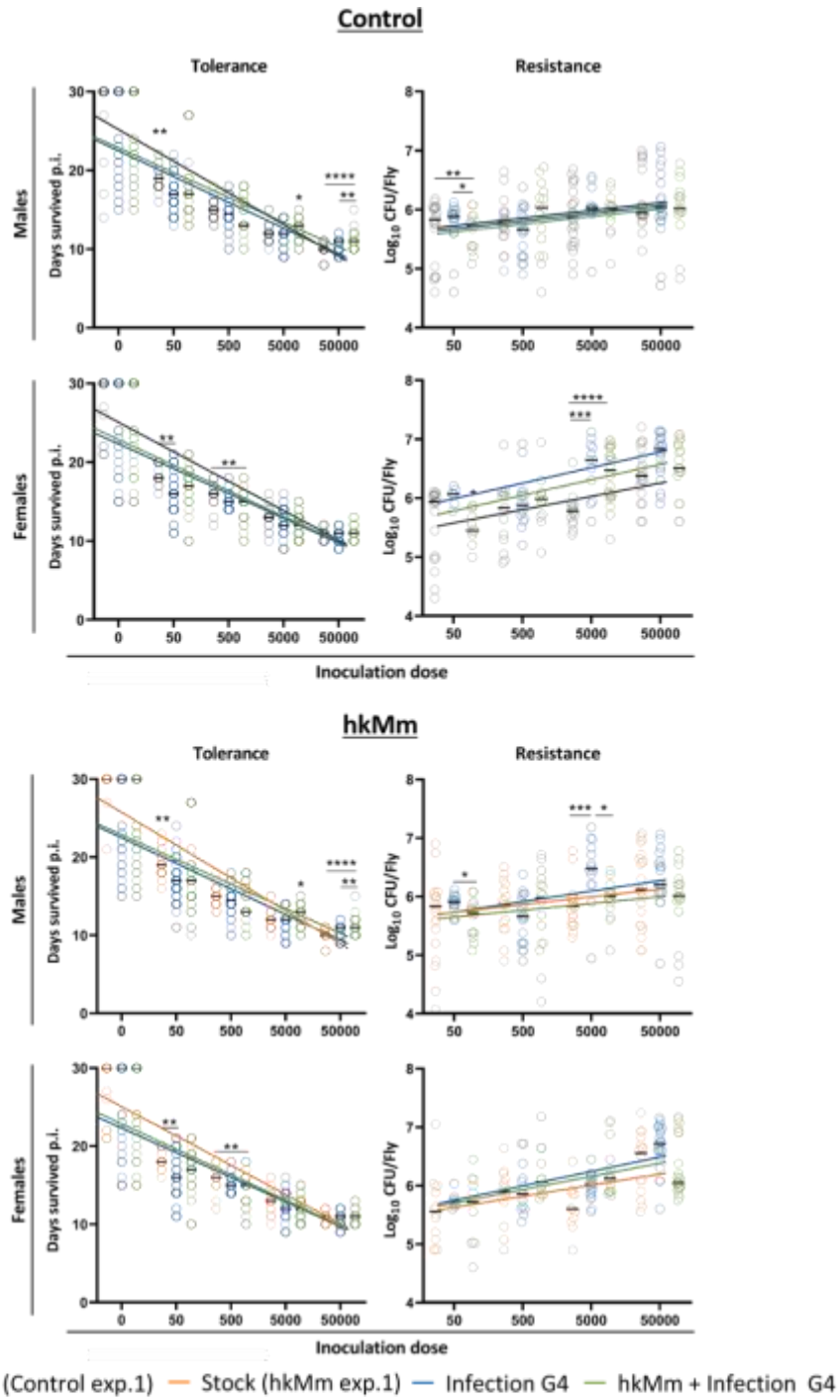


Figure 41| Effect of coevolved *M. marinum* infection on tolerance and resistance of not adapted *D. melanogaster* on the 5th generation. Lines represent the regression lines fitted for each group and each circle represents an individual fly. Both survival and bacillary load between groups were analysed independently for each inoculation dose and data was tested for normality. Statistically significant differences were represented as follow: *p≤0.05, **p≤0.01, ***p≤0.001, ****p≤0.0001 (Kruskal-Wallis test).

Table 14| Tolerance to *M. marinum* infection in the 5th generation of *D. melanogaster*

	Group	Slope	Comparison	Slope	p value
Control	Stock (exp.1) Males	-4.160	Infection G4	-3.219	<0.0001
			hkMm+Inf. G4	-3.087	<0.0001
	Stock (exp.1) Females	-3.631	Infection G4	-3.067	0.0073
			hkMm+Inf. G4	-3.079	0.0329
hkMm	Stock (exp.1) Males	-4.123	Infection G4	-3.212	<0.0001
			hkMm+Inf. G4	-3.094	<0.0001
	Stock (exp.1) Females	-3.667	Infection G4	-3.083	0.0073
			hkMm+Inf. G4	-3.188	0.0329

Table 15| Resistance to *M. marinum* infection in the 5th generation of *D. melanogaster*

	Group	Slope	Y-inter	Comparison	Slope	Y-inter	p value slope	p value Y-inter.
Control	Stock (exp.1) Males	0.168	5.319	Infection G4	0.139	5.430	ns	ns
				hkMm+Inf. G4	0.129	5.454	ns	ns
	Stock (exp.1) Females	0.247	5.095	Infection G4	0.262	5.467	ns	0.0191
				hkMm+Inf. G4	0.299	5.195	ns	0.0459
hkMm	Stock (exp.1) Males	0.149	5.389	Infection G4	0.209	5.396	ns	ns
				hkMm+Inf. G4	0.115	5.453	ns	ns
	Stock (exp.1) Females	0.225	5.147	Infection G4	0.279	5.196	ns	ns
				hkMm+Inf. G4	0.215	5.387	ns	ns

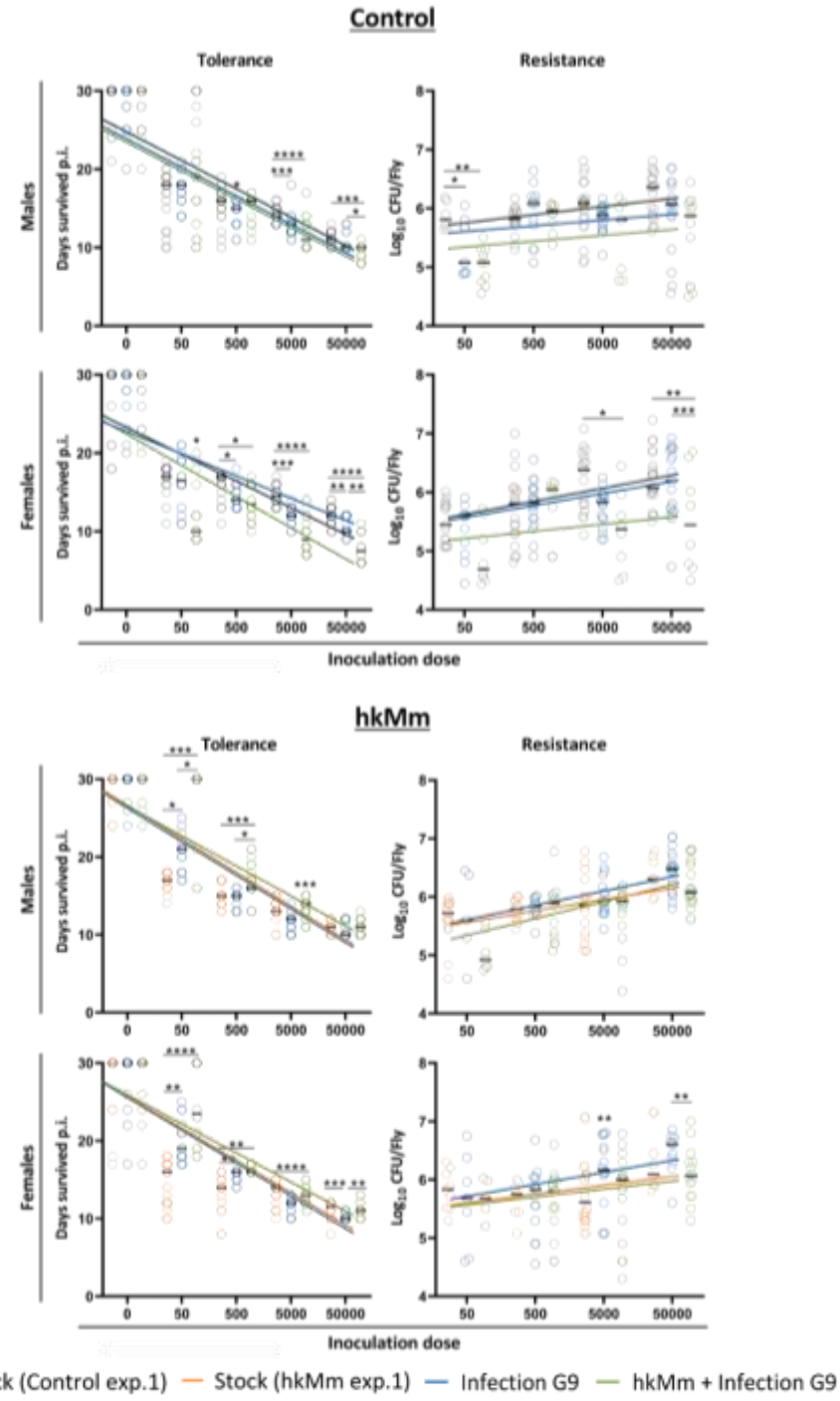


Figure 42| Effect of coevolved *M. marinum* infection on tolerance and resistance of not adapted *D. melanogaster* on the 10th generation. Lines represent the regression lines fitted for each group and each circle represents an individual fly. Both survival and bacillary load between groups were analysed independently for each inoculation dose and data was tested for normality. Statistically significant differences were represented as follow: *p≤0.05, **p≤0.01, ***p≤0.001, ****p≤0.0001 (Kruskal-Wallis test).

Table 16| Tolerance to *M. marinum* infection in the 10th generation of *D. melanogaster*

	Group	Slope	Comparison	Slope	p value
Control	Stock (exp.1) Males	-3.371	Infection G9	-3.482	ns
			hkMm+Inf. G9	-3.947	ns
	Stock (exp.1) Females	-2.790	Infection G9	-3.361	0.0321
			hkMm+Inf. G9	-3.921	0.0005
hkMm	Stock (exp.1) Males	-4.239	Infection G9	-4.242	ns
			hkMm+Inf. G9	-3.787	0.0175
	Stock (exp.1) Females	-4.428	Infection G9	-4.258	ns
			hkMm+Inf. G9	-3.671	0.0119

Table 17| Resistance to *M. marinum* infection in the 10th generation of *D. melanogaster*

	Group	Slope	Y-inter	Comparison	Slope	Y-inter	p value slope	p value Y-inter.
Control	Stock (exp.1) Males	0.125	5.576	Infection G9	0.139	5.430	ns	ns
				hkMm+Inf. G9	0.129	5.454	ns	0.046
	Stock (exp.1) Females	0.248	5.101	Infection G9	0.228	5.038	ns	ns
				hkMm+Inf. G9	0.136	4.956	ns	0.0401
hkMm	Stock (exp.1) Males	0.238	5.100	Infection G9	0.331	4.852	ns	ns
				hkMm+Inf. G9	0.384	4.466	ns	0.0175
	Stock (exp.1) Females	0.162	5.371	Infection G9	0.173	5.524	ns	ns
				hkMm+Inf. G9	0.136	5.353	ns	ns

Results from the first approach revealed similar behaviour of both mycobacterial isolates at generation 5, but suggested a higher attenuation of the pathogen isolated from the treated hosts by generation 10. Thus, the second approach was meant to evaluate the coevolution of both the host and the pathogen by assessing the tolerance and resistance levels of those groups that did coevolve with the pathogen against its own isolate and the frozen stock of *M. marinum* (Figure 43 and Figure 44).

As observed in experiment 1, and contrary to the first approach of this second experiment, results here showed a continuity in the tolerance and resistance levels throughout all generations for both groups. However, while those hosts that coevolved with the pathogen showed increased resistance to the isolate compared with the frozen stock but no changes on the tolerance levels (Table 18-19), those

hosts that were also orally treated with hkMm showed increased levels of both tolerance and resistance to the isolate in comparison with the frozen stock (Table 20-21).

Table 18| Tolerance to coevolved *M. marinum* in the Infection group of *D. melanogaster*

	Group	Slope Stock	Slope Infection	p value
5th Generation	Males	-2.890	-2.915	ns
	Females	-2.736	-2.649	ns
10th Generation	Males	-3.275	-3.566	ns
	Females	-3.394	-3.462	ns

Table 19| Resistance to coevolved *M. marinum* in the Infection group of *D. melanogaster*

	Group	Slope Stock	Slope Infection	p value	Y-inter stock	Y-inter Infection	p value
5th Generation	Males	0.203	0.207	ns	5.150	4.950	0.0248
	Females	0.224	0.172	ns	5.428	5.291	0.0374
10th Generation	Males	0.239	0.207	ns	5.293	5.102	0.0436
	Females	0.149	0.317	ns	5.691	4.867	0.0495

Table 20| Tolerance to coevolved *M. marinum* in the orally treated group of *D. melanogaster*

	Group	Slope Stock	Slope hkMm + Inf.	p value
5th Generation	Males	-3.360	-2.960	<0.0001
	Females	-2.897	-2.474	<0.0001
10th Generation	Males	-3.398	-2.970	0.0087
	Females	-4.111	-3.792	0.0455

Table 21| Resistance to coevolved *M. marinum* in the orally treated group of *D. melanogaster*

	Group	Slope Stock	Slope hkMm + Inf.	p value	Y-inter stock	Y-inter hkMm + Inf.	p value
5th Generation	Males	0.218	0.219	ns	5.334	5.051	0.0447
	Females	0.328	0.439	ns	5.274	4.608	0.0253
10th Generation	Males	0.173	0.247	ns	5.509	4.762	0.0033
	Females	0.058	0.057	ns	6.270	5.623	0.0056

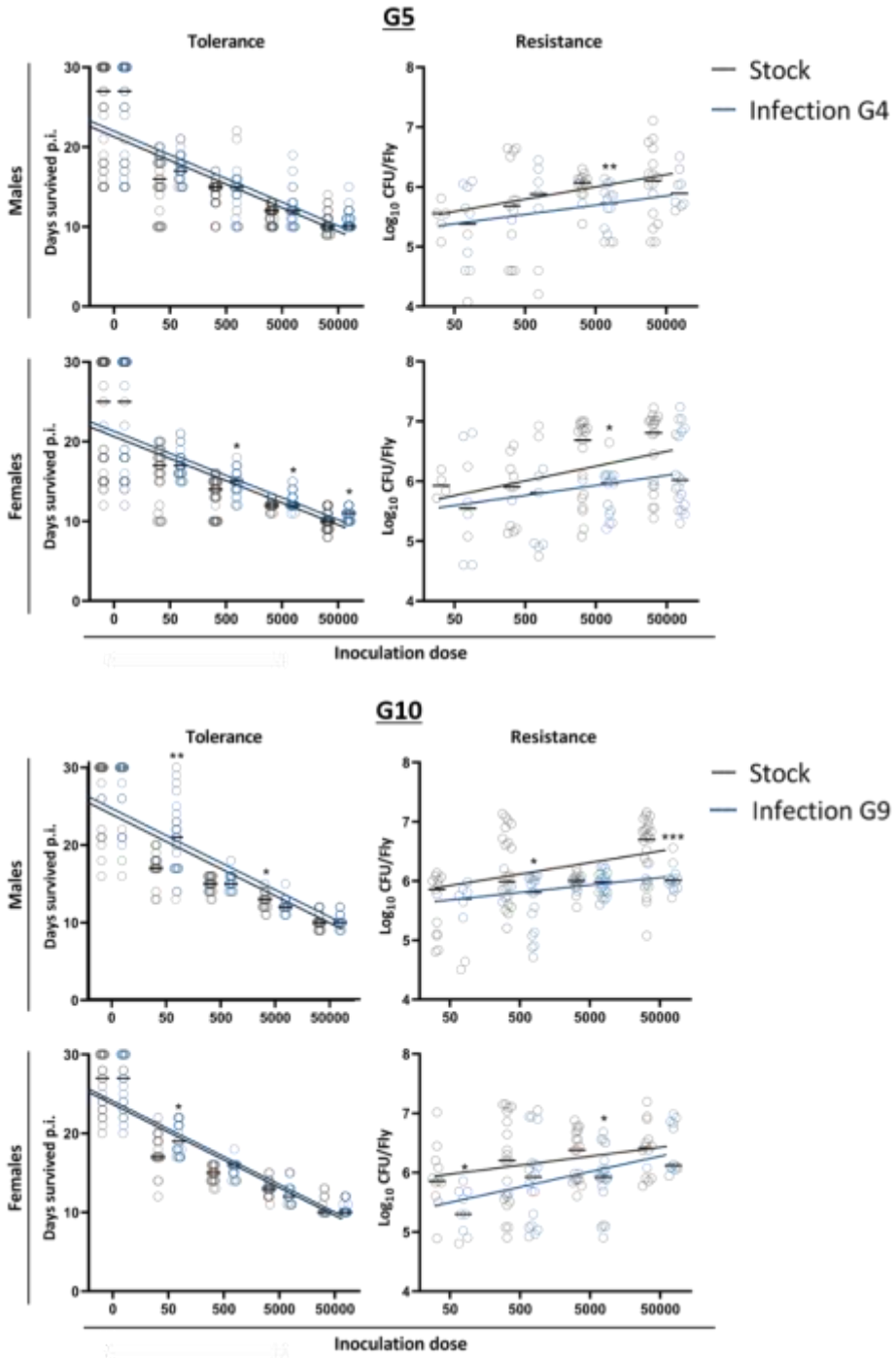


Figure 43| Effect of coevolving with *M. marinum* on tolerance and resistance to subsequent infections of *D. melanogaster*. Lines represent the regression lines fitted for each group and each circle represents an individual fly. Both survival and bacillary load between groups were analysed independently for each inoculation dose and data was tested for normality. Statistically significant differences were represented as follow: *p≤0.05, **p≤0.01, ***p≤0.001 (Kruskal-Wallis test).

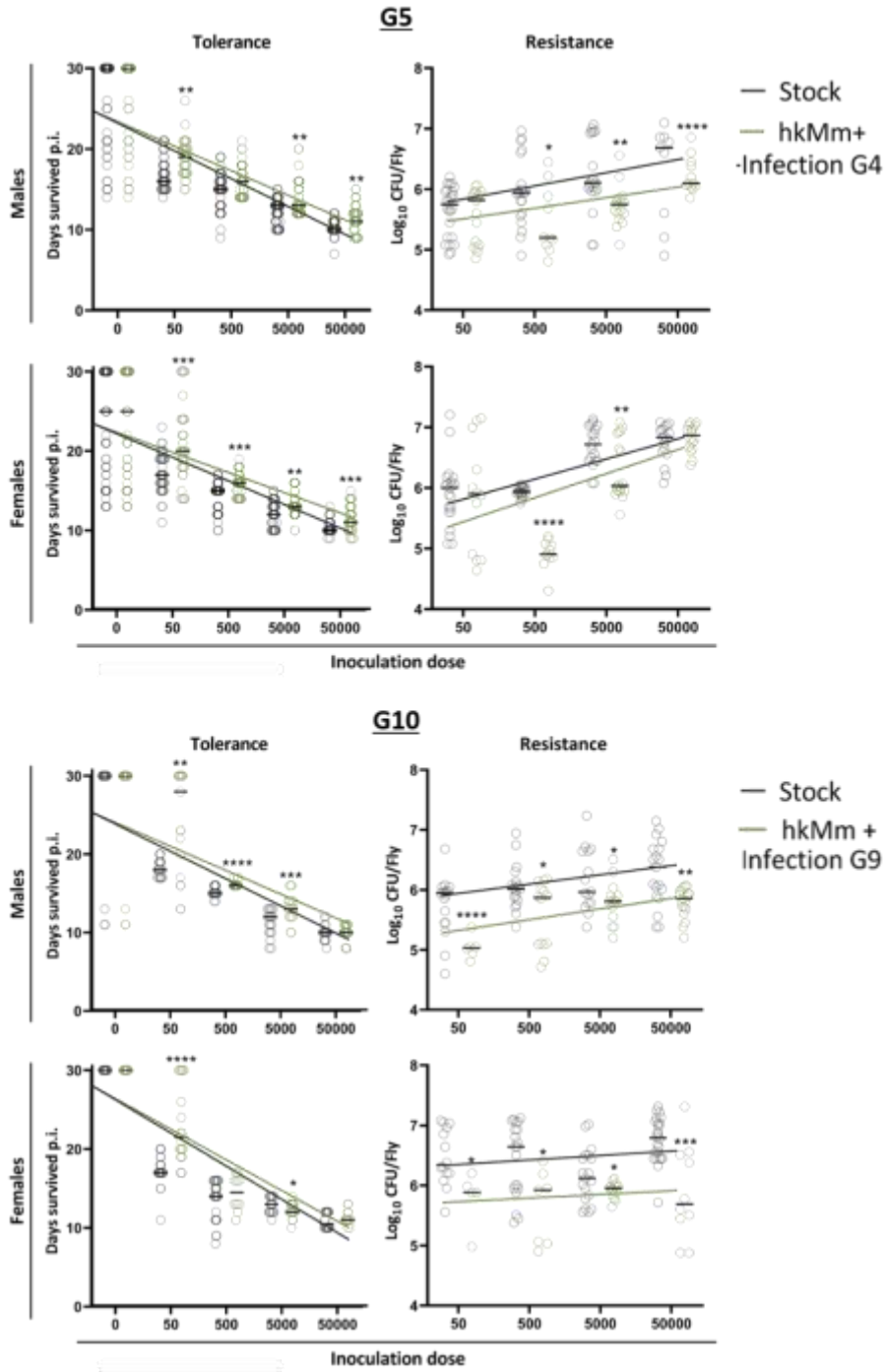


Figure 44| Effect of coevolving with *M. marinum* on tolerance and resistance to subsequent infections of *D. melanogaster* treated with hkMm. Lines represent the regression lines fitted for each group and each circle represents an individual fly. Both survival and bacillary load between groups were analysed independently for each inoculation dose and data was tested for normality. Statistically significant differences were represented as follow: *p≤0.05, **p≤0.01, ***p≤0.001, ****p≤0.0001 (Kruskal-Wallis test).

6.2.2| Differential expression analysis of *D. melanogaster*

In order to characterize the differences in terms of gene expression of flies exposed either to the oral treatment with hkMm and/or to the systemic infection with *M. marinum*, as well as the effect that these exposures had over generations of the host, exposed flies were compared to their corresponding control groups. The same strategy was used for all the comparisons. First, all differentially expressed genes (DEGs) obtained with the *voom* transformation from the lima package of R were used to perform the Gene Set Enrichment Analysis (GSEA) ranked by decreasing values of the t-statistic value. Then, from all the pathways included in the gene matrix used, the more relevant were selected in order to prevent overrepresentation of some constituent pathways such as those related with protein or DNA/RNA processing, and only those pathways with a nominal p value <0.05 and a False Discovery Rate (FDR) <0.25 were considered significant and plotted. Then we proceed to select the more relevant DEGs. For this, first we selected those genes with an adjusted p value <0.05 and an absolute $\log_2$ fold change >1. Then we selected those significant DEGs that belong to the significantly altered pathways and ranked them by decreasing adjusted p value. The top 30 DEGs were selected for the heatmap representation. Exceptionally, some genes with extreme $\log_2$ fold change values or genes considered of interest for the study were also selected.

6.2.2.1| Effect of the oral administration of hkMm in *D. melanogaster*

Previously in this study we observed that both males and females orally treated with hkMm showed similar levels of tolerance to the infection with *M. marinum* but significantly higher levels of resistance to the infection compared to untreated flies (Figure 39). Thus, first we aimed to evaluate the genotypic changes that flies experienced due to the oral treatment with hkMm in the absence of infection (Figure 45).

We observed that the oral administration of hkMm had a short-time effect on flies, as both sexes had a high number of significant DEGs after 24h but very few or none 10 days after although, as explained in section 1, treatment was re-administrated at day 5 (Figure 45A).

The GSEA analysis (Figure 45B) revealed that, even in the absence of infection, treated flies presented up-regulation of many pathways involved in the organism homeostasis: the Hippo signalling pathway, which plays a major role in growth control (384); the Mithogen-Activated Protein Kinase (MAPK) signalling pathway that regulates cell proliferation, differentiation and survival in response to both intra- and extracellular stressors (385); and the mammalian Target of Rapamycin (mTOR) signalling pathway that also regulates cell fate as well as immune cells differentiation

in response to insulin levels and it has also been related to neuronal differentiation (386,387).

Both males and females also showed same levels of up-regulation of the transforming growth factor beta (TGF-β) and the wingless (Wnt) signalling pathways. The TGF-β signalling pathway also regulates developmental processes, as well as hormonal, physiological, immune and tissue homeostatic processes (388). On the other hand, the Wnt signalling pathway is also related with development, the maintenance of immune surveillance and, during infections by pathogenic microbes, helps mount host resistance to infection (389,390). The oral administration of hkMm also induced the expression of the Toll pathway in males, and both humoral responses in females. Another unexpected finding was the sexual dimorphism observed in the Foxo signalling pathway, which was overall repressed in males but triggered in females.

Finally, we analysed the top significant DEGs based on their adjusted P value and their involvement on the selected pathways only at 24h after the administration of the treatment, based on the absence of significant DEGs at 10d post-treatment (Table 22 and Figure 45C). In concordance with results obtained in the GSEA, in both sexes we observed significally up-regulated several genes related with apoptosis, autophagy and endocytosis and genes related with the Hippo and the Wnt signalling pathways. In both sexes we also found significantly increased expression of genes related with the metabolism of fatty acids, although this pathway was not significantly altered in the GSEA. In addition, in both sexes was the up-regulation of genes related with the mitochondrial respiration genes.

Also, in concordance with results from the GSEA, we found glycolysis-related gene significantly repressed in females, which were not found in males. In addition, several Toll- and Imd-repressors were significantly repressed in females, while males showed significantly increased expression of the Toll receptor.

Overall, results of the differential expression analysis suggested that the oral administration of hkMm induced the up-regulation of several signalling pathways related with cell proliferation and differentiation, and tissue homeostasis, while at the same time activated the Toll signalling pathway in males and the Imd pathway in females of *Drosophila*.

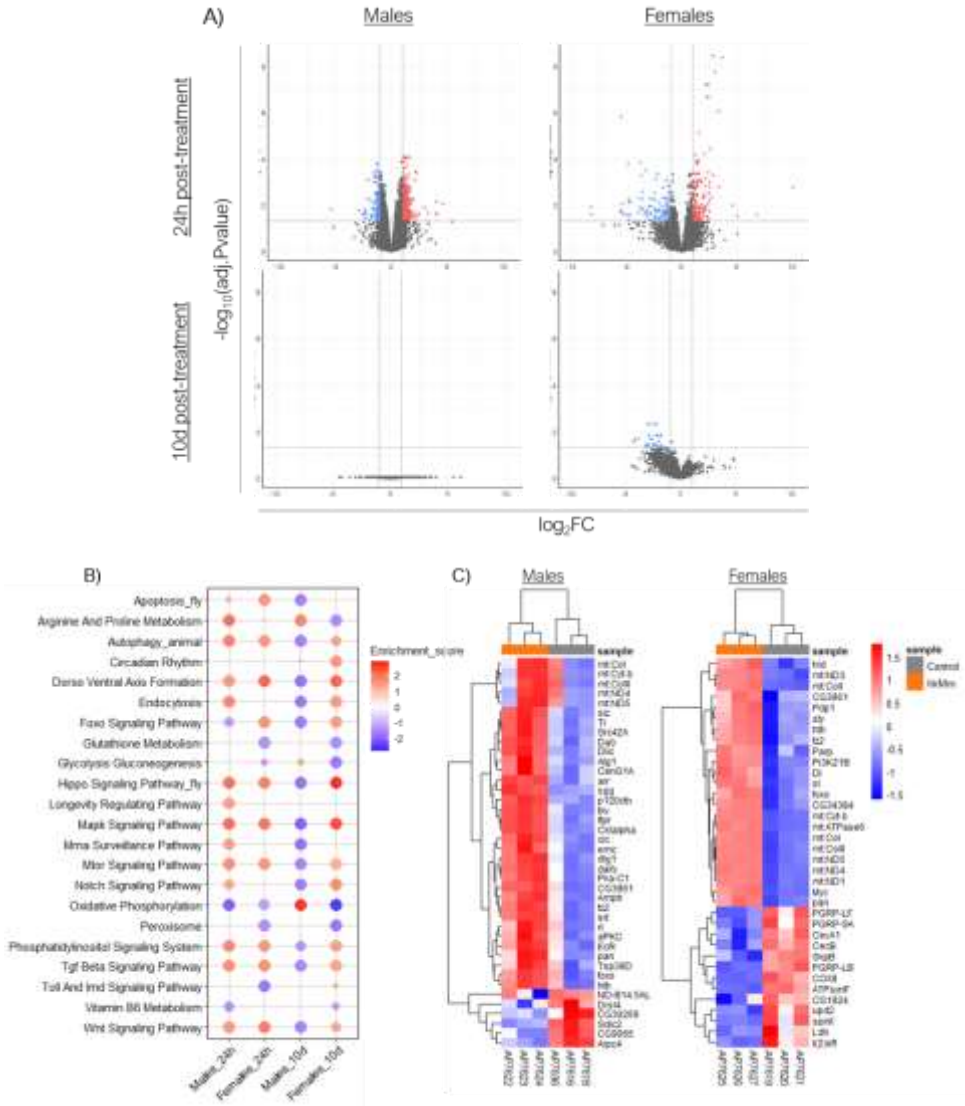


Figure 45| Differential expression analysis of male and female flies treated orally with hkMm. **(A)** Volcano plots of DEGs (genes with adjusted Pvalue <0.05 were considered significantly down-regulated when the log_2 fold change was <-1 (blue) and up-regulated when the log_2 fold change was >1 (red)). **(B)** Modular transcriptional analysis. Red and blue indicate modules over- or under-abundant compared with control; size of dots represents the reverse of the nominal Pvalue (lower values are represented as bigger dots). Only modules with FDR <25% and nominal Pvalue <0.05 were considered significant. **(C)** Heatmap of the top 30 DEGs. Only genes with adjusted Pvalue <0.05 and absolute log_2 fold change >1were considered and were then selected based on decreasing p value. Results for 10d after treatment were not presented as no DEGs passed the cut-off.

Table 22| Top DEGs in flies 24h after treatment with hkMm

Top DEGs Males				Top DEGs Females			
Pathway	Gene	Log2FC	Adj.Pval	Pathway	Gene	Log2FC	Adj.Pval
Apoptosis	rl	1.014	0.003	Apoptosis	Parp	1.018	0.003
	EcR	1.170	0.037		hid	1.274	0.007
Autophagy	Atg1	1.053	0.016	Circadian rhythm	Pdp1	1.182	0.015
Endocytosis	Amph	1.008	0.000	Fatty acid degradation	CG3961	1.096	0.032
	Arpc4	-1.005	0.002	Foxo signalling pathway	foxo	1.384	0.000
	CenG1A	1.043	0.007		Pi3K21B	1.368	0.001
	siz	1.040	0.009	Glycolysis metabolism	Ldh	-1.363	0.023
	Dab	1.259	0.013	Hippo signalling pathway	hth	1.709	0.032
	aPKC	1.070	0.013		upd2	-4.842	<0.001
Fatty acid degradation	CG3961	1.291	0.002	Longevity regulation	l(2)efl	-1.098	0.029
Foxo signalling pathway	foxo	1.357	0.000	MAPK signalling pathway	sty	1.344	0.045
Hippo signalling pathway	hth	1.458	0.017	mTor signaling pathway	fz2	1.572	0.018
	dlg1	1.152	0.002	Notch signalling pathway	Dl	1.248	0.023
	ed	1.554	0.018	Oxidative phosphorylation	mt:Col	2.429	<0.001
Lysosome	Tsp39D	1.125	0.005		mt:ColIII	2.883	<0.001
	CG30269	-1.151	0.009		mt:Cyt-b	3.684	<0.001
MAPK signaling pathway	tkv	1.149	0.002		mt:ND4	3.316	<0.001
	Src42A	1.258	0.002		mt:ND5	2.596	<0.001
	cic	1.288	0.022		mt:ATPase6	3.039	<0.001
mTor signalling pathway	fz2	1.491	0.006		mt:ND1	2.062	<0.001
	arr	1.150	0.010		mt:ND3	2.412	<0.001
	sgg	1.139	0.015		mt:ColI	1.761	0.001
Oxidative phosphorylation	mt:Col	1.267	0.007		ATPsynF	-1.348	0.005
	mt:ColIII	1.288	0.013		COX8	-1.259	0.023
	mt:Cyt-b	1.786	0.005	Phagosome	CG1924	-2.015	0.008
	mt:ND4	1.575	0.022	Phosphatidylinositol	sl	1.937	0.004
	mt:ND5	1.530	0.019		CG34384	2.481	0.006
	CG9065	-1.027	0.019	TGF-β signalling pathway	SkpB	-1.445	0.015
	ND-B14.5AL	-2.418	0.024		Myc	1.810	0.009
Phagosome	Dlic	1.126	0.005	Toll and Imd signalling pathways	spirit	-2.882	0.005
	Sdic2	-1.700	0.014		PGRP-LB	-2.735	0.012
Phosphatidylinositol	Itpr	1.263	0.015		CecA1	-5.496	0.017
TGF-β signalling pathway	emc	1.166	0.001		PGRP-SA	-1.820	0.019
Toll and Imd signalling pathways	Drsl4	-1.486	0.022		CecB	-5.469	0.020
	Tl	1.157	0.029		PGRP-LF	-1.587	0.048
Wnt signalling pathway	pan	1.336	0.008	Wnt signalling pathway	pan	1.425	0.029
	Cklalpha	1.166	0.001				
	Pka-C1	1.107	0.003				
	p120ctn	1.005	0.011				
	dally	1.267	0.018				

We then proceed to evaluate the effect that the oral administration of hkMm had on *D. melanogaster* infected with *M. marinum*. For this, we did the differentail expression analysis of infected flies and treated and infected flies compared with the control group. The GSEA was performed for each group independently. Heatmaps were constructed with those genes that were significantly differentially expressed in those flies that were treated and infected, but were not in untreated infected flies. We also

included those genes that were present in both condition but inversally expressed to validate which genes and pathways might be involved in the increased resistance of treated flies.

Results revealed that, as happened with treated uninfected flies, the differential expression profile 10d after the infeciton showed very few significantly altered genes, suggesting again that an intermediate time-point would be more suitable for the study of this host's response, although a slightly increase in DEGs on the treated group was observed (Figure 46A).

Here, results from the GSEA (Figure 46B) showed that treated infected males had increased levels of apoptosis and phagosome, lysosome and peroxisome activity immediately after the infection (24h p.i.), but repression of the hormone biosynthesis and oxidative phosphorilation pathways, compared to untreated infected flies. The GSEA also revealed a lower repression of the fatty acid degradation module in treated males, which might correlate with a decrease in the lipid droplets accumulation and, thus, the reduction of the bacillary load. As expected, in both cases the main signalling pathways (TGF-β, Hippo, Wnt and mTOR) related with cell proliferation were induced immediately after the infection, but no significant alteration of the main immune pathways in either group was observed.

The analysis of the top DEGs (Figure 46C and Table 23) confirmed the up-regulation of the apoptosis, as well as the increased phagosome, lysosome and peroxisome activites in treated males and the down-regulation of the oxidative phosphorilation pathway in this group, mainly mediated by a significant repression of the genes encoding for the mitochondrial respiration machinery, process that has been highly linked with the induction of a pro-inflammatory immune response (391). In addition, this analysis also revealed induction of both humoral immune pathways in both groups.

Finally, the analysis of the response to the infection at day 10^{th} (Figure 46C and Table 24) revealed that almost all pathways up-regulated immediatlely after the infection were either repressed or not altered in both groups at this time point, with the exception of the oxidative phosphorilation and the immune pathways in the untreated, and the arginine- and glycolysis-related pathways in the treated groups. The anlysis of the DEGs was only possible for the treated group and results revealed the presence of several turandots up-regulated, while reproduction-related genes were repressed.

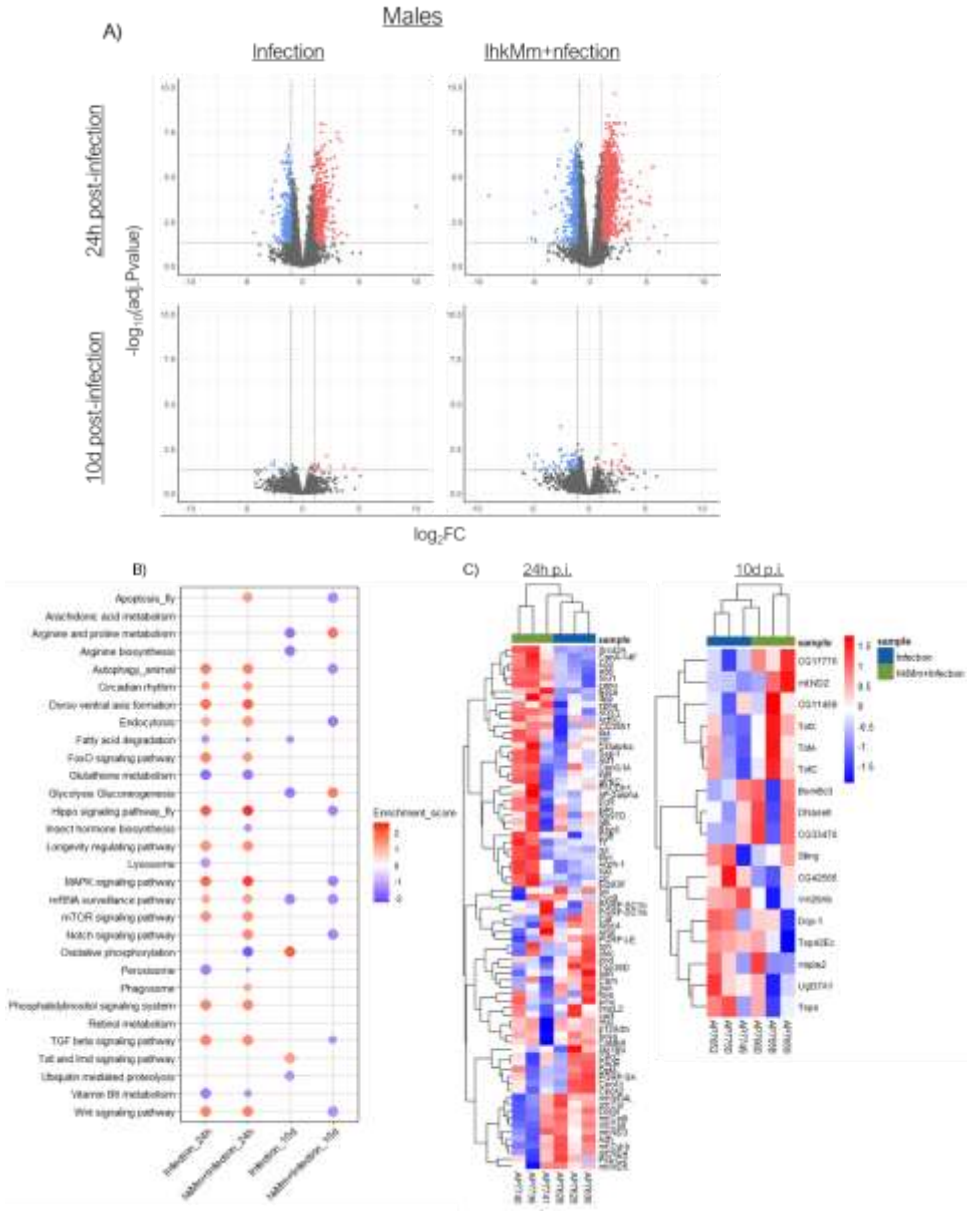


Figure 46| Differential expression analysis of male flies infected with *M. marinum*. (A) Volcano plots of DEGs (genes with adjusted Pvalue <0.05 were considered significantly down-regulated when the log_2 fold change was <-1 (blue) and up-regulated when the log_2 fold change was >1 (red)). **(B)** Modular transcriptional analysis. Red and blue indicate modules over- or under-abundant compared with control; size of dots represents the reverse of the nominal Pvalue (lower values are represented as bigger dots). Only modules with FDR <25% and nominal Pvalue <0.05 were considered significant. **(C)** Heatmap of the top 30 DEGs. Only genes with adjusted Pvalue <0.05 and absolute log_2 fold change >1were considered and were then selected based on decreasing p value.

Table 23| Top DEGs in male flies 24h after infection with *M. marinum*

Top DEGs Infection				Top DEGs hkMm+Infection 24h p.i.			
Pathway	Gene	Log2FC	Adj.Pval	Pathway	Gene	Log2FC	Adj.Pval
Apoptosis	Ejp93F	1.914	0.001	Apoptosis	bic	1.478	2E-07
	EcR	1.536	0.003		bsk	-1.117	3E-07
Arginine biosynthesis	P5CDh1	1.031	<0.001		crc	1.958	7E-07
Autophagy	Bace	-1.266	0.049		EcR	-1.088	<0.001
Endocytosis	Arpc4	-1.117	<0.001	Arginine biosynthesis	Argk	1.403	1E-07
	CenG1A	1.242	<0.001	Autophagy	Atg18b	-1.064	3E-02
	AP-2alpha	1.095	0.001		Bace	1.163	0.016
	aPKC	1.266	0.001	Notch	brn	1.704	3E-07
Fatty acid degradation	CG3961	1.524	<0.001	Endocysotis	baz	2.413	2E-07
Foxo signalling pathway	foxo	1.614	<0.001		capu	1.188	4E-07
	InR	1.526	0.007		Egfr	2.262	2E-05
Hippo signalling pathway	emc	1.506	<0.001	Foxo signalling pathway	Cat	1.076	4E-07
	hth	1.875	0.001		CycB	1.739	8E-07
Hormone biosynthesis	CKIα	1.320	<0.001		Akt1	1.076	6E-05
Longevity regulation	rut	1.079	0.014	Hippo signalling pathway	emc	-1.255	<0.001
Lysosome	Tsp39D	1.285	<0.001		hth	-1.513	<0.001
MAPK signalling pathway	cic	1.752	0.001	Hormone biosynthesis	CKIα	-3.068	<0.001
	Src42A	1.238	0.001	Longevity regulation	Pka-C2	2.196	5E-07
mRNA surveillance	msi	1.655	<0.001		ImpL2	-1.080	3E-06
	Rbp6	1.527	0.001		rut	-1.011	<0.001
	Rb97D	1.228	<0.001	Lysosome	Acph-1	3.104	1E-08
Oxidative phosphorylation	mt:CoIII	2.400	<0.001		Sap-r	1.973	9E-07
	mt:ND4	3.055	<0.001		Mvl	-8.962	1E-04
	mt:ND4L	2.676	0.001	MAPK signallinfg pathway	aop	1.348	1E-07
	mt:CoI	2.414	<0.001		boss	1.532	3E-07
	mt:CoIII	1.106	0.030		chic	1.728	6E-07
	mt:Cyt-b	3.314	<0.001	mRNA surveillance	Rb97D	-1.189	<0.001
	mt:ND3	1.746	0.005	Oxidative phosphorylation	mt:CoI	-1.146	<0.001
Peroxisome	AOX1	-1.153	0.001		mt:CoIII	1.209	<0.001
Toll and Imd signalling pathways	sphe	-1.171	0.003		mt:Cyt-b	-1.059	<0.001
	Tl	1.384	0.005		mt:ND3	-1.079	<0.001
	Drsl4	-1.459	0.016		mt:ND5	-1.213	<0.001
	PGRP-SC1a	-2.215	0.026	Phagosome	Act5C	1.689	2E-08
	PGRP-SC1b	-1.958	0.037		bru1	2.059	1E-07
Wnt signalling pathway	CaMKII	1.349	<0.001	Retinol metabolism	Adh	2.883	4E-08
	pan	2.122	<0.001	TGF-β signalling pathway	dpp	-2.780	1E-06
	CanA-14F	1.059	0.001	Toll and Imd signalling pathways	ben	2.057	2E-07
	sgg	1.315	0.002		cact	-1.015	4E-07
	p120ctn	1.107	0.002		CecA1	1.377	5E-07
	Myc	2.142	0.003		CecA2	2.244	5E-07
					CecB	1.685	5E-07
					CecC	1.816	5E-07
					DptA	-1.913	2E-05
					imd	1.817	2E-04
					PGRP-SA	1.243	4E-03
					PGRP-LE	1.303	6E-03

Table 24| Top DEGs in male flies 10d after infection with *M. marinum*

Top DEGs hkMm+Infection 10d p.i.			
Pathway	Gene	Log2FC	Adj.Pval
Apoptosis	Dcp-1	-1.176	0.006
	Tspo	-1.036	0.011
JAK/STAT signalling pathway	TotX	3.103	0.007
	TotA	3.112	0.030
	TotC	3.635	0.038
JNK signalling pathway	Tsp42Ec	-1.594	0.015
Longevity regulation	miple2	-1.117	0.004
Lysosome	DNaseII	2.054	0.027
	CG42565	-1.113	0.047
Oxidative phospho.	mt:ND2	2.082	0.015
Phagosome	CG11459	2.828	0.014
Phosphatidylinositol	CG17770	1.136	0.048
Reproduction	Vm26Ab	-5.311	0.007
Retinol matebolism	Ugt37A1	-3.005	0.042
Toll and Imd signalling pathways	CG33470	2.856	0.016
	BomBc3	2.572	0.027
	Sting	-1.196	0.038

In female flies we observed the same phenotype as in males, with same levels of tolerance between groups, but higher resistance to *M. marinum* infection in treated females. Results of the differential expression analysis also showed several similarities between sexes.

As in males, volcano plots showed the same reduction in the response against the infection in both female groups, but treated females seemed to have a few more up-regulated genes 10d post-infection in comparison with untreated flies (Figure 47A).

Also similar to results obtained for males, the GSEA showed that immediately after the infection treated females presented increased induction of apoptotic, autophagic and endocytic pathways, as well as a reduction in the repression of the immune and lysosomal pathways when compared with untreated females (Figure 47B). Also, as previously mentioned, 10d after the infection the main signalling pathways related with the control of cell growth and proliferation were down-regulated in both groups, while the arginine-related pathways were down-regulated in untreated females but up-regulated in the treated group. Surprisingly, the retinol metabolism was also up-regulated in treated females, which was found repressed in males.

The anlysis of the top DEGs (Figure 47C) also showed a high concordance with the results from the GSEA, as hapenned in males. Immediately after the infection (Table 25), untreated females presented several down-regulated genes belonging to the peroxisome and phagosome activity, as well as increased expression of some mitochondrial reporation-related genes as observed in males. We also observed a significant repression of several genes related with the glutathione metabolism and the glycolysis pathways. On the other hand, treated females showed significant induction of several genes related with autophagy, endocytosis, lysosome and phagosome activity. Also as observed in males, treated females had highly repressed several mitochondrial respiration-related genes, but opposite to treated males, females showed significant repression of the negative regulators of both immune pathways. Surprisingly, 10d after the infection, untreated females presented significantly repressed several genes related with cellular immune response, while treated females showed significant up-regulation of several digestive enzymes, lysosomal proteins and also Turandots and Bomacins (Table 26).

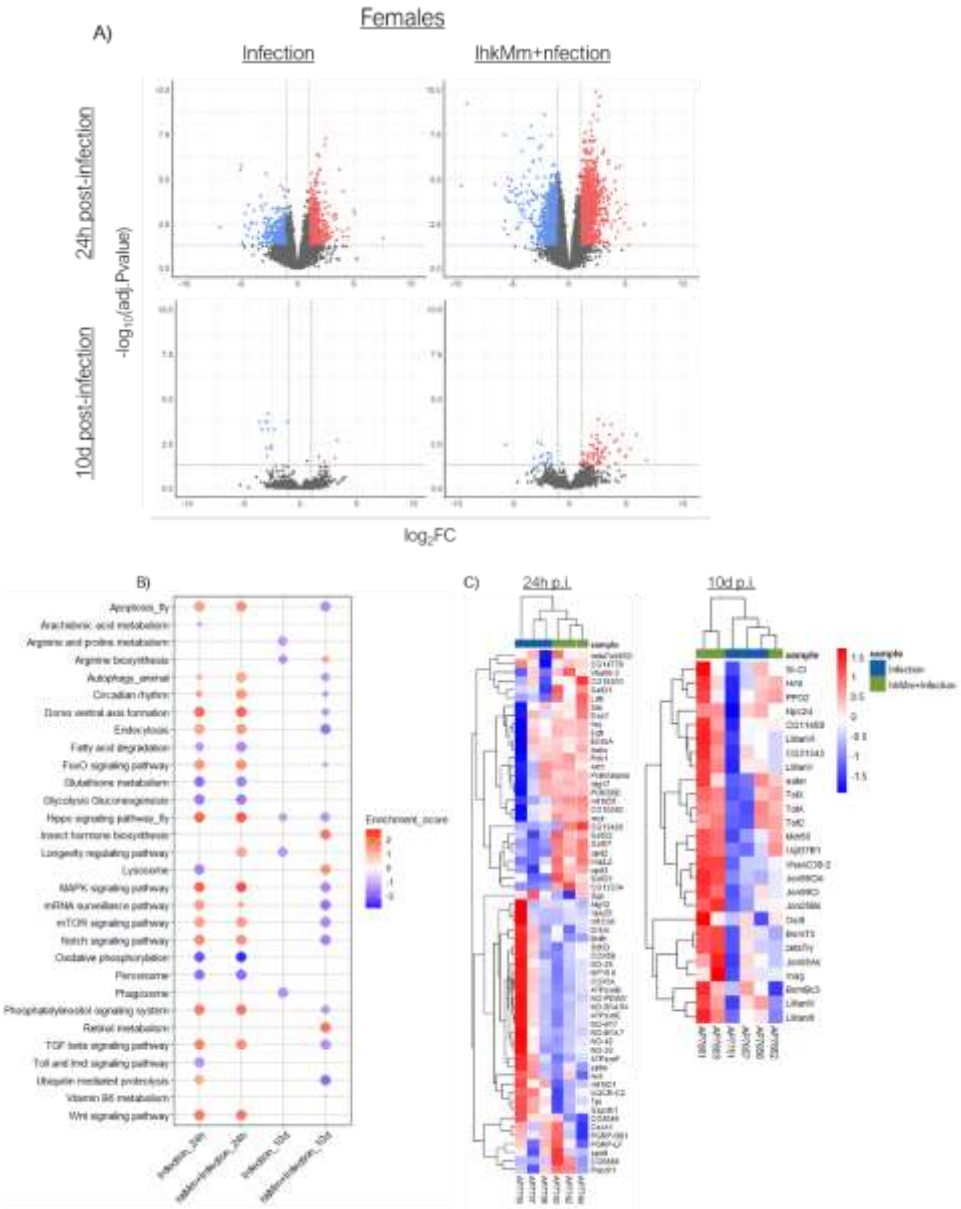


Figure 47| Differential expression analysis of female flies infected with *M. marinum*. (A) Volcano plots of DEGs (genes with adjusted Pvalue <0.05 were considered significantly down-regulated when the log_2 fold change was <-1 (blue) and up-regulated when the log_2 fold change was >1 (red)). **(B)** Modular transcriptional analysis. Red and blue indicate modules over- or under-abundant compared with control; size of dots represents the reverse of the nominal Pvalue (lower values are represented as bigger dots). Only modules with FDR <25% and nominal Pvalue <0.05 were considered significant. **(C)** Heatmap of the top 30 DEGs. Only genes with adjusted Pvalue <0.05 and absolute log_2 fold change >1were considered and were then selected based on decreasing p value.

Table 25| Top DEGs in female flies 10d after infection with *M. marinum*

Top DEGs Infection				Top DEGs hkMm+Infection 24h p.i.			
Pathway	Gene	Log2FC	Adj.Pval	Pathway	Gene	Log2FC	Adj.Pval
Arginine and proline metabolism	CG4546	1.106	0.031	Apoptosis	Buffy	-1.348	<0.001
Glutathione metabolism	GstD1	-1.377	<0.001	Autophagy	Atg12	-1.252	0.001
	GstE7	-1.198	0.004		Dsp1	1.151	<0.001
	GstD3	-1.385	0.005		Atg17	1.345	<0.001
	GstD2	-2.579	0.039	Endocytosis	EndoA	1.149	<0.001
Glycolysis gluconeogenesis	Pepck1	-1.333	0.034		babo	1.033	<0.001
	Ldh	-1.283	0.012		baz	1.026	<0.001
	CG13334	-1.528	0.029		Vps25	-1.251	<0.001
Hormone biosynthesis	mvd	1.179	0.007		Egfr	1.014	0.012
JAK/STAT signalling pathway	upd2	-2.975	0.015	Foxo signalling pathway	Akt1	1.045	<0.001
Oxidative phosphorilation	mt:ND1	1.217	0.011	Glycolysis gluconeogenesys	Gapdh1	-1.463	<0.001
	mt:CoII	1.015	0.033		Tpi	-1.156	<0.001
Peroxisome	CG6888	-2.341	0.009	JAK/STAT signalling pathway	upd3	1.991	0.025
	CG18003	-1.078	0.017	Lonevity regulation	ImpL2	1.285	0.003
	Sgp	1.733	0.027	Lysosome	Pdk1	1.137	<0.001
Phagosome	CG14778	-1.002	0.043	Oxidative phosphorylation	NP15.6	-1.555	<0.001
	CG13426	-1.887	0.028		UQCR-C2	-1.181	<0.001
	betaTub85D	-2.485	0.003		mt:ND5	2.479	<0.001
	Vha68-3	-2.691	0.007		ATPsynB	-1.457	<0.001
Toll signalling pathway	spirit	-1.603	0.031		ATPsynF	-1.719	<0.001
					ND-B17	-1.437	<0.001
					SdhD	-1.293	<0.001
					ND-30	-1.215	<0.001
					ATPsynE	-1.494	<0.001
					ND-42	-1.020	<0.001
					ND-B14.7	-1.545	<0.001
					ND-B14.5A	-1.623	<0.001
					COX5A	-1.517	<0.001
					ND-PDSW	-1.405	<0.001
					COX6B	-1.321	<0.001
					ND-24	-1.300	<0.001
				Phagosome	mys	1.710	<0.001
				Phosphatidylinositol	CG10082	1.142	<0.001
					PI4KIIalpha	1.131	<0.001
					PI3K68D	1.298	<0.001
				Toll and Imd signalling pathways	sphe	-1.373	<0.001
					kay	1.189	0.001
					PGRP-SB1	-2.808	0.006
					Drsl4	-1.761	0.017
					PGRP-LF	-1.099	0.044
					CecA1	-2.915	0.045

Table 26| Top DEGs in female flies 10d after infection with *M. marinum*

Top DEGs Infection 10d p.i.				Top DEGs hkMm+Infection 10d p.i.			
Pathway	Gene	Log2FC	Adj.Pval	Pathway	Gene	Log2FC	Adj.Pval
Endocytosis	Sr-CI	-2.516	0.006	Chitin regulation	Osi9	4.067	0.038
Melanization	PPO2	-2.935	0.015	Digestion enzymes	Jon99Ci	2.524	0.015
Phagosome	ester	-3.004	0.006		Jon99Cii	1.787	0.046
	Hml	-3.403	0.001		zetaTry	1.935	0.004
ROS	Karl	-2.298	0.001		mag	2.889	0.008
				Efflux transporter	Mdr50	3.517	0.001
				JAK/STAT signalling pathway	TotX	3.215	0.005
					TotC	4.360	0.009
					TotA	3.493	0.011
				Lysosome	LManIII	4.895	0.018
					LManIV	5.004	0.015
					LManV	5.331	0.006
					LManVI	4.410	0.006
					VhaAC39-2	1.829	0.004
					CG11459	2.597	0.029
					Jon65Aii	2.413	0.002
					Jon25Bii	2.490	0.039
				Retinol metbolism	Ugt37B1	2.540	0.027
				Sterol transport	Npc2d	2.462	0.008
				Toll signalling pathway	BomBc3	2.447	0.047
					BomT3	1.727	0.048

6.2.2.2| Differential expression analysis of evolved *D. melanogaster*

The differential gene expression of flies from the 5th generation of this study was also evaluated to determine which pathways and genes might be playing a key role in the phenotypic adaptation we observed previously. Although all evolutive conditions showed similar phenotypes with increased tolerance but not resistance to the infection, we also found the exception of males that had been exposed to the combination of oral treatment with hkMm and infection with *M. marinum* throughout generations, which also presented increased resistance. Thus, we hypothesised that, although the phenotypic outcome was the same for all groups, tolerance could be achieved by different mechanisms in each condition. Therefore, each group was analysed separately in order to determine similarities and differences among them. We also analysed males and females separately as we did not compare tolerance and resistance levels between them.

6.2.2.2.1| Differential expression analysis of evolved males

The differential expression analysis of the 5th generation of males revealed no major differences among groups in the amount of significantly differentially expressed genes, neither at 24h nor at 10d post-infection, with the exception of those flies exposed to oral hkMm, which had less significantly altered genes immediately after the infection (Figure 48A). The GSEA analysis at 24h p.i. was consistent with this finding, as males treated with hkMm throughout generations presented no significant induction of apoptosis and endocytosis, as well as less repression of the peroxisome and longevity regulation and glycolysis-related genes. On the other hand, these flies presented up-regulation of the phagosome activity and down-regulation of the arachidonic acid metabolism and the immune signalling pathways. Flies exposed to *M. marinum* over generation in presence or absence of hkMm, showed no differences at this level with the Control group (exposed to PBS).

The GSEA analysis at 10d p.i. revealed that all groups, whether they were more tolerant or not to the *M. marinum* infection, showed significant up-regulation of the Hippo, MAPK, TGF-β and immune pathways, as well as increased retinol metabolism. The tolerant groups showed some common traits: increase in arachidonic acid metabolism and reduction of arginine-related pathways, apoptosis, autophagy, TGF-β signalling pathway and ubiquitin mediate proteolysis. However, those flies exposed to the combination of hkMm and infection, showed no repression of the arginine-related pathways, but of the glycolysis biosynthesis, peroxisome and fatty acid degradation, as well as a significant induction of the innate immune pathways (Figure 48B).

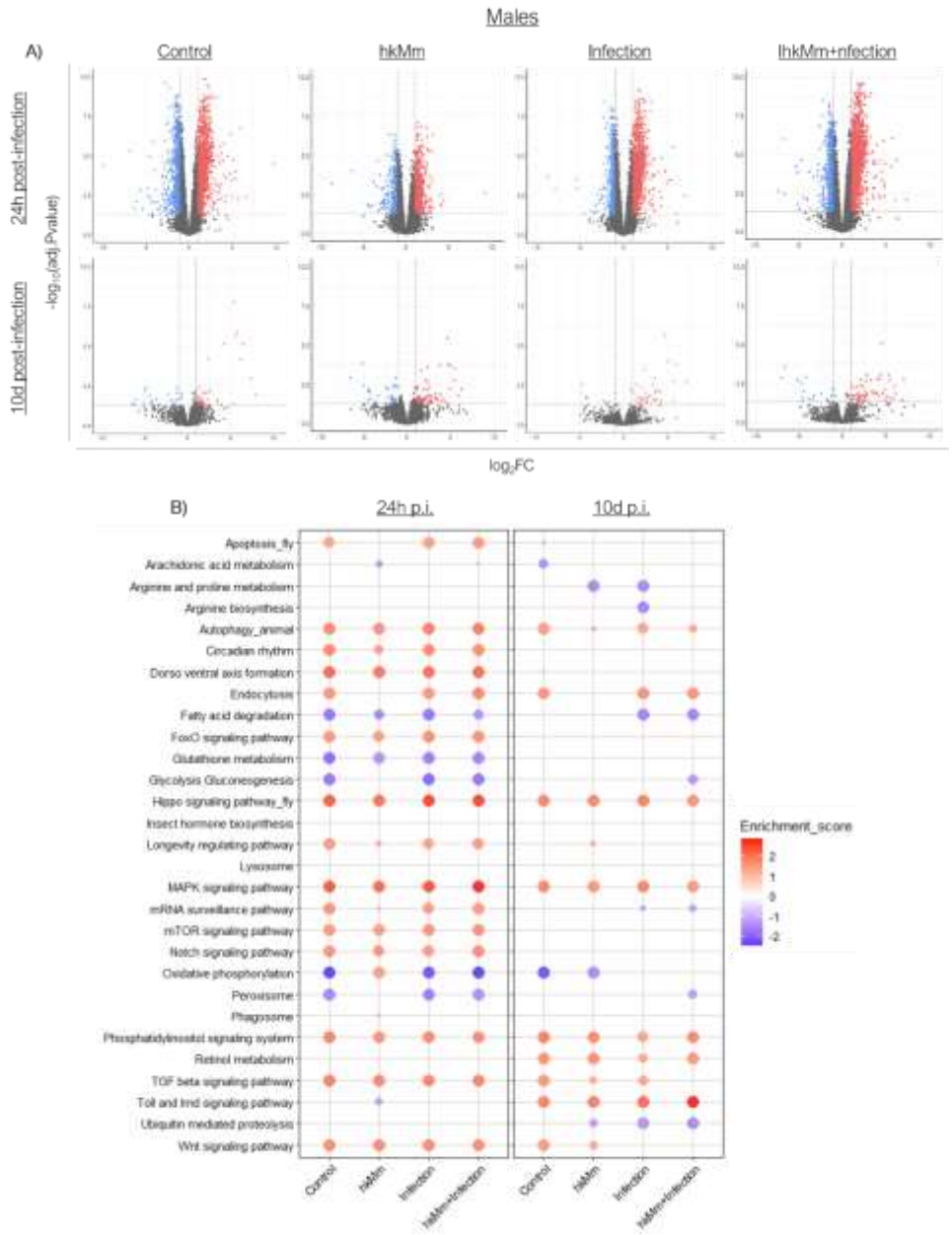


Figure 48| Differential expression analysis of male flies from generation 5 infected with *M. marinum*. (A) Volcano plots of DEGs (genes with adjusted Pvalue <0.05 were considered significantly down-regulated when the log_2 fold change was <-1 (blue) and up-regulated when the log_2 fold change was >1 (red)). **(B)** Modular transcriptional analysis. Red and blue indicate modules over- or under-abundant compared with control; size of dots represents the reverse of the nominal Pvalue (lower values are represented as bigger dots). Only modules with FDR <25% and nominal Pvalue <0.05 were considered significant.

Based on these results, we initially performed the differential gene expression analysis for each of the adapted conditions versus their respective infected control from this same generation. However, as observed in the GSEA, very few significant genes or none were found. Thus, we rather performed the differential expression analysis for each group versus their respective group from the G0, as always, and we then identified those genes that were exclusively differentially expressed in each condition.

Males that had been exposed to oral administration of hkMm or to *M. marinum* infection over, presented very few unique genes, while we found many genes differentially expressed in the control that were not present in these conditions. Although for each group these lists of genes differ, in both cases genes were mainly involved in apoptosis, increased endocytosis and insulin consumption, repression of the metabolic pathways and down-regulation of the main immune suppressors. In addition, control flies presented repressed phagosome activity in comparison with flies from the hkMm group (Figure 49 and Table 27). These results correlate with those observed in the GSEA and might suggest that these flies generate a more controlled and milder response to infection, which might correlate with their tolerant phenotype.

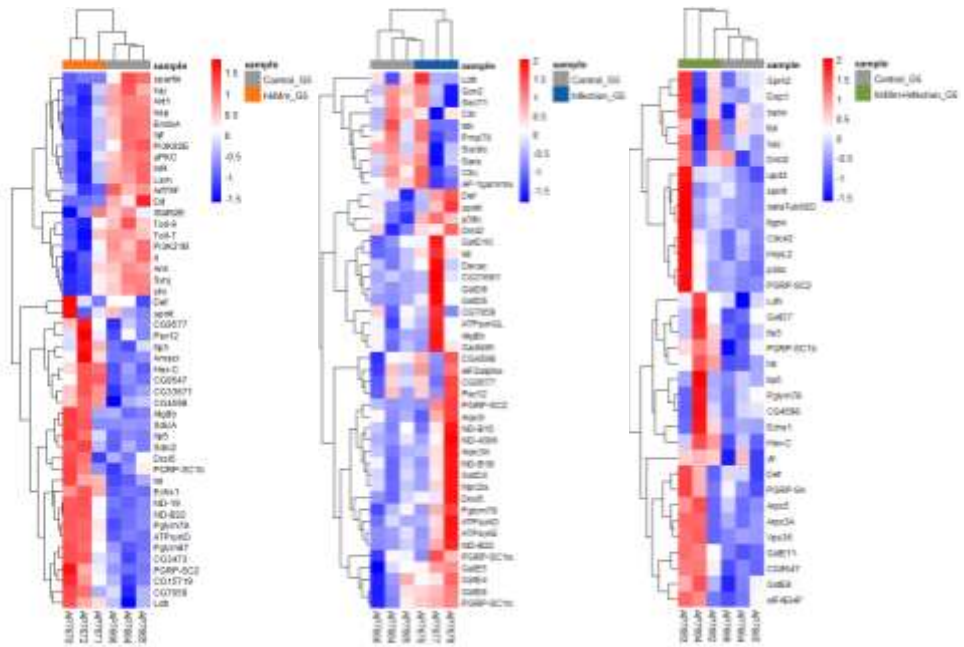


Figure 49| Differential expression analysis of male flies from generation 5 24h p.i. with *M. marinum*. Heatmap of the top significant DEGs on the Control group that were not present in the hkMm and the Infection groups. Only genes with adjusted Pvalue <0.05, absolute log_2 fold change >1 and present only in one of the conditions were considered and were then selected based on decreasing p value and function.

Table 27| Top DEGs exclusive for generation 5 males of the Control group 24h post-infection

hkMm G5				Infection G5			
Pathway	Gene	Log2FC	Adj.Pval	Pathway	Gene	Log2FC	Adj.Pval
Apoptosis	hep	1.236	<0.001	Apoptosis	Decay	-1.143	0.004
	kay	1.041	0.001	Autophagy	eIF2alpha	-1.013	<0.001
	Lam	1.461	0.001		htk	1.031	0.003
Endocytosis	EndoA	1.117	<0.001		Gcn2	1.176	0.008
	Arf79F	1.077	<0.001		Atg8b	-1.067	0.013
	lqf	1.210	<0.001		CG31661	-2.524	0.022
	shi	1.316	<0.001	Endocytosis	Arpc3A	-1.272	<0.001
	spartin	1.367	<0.001		Arpc5	-1.051	<0.001
	aPKC	1.471	<0.001		Chc	1.074	<0.001
Fatty acid degradation	CG4598	-1.055	<0.001		Sara	1.012	<0.001
	CG9547	-1.176	<0.001		Su(dx)	1.048	<0.001
	hll	-3.978	0.008		Cbl	1.087	0.003
	Echs1	-1.101	<0.001		Sec71	1.059	0.004
Foxo signalling pathway	Akt1	1.008	<0.001	Fatty acid degradation	CG4598	-1.055	<0.001
	Atg8b	-1.067	0.013		hll	-3.978	0.008
	Ilp3	-1.479	0.005	Foxo signalling pathway	Gadd45	-1.853	0.003
	Ilp5	-1.082	0.028		p38c	-1.180	0.012
	InR	1.822	<0.001	Glutathione metabolism	GstD9	-1.321	0.001
	PI3K21B	1.126	0.001		GstE8	-1.080	0.002
	PI3K92E	1.251	0.001		GstD10	-1.295	0.003
	rl	1.184	<0.001		GstD3	-1.035	0.005
Glycolysis gluconeogenesys	CG7059	-1.024	<0.001		GstE5	-1.069	0.011
	Pglym78	-1.069	0.001		GstD5	-1.513	0.031
	Hex-C	-1.262	0.001		GstE4	-1.741	0.037
	Ldh	-1.038	0.007	Glycolysis gluconeogenesys	CG7059	-1.024	<0.001
	Pglym87	-1.106	0.008		Pglym78	-1.069	0.001
Toll and Imd signalling pathways	Ank	1.247	<0.001		Ldh	-1.038	0.007
	CG3473	-1.178	<0.001	Toll and Imd signalling pathways	PGRP-SC2	-2.077	0.006
	Def	-3.360	0.050		Drsl5	-1.276	0.006
	Dif	1.452	<0.001		PGRP-SC1b	-2.204	0.007
	Drsl5	-1.276	0.006		Drsl2	-3.614	0.008
	PGRP-SC1b	-2.204	0.007		spirit	-1.548	0.018
	PGRP-SC2	-2.077	0.006		PGRP-SC1a	-1.672	0.034
	spirit	-1.548	0.018		Def	-3.360	0.050
	Toll-9	1.153	0.030	Lysosome	Npc2a	-1.312	<0.001
	Toll-7	1.093	0.002		AP-1gamma	1.072	0.001
Oxidative phosphorylation	ATPsynD	-1.115	0.001	Oxidative phosphorilation	ATPsynE	-1.196	<0.001
	ND-19	-1.018	0.001		ND-ASHI	-1.033	0.001
	ND-B22	-1.064	0.001		ND-B18	-1.097	0.001
Peroxisome	Amacr	-1.155	<0.001		ATPsynD	-1.115	0.001
	CG9577	-1.026	<0.001		ATPsynGL	-1.159	0.001
	CG33671	-1.122	<0.001		ND-B22	-1.064	0.001
	Pex12	-1.047	<0.001		ND-B15	-1.208	0.001
Phagosome	SdicA	-9.901	<0.001	Peroxisome	CG9577	-1.026	<0.001
	Sdic2	-2.403	<0.001		Pex12	-1.047	<0.001
	CG15719	-1.261	<0.001		Pmp70	1.038	<0.001
JAK/STAT signalling pathway	Stat92E	1.009	<0.001				

Although only genes of the control group are shown here, as they are the most relevant, it is worth mentioning that the hkMm-exposed males exhibited significant up-regulation of genes encoding for relevant proteins: the juvenile hormone (Jhe; log_2FC 1.077, adj.Pvalue 0.035), which favours reproduction over immunity; sestrin (Sesn; log_2FC 1.093, adj.Pvalue <0.001) that has been described to supress accumulation of ROS and, thus, regulate oxidative damage; and stress-sensitive B (SesB; log_2FC 1.389, adj.Pvalue <0.001) that mediates the export of ATP from the mitochondria to fuel the cell.

In the infection group we also found two relevant genes over-expressed, one involved in hemocytes differentiation, *yantar* (ytr; log_2FC 1.134, adj.Pvalue <0.001), and the *CG6424* gene, which human ortholog *FAM13A* has been linked with chronic obstructive pulmonary disease (COPD).

When we looked at the differences on the DEGs of the control group in comparison with the group that had been exposed to both stimuli over time (Table 28), we observed that the hkMm+Infection group did have several significantly altered genes that were not present in the control group, mainly related with increased endocytosis and phagosome activity. Interestingly, we also observed some genes related with increased cellular immune response (*impl2* and *ytr*). Altogether, these results might indicate that this group presented a differentiated response with respect to the control and not a milder one like the other two groups.

Table 28| Top DEGs exclusive for generation 5 males of the Control and hkMm+Infection groups 24h post-infection

Top DEGs Control G5

Pathway	Gene	Log2FC	Adj.Pval
Endocytosis	Vps36	-1.134	<0.001
	Arpc3A	-1.272	<0.001
	Arpc5	-1.051	<0.001
Fatty acid degradation	CG9547	-1.176	<0.001
	Echs1	-1.101	<0.001
	CG4598	-1.055	<0.001
	hll	-3.978	0.008
Foxo signalling pathway	Ilp3	-1.479	0.005
	p38c	-1.180	0.012
	Ilp5	-1.082	0.028
Glutathione metabolism	GstE11	-1.416	0.000
	GstE8	-1.080	0.002
	GstD7	-1.424	0.005
Glycolysis gluconeogenesis	Pglym78	-1.069	0.001
	Hex-C	-1.262	0.001
	Ldh	-1.038	0.007
Toll and Imd signalling pathways	PGRP-SA	-1.542	0.003
	PGRP-SC2	-2.077	0.006
	PGRP-SC1b	-2.204	0.007
	Drsl2	-3.614	0.008
	spirit	-1.548	0.018
	Def	-3.360	0.050
JAK/STAT signalling pathway	upd3	-2.897	0.006

Top DEGs hkMm+Infection G5

Pathway	Gene	Log2FC	Adj.Pval
Autophagy	Dsp1	1.000	<0.001
Endocytosis	baz	1.205	<0.001
	Cdc42	1.195	<0.001
	babo	1.082	<0.001
	Gprk2	1.137	<0.001
Longevity regulation	ImpL2	1.125	0.008
mTOR signalling pathway	eIF4EHP	1.018	<0.001
	fz4	1.047	0.020
Phagosome	betaTub60D	1.367	0.001
	Itgbn	1.183	0.029
Cellular immune response	ytr	1.041	<0.001

Surprisingly, at 10d all three stimulated groups throughout generations showed similar differential expression profile when compares with the control group (Figure 50 andTable 29). These groups showed significant up-regulation of several Bomanins (Boms, IM14 and IM4), which are a family of a dozen secreted peptides that participate in the Toll-mediated innate immune response in *Drosophila* (392). We also observed common induction of the Toll pathway inducer GNBP-3 and related AMP, Drosomycin (Drs), as well as the Imd-related AMP, Drosocin (Dro).

However, each group also presented some exclusive relevant genes. The hkMm group showed up-regulation of the iron binding protein Transferrin 1 (Tsf1), while the Infection group revealed down-regulation of genes involved in proteolysis (Jon65Aii) and digestion (Jon99Cii). Finally, the resistant group that combined exposure to hkMm and infection, showed significant induction of several Imd-related AMPs (DptA, DptB and Mtk) as well as the positive Imd-regulator, PGRP-SD.

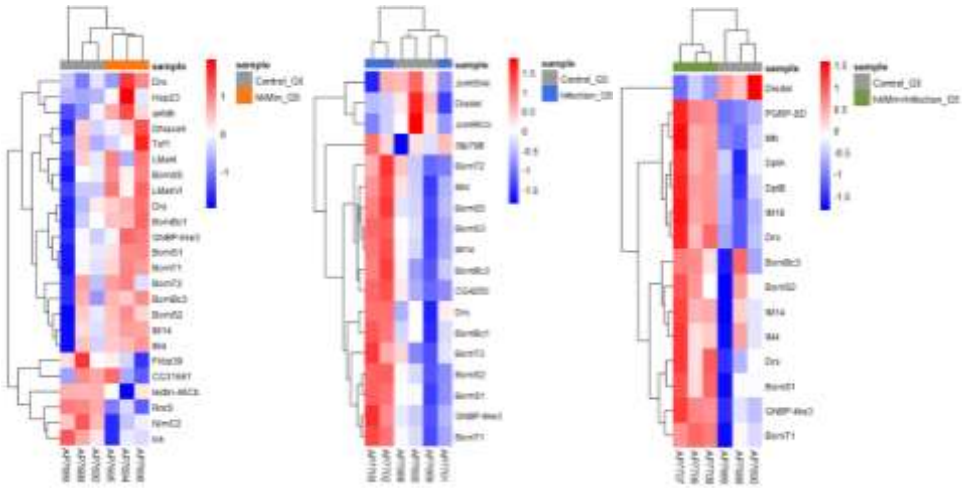


Figure 50| Differential expression analysis of male flies from generation 5 10d p.i. with *M. marinum*. Heatmap of the top significant DEGs on the Control group that were not present in the hkMm and the Infection groups. Only genes with adjusted Pvalue <0.05, absolute log_2 fold change >1 and present only in one of the conditions were considered and were then selected based on decreasing p value and function.

Table 29| Top DEGs exclusive for generation 5 males of the stimulated groups 10d post-infection

Top DEGs 10d p.i.							
Pathway	Gene	hkMm G5		Infection G5		hkMm+Infection G5	
		Log2FC	Adj.Pval	Log2FC	Adj.Pval	Log2FC	Adj.Pval
Toll and Imd signalling pathways	BomBc1	4.257	0.031	4.264	0.048		
	BomBc3	2.787	0.015	3.277	0.003	2.582	0.027
	BomS1	3.872	0.013	3.716	0.027	3.868	0.013
	BomS2	2.448	0.031	2.720	0.020	2.349	0.046
	BomS5	2.939	0.047	3.408	0.027		
	BomT1	3.909	0.034	4.182	0.032	4.200	0.020
	BomT2	1.855	0.031	1.919	0.035		
	Dro	4.545	0.044			5.998	0.005
	Drs	3.167	0.022	3.558	0.009	4.021	0.002
	GNBP-like3	3.393	0.031	3.623	0.030	3.883	0.011
	IM14	3.322	0.012	3.603	0.007	3.397	0.010
	IM4	2.529	0.034	2.706	0.032	2.479	0.046

hkMm G5			Infection G5			hkMm+Infection G5		
Gene	Log2FC	Adj.Pval	Gene	Log2FC	Adj.Pval	Gene	Log2FC	Adj.Pval
Fkbp39	-1.071	0.005	BomS3	2.505	0.048	DptA	6.163	0.013
Hsp23	2.241	0.007	BomT3	1.982	0.020	DptB	5.152	0.021
lectin-46Cb	-1.448	0.004	CG4250	1.690	0.020	IM18	4.951	0.038
NimC2	-2.200	0.006	Jon65Aii	-2.057	0.047	Mtk	4.626	0.013
Tsf1	2.155	0.021	Jon99Cii	-1.710	0.049	PGRP-SD	2.849	0.013
			Sfp79B	3.106	0.048			

6.2.2.2.1| Differential expression analysis of evolved females

The analysis of the differential expression profile of the different groups in females, revealed similar expression levels as in males 24h after the infection, but increased numbers of significant DEGs at 10d post-infection. However, an opposite at what we observed in males, females that had been exposed to hkMm over time had increased numbers of significant DEGs than the rest of the groups (Figure 51A).

The GSEA analysis of infected females revealed that those flies that were unstimulated (only sterile PBS injections) over the course of 5 generations showed the same profile as unstimulated males at 24h p.i., but not at 10d, where females showed no significant alteration of many of the pathways that were still up-regulated in males at that time point. As in males, this analysis also revealed no major differences among groups, with the metabolic pathways mainly down-regulated, as well as the innate immune pathways, while tissue repair and cell proliferation pathways up-regulated. However, stimulated groups showed immediate repression of the arginine- and retinol-related pathways after the infection and significant induction of the ubiquitin mediate proteolysis, while the control group had immediate up-regulation of the phagosome activity.

At 10d p.i. we observed several differences not only between the control and stimulated groups, but also among these last ones. Overall, all females showed repression of the metabolic pathways and significant induction of the MAPK, Hippo and Foxo signalling pathways. However, only female flies exposed to hkMm over time showed significant increased autophagy and endocytosis and increased induction of the mTOR and Notch signalling pathways.

Finally, opposite of what we observed in males which presented high induction of phosphatidylinositol, TGF-β, retinol and innate immune pathways in all groups 10d after the infection, only stimulated females showed up-regulation of the Toll and Imd signalling pathways at this time point, but not the rest of the pathways.

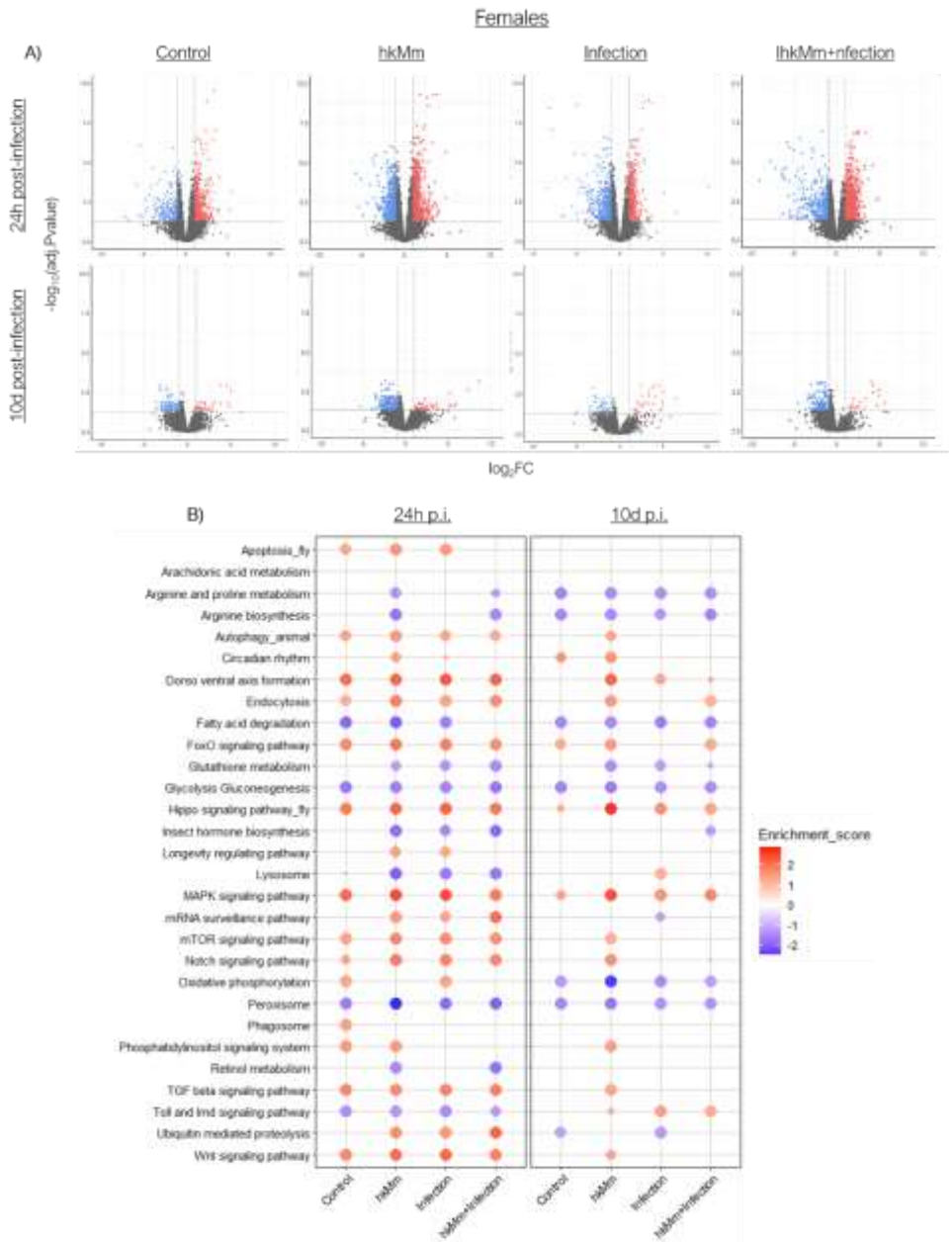


Figure 51| Differential expression analysis of female flies from generation 5 infected with *M. marinum*. (A) Volcano plots of DEGs (genes with adjusted Pvalue <0.05 were considered significantly down-regulated when the log_2 fold change was <-1 (blue) and up-regulated when the log_2 fold change was >1 (red)). **(B)** Modular transcriptional analysis. Red and blue indicate modules over- or under-abundant compared with control; size of dots represents the reverse of the nominal Pvalue (lower values are represented as bigger dots). Only modules with FDR <25% and nominal Pvalue <0.05 were considered significant.

As in males, we performed the differential expression analysis for each group versus their respective group from the G0, and we then identified those genes that were exclusively expressed in each condition. However, opposite to what we found in males, in females all three stimulated groups presented several unique genes significantly differentially expressed (Figure 52 and Table 30).

In all conditions, the top DEGs coincided with the results obtained in the GSEA and were highly similar among them, sharing a wide number of genes, although not all of the genes made it to the top DEGs in all conditions. Overall, the metabolic pathways were mainly repressed in all three groups, as well as lysosome and peroxisome-related genes. At the same time, we observed an up-regulation in the apoptosis, autophagy, endocytosis and ubiquitin mediated proteolysis.

Interestingly, the hkMm and the Infection groups also showed up-regulation of the gene related with hemocytes differentiation, which was not observed in the combined group. In males, we also observed up-regulation of this gene in the Infection group.

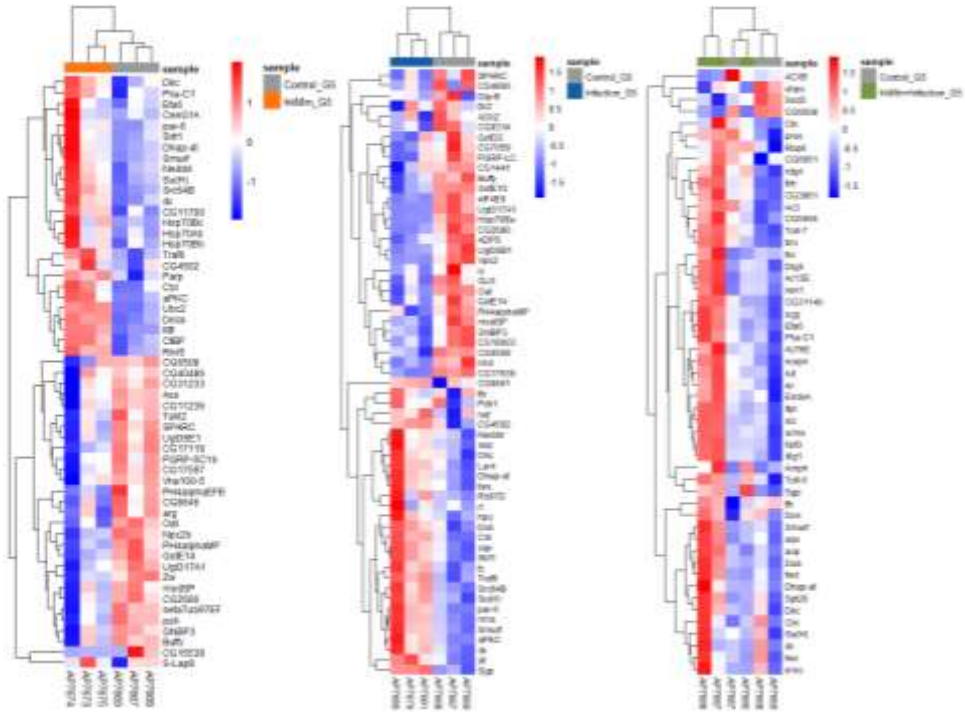


Figure 52| Differential expression analysis of female flies from generation 5 24h p.i. with *M. marinum*. Heatmap of the top significant DEGs on the Control group that were not present in the hkMm and the Infection groups. Only genes with adjusted Pvalue <0.05, absolute log_2 fold change >1 and present only in one of the conditions were considered and were then selected based on decreasing p value and function.

Table 30| Top DEGs for generation 5 stimulated females 24h post-infection

hkMm G5

Pathway	Gene	Log2FC	Adj.Pval
Apoptosis	SPARC	-1.640	<0.001
	Bully	-1.347	<0.001
	Drice	1.123	0.001
	Parp	1.010	0.001
Arginine biosynthesis	atg	-1.430	0.002
	Ass	-1.405	0.002
	PHalphaMP	-1.877	<0.001
	Oat	-1.124	0.004
	PHalphaMPB	-1.020	0.010
	CG15539	-2.219	0.029
Endocytosis	par-6	1.152	0.001
	Smurf	1.322	0.001
	CenG1A	1.240	0.002
	Src64B	1.365	0.004
	aPKC	1.116	0.005
	Eps8	1.043	0.011
Glutathione metabolism	Zw	-1.038	<0.001
	GstS14	-1.509	0.004
	S-Lap8	3.315	0.006
	Rnr8	1.168	0.011
	CG31233	-1.328	0.017
Insect hormone biosynthesis	CG40486	-1.318	0.001
	CG32680	-1.305	0.003
Longevity regulation	Sirt1	1.460	0.000
	Pka-C1	1.115	0.007
	Hsp70Bb	2.702	0.010
	Hsp70Bc	2.353	0.035
	Hsp70Ab	1.841	0.039
Lysosome	CG6506	-5.671	<0.001
	CG8646	-1.538	0.002
	CG17119	-1.159	0.005
	Npc2b	-1.001	0.008
Notch signalling pathway	CtBP	1.096	<0.001
	Su(H)	1.825	<0.001
	dx	1.694	0.001
Peroxisome	CG17597	-1.223	<0.001
	CG11236	-1.563	<0.001
	Dhap-at	1.302	0.001
Phagosome	betaTubSTEF	-1.202	<0.001
	Mf	1.016	0.001
	Dlc	1.099	0.008
	Vha100-5	-1.069	0.012
Retinol metabolism	Ugt317A1	-1.461	0.003
	Ugt35E1	-1.845	0.007
Toll and Imd signalling pathways	modSP	-1.128	<0.001
	GNBP3	-1.592	<0.001
	psh	-1.215	0.001
	PGRP-SC1b	-2.464	0.002
	Tak2	-1.032	0.039
Ubiquitin mediated proteolysis	CG4502	1.072	<0.001
	Traf6	1.103	<0.001
	Cbl	1.426	<0.001
	Ubc2	1.068	0.001
	Nedd4	1.157	0.005
	CG11700	1.094	0.014
Cellular immune response	ytr	1.049	<0.001

Infection G5

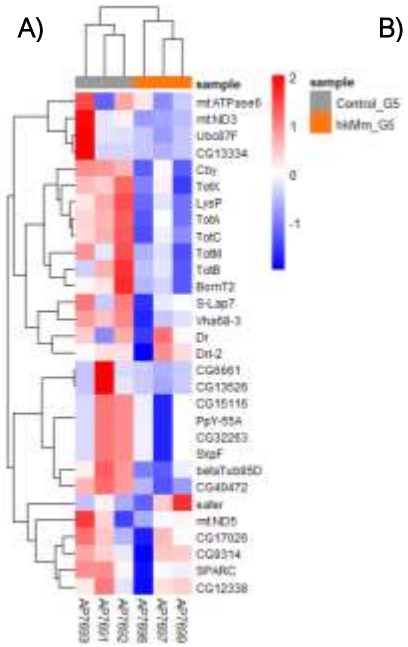
Pathway	Gene	Log2FC	Adj.Pval
Apoptosis	Traf6	1.146	<0.001
	Bully	-1.239	<0.001
	dl	1.072	0.001
	Lam	1.381	0.004
	tgo	1.025	0.005
	SPARC	-1.123	0.001
Arginine biosynthesis	Oat	-1.525	<0.001
	PHalphaMP	-1.783	0.001
	GLS	-1.072	0.002
Endocytosis	CG6661	2.484	0.010
	nuf	1.017	<0.001
	par-6	1.247	<0.001
	Cbl	1.516	<0.001
	Vps2	-1.026	<0.001
	Smurf	1.237	0.003
	Src64B	1.230	0.009
	aPKC	1.021	0.012
	Nedd4	1.052	0.013
Glutathione metabolism	CG17636	-1.286	0.002
	GstE10	-1.289	0.006
	GstD2	-3.222	0.016
	GstE14	-1.032	0.048
Insect hormone biosynthesis	CG2660	-1.172	0.006
Longevity regulation	Sirt1	1.349	<0.001
	Hsp70Ba	-5.568	0.006
	CG5514	-1.878	0.009
MAPK signalling pathway	tik	1.050	0.001
	Dok	1.173	0.002
	slpr	1.343	0.008
Notch signalling pathway	Su(H)	1.680	<0.001
	dx	1.605	0.002
Peroxisome	Sqs	2.668	0.001
	eIF4E6	-1.879	0.001
	CG18005	-1.469	0.002
	ACX2	-2.581	0.002
	Dhap-at	1.098	0.005
	ADPS	-1.409	0.006
	fy	-1.268	0.007
	CG1441	-1.195	0.009
Phagosome	Dlc	1.041	0.013
Retinol metabolism	Ugt317A1	-1.540	0.002
	Ugt35B1	-1.016	0.015
Toll and Imd related pathways	modSP	-1.019	0.001
	imd	-1.200	0.001
	PGRP-LC	-1.426	0.005
	GNBP3	-1.223	0.006
Ubiquitin mediated proteolysis	CG4502	1.080	<0.001
	fbl2	-1.445	0.015
Cellular immune response	ytr	1.056	<0.001

hkMm+Infection G5

Pathway	Gene	Log2FC	Adj.Pval
Arginine metabolism	CG6661	2.317	0.013
	shps	-1.940	0.025
Autophagy	CG6508	-10.316	<0.001
	Pka-C1	1.128	0.005
	Ppr	1.396	0.007
	sima	1.109	0.026
	Sp20	1.073	0.037
Circadian rhythm	sgg	1.558	0.003
	tim	1.245	0.008
	Clk	1.551	0.014
Endocytosis	Arph	1.128	<0.001
	EndoA	1.001	<0.001
	Smurf	1.134	0.005
	Eps6	1.119	0.006
	Cbl	1.096	0.006
	stz	1.137	0.007
	Dab	1.069	0.026
Fatty acid degradation	CG3961	1.375	0.001
Hippo signalling pathways	dlg1	1.318	0.001
	ex	1.890	0.003
	Dg5	1.343	0.005
	tws	1.017	0.008
Longevity regulation	Sod3	-1.097	<0.001
	5ch	1.701	0.006
	Ac13E	1.147	0.011
	rst	1.257	0.016
	ACXB	-1.632	0.016
	Ac3	1.730	0.023
	Ac78F	1.000	0.028
MAPK signalling pathway	tik	1.086	0.001
	9v	1.148	0.002
	kek1	1.396	0.002
	Dok	1.038	0.006
	pros	1.696	0.009
	slpr	1.219	0.014
mRNA surveillance	Rpb8	1.397	0.007
	Not3	2.893	0.044
Notch signalling pathway	Su(H)	1.105	0.013
	dx	1.076	0.030
Peroxisome	Sqs	2.379	0.002
	Dhap-at	1.067	0.005
	CG5085	1.370	0.013
Phagosome	Dlc	1.001	0.013
Phosphatidylinositol signaling	rdgA	1.242	0.001
	fwd	1.064	0.007
	norpA	1.320	0.011
Toll and Imd signalling pathways	CG31140	2.563	0.028
	Toll-6	2.364	0.011
	Toll-7	1.470	0.016

As observed in the GSEA, only in the hkMm exposed females we could found differentially expressed genes compared to the control group at 10d p.i. (Figure 53). Flies that were either exposed to the infection or to the combination of both mycobacteria for 5 generation, showed no different gene expression profile compared to unstimulated flies. Only in the Infection group we observed significantly increased expression of Transferrin 1 (Tsf1). In addition, the control group presented significant down-regulation of the gene *eater*, which encodes a transmembrane receptor specifically expressed in hemocytes and required for the phagocytosis of Gram-positive bacteria and the attachment of hemocytes to sessile niches. Thus, these results reinforce the idea that an intermediate time point would be required to make a clearer picture of which mechanisms might be involved in increasing tolerance to infection.

A)



B)

Top DEGs Control 10d p.i.

Pathway	Gene	Log2FC	Adj.Pval
Cellular immune response	eater	-2.368	0.029
Toll and Imd signalling pathways	BomT2	1.977	0.018
	Dr	1.203	0.028
	Drl-2	1.508	0.031
JAK/STAT signalling pathway	TotA	4.492	0.001
	TotB	4.618	0.014
	TotC	5.018	0.002
	TotM	4.816	0.025
	TotX	3.849	0.001

Top DEGs hkMm 10d p.i.

Pathway	Gene	Log2FC	Adj.Pval
Arginine biosynthesis	CG6661	-2.931	0.017
Glutathione metabolism	S-Lap7	-3.449	0.036
Glycolysis	CG13334	-1.722	0.047
mTOR	PpY-55A	-1.477	0.028
Oxidative phosphorilation	CG40472	-1.085	0.030
	mt:ATPase6	-1.436	0.023
	mt:ND3	-1.983	0.009
	mt:ND5	-1.603	0.026
Peroxisome	CG12338	-1.004	0.044
	CG32263	-1.194	0.036
	CG9314	-1.795	0.047
	CG15116	-1.573	0.049
Phagosome	Vha68-3	-3.077	0.013
	betaTub85D	-2.349	0.020
Phosphatydylinositol	CG13526	-1.356	0.039
	CG17026	-1.061	0.036
Ubiquitin mediated proteolysis	SkpF	-1.477	0.021
	Ubc87F	-2.647	0.028
Wnt signalling pathway	Cby	-2.941	0.020

Figure 53| Differential expression analysis of female flies exposed to hkMm for 5 generations 10d p.i. with *M. marinum*. (A) Heatmap of the top significant DEGs on the Control group that were not present in the hkMm and the Infection groups. Only genes with adjusted Pvalue <0.05, absolute log_2 fold change >1 and present only in one of the conditions were considered and were then selected based on decreasing p value and function. **(B)** List of the top relevant DEGs.

6.2.2.3| Differential expression analysis of *D. melanogaster* that coevolved with *M. marinum*

We also wanted to validate the differences on the genetic profile of those flies that had coevolve together with *M. marinum*. For this, we compared the gene expression of these groups versus the same group from experiment 1 in order to find which genes were differently regulated due to the coevolution.

Results showed that a large number of genes were differentially expressed in both sexes and at both time points when the host and the pathogen evolved together, compared to when the host evolved alone (Figure 54A). The GSEA analysis revealed that overall, coevolved flies presented a high repression of all of the selected pathways both at 24h and 10d after the infection, with the exception of the up-regulation of the oxidative phosphorylation and glutathione metabolism modules in males and the ubiquitin-mediated proteolysis, endocytosis and fatty acid degradation modules in females (Figure 54B).

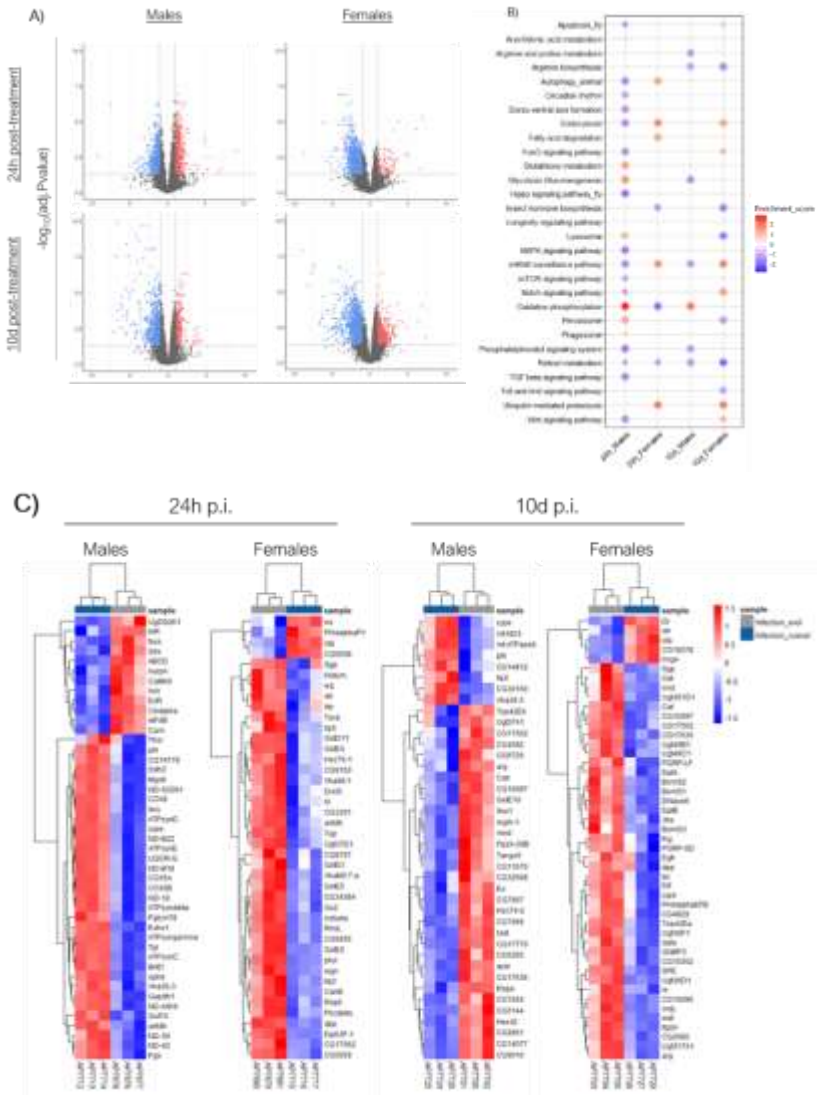


Figure 54| Differential expression analysis of coevolved flies infected with *M. marinum*. (A) Volcano plots of DEGs (genes with adjusted Pvalue <0.05 were considered significantly down-regulated when the $\log_2$ fold change was <-1 (blue) and up-regulated when the $\log_2$ fold change was >1 (red)). **(B)** Modular transcriptional analysis. Red and blue indicate modules over- or under-abundant compared with control; size of dots represents the reverse of the nominal Pvalue (lower values are represented as bigger dots). Only modules with FDR <25% and nominal Pvalue <0.05 were considered significant. **(C)** Heatmap of the top DEGs. Only genes with adjusted Pvalue <0.05 and absolute $\log_2$ fold change >1 were considered and were then selected based on decreasing p value.

The analysis of the top significantly expressed genes showed great concordance with the results from the GSEA. Immediately after the infection, both males and females showed several genes related with the Foxo signalling pathway significantly repressed. In females, almost all genes belonging to the selected pathways were

significantly repressed while, in males, genes belonging to the metabolic pathways were up-regulated in comparison with flies that were stimulated with new pathogenic mycobacteria in the experiment 1 (Figure 54C and Table 31). Phenotypically, these flies were more tolerant but not more resistant to infections with *M. marinum*. Thus, these results might suggest that the tolerance is driven by an unresponsiveness state on the host.

Table 31| Top DEGs for generation 5 of coevolved flies 24h post-infection

24h Males			
Pathway	Gene	Log2FC	Adj.Pval
Autophay	S6k	-1.188	<0.001
Fatty acid degradation	Echs1	1.234	<0.001
Foxo signalling pathway	bsk	-1.038	<0.001
	InR	-1.562	0.011
	foxo	-1.010	0.004
	Thor	1.070	0.025
Glutathione biosynthesis	Mgstl	1.603	<0.001
	GstT4	1.154	<0.001
Glycolysis	Gapdh1	1.648	<0.001
	Pgk	1.178	<0.001
	Tpi	1.182	<0.001
	Pglym78	1.448	<0.001
Hormone biosynthesis	EcR	-1.326	0.014
	antdh	1.751	<0.001
mTOR signalling pathway	Vha36-3	1.416	<0.001
	eIF4B	-1.131	<0.001
	BHD	1.019	<0.001
Oxidative phosphorylation	ND-ASHI	1.682	<0.001
	ATPsyngamma	1.461	<0.001
	ND-42	1.185	<0.001
	cype	1.937	<0.001
	SdhC	1.693	<0.001
	ATPsynD	1.619	<0.001
	ATPsynC	1.232	<0.001
	ATPsyndelta	1.325	<0.001
	ND-B22	1.539	<0.001
	UQCR-Q	1.690	<0.001
	ND-B18	1.492	<0.001
	ND-39	1.059	<0.001
	ATPsynE	1.508	<0.001
	ND-18	1.443	<0.001
	COX5A	1.543	<0.001
	COX6B	1.346	<0.001
	levy	1.648	<0.001
	COX8	1.797	<0.001
	ND-SGDH	1.271	<0.001
Peroxisome	CG14778	1.517	<0.001
	ABCD	-1.001	<0.001
	plh	1.704	<0.001
Retinol biosynthesis	Ugt302K1	-2.099	<0.001
Toll signalling pathway	sphe	1.025	<0.001
Wnt signalling pathway	Cklalpha	-1.647	<0.001
	norpA	-2.205	<0.001
	CaMKII	-1.156	<0.001

24h Females			
Pathway	Gene	Log2FC	Adj.Pval
Apoptosis	wgn	-1.097	0.002
Arginine metabolism	PH4alphaPV	1.846	0.001
	Gs2	-1.325	<0.001
Autophay	CG6508	6.870	<0.001
	CG5059	-1.142	0.005
	Pkcdelta	-1.221	0.003
Endocytosis	Hsc70-1	-1.059	0.001
Foxo signalling pathway	Ilp2	-1.655	0.007
	Ilp5	-1.613	0.010
Glutathione metabolism	GstD11	-2.663	<0.001
	GstE2	-1.940	<0.001
	GstE4	-2.667	0.005
	GstE3	-1.189	0.005
	GstE5	-1.930	0.009
Hormone biosynthesis	CG8757	-3.516	0.000
	CG3301	-1.193	0.003
	amtdh	-1.434	0.007
	CG9150	-1.630	0.009
Lysosome	CG6656	-1.064	0.008
MAPK signalling pathway	phyl	-2.549	<0.001
	bi	-2.064	0.002
Peroxisome	Sgp	-1.999	0.007
	CG17562	-1.164	0.008
Phagosome	VhaM9.7-a	-2.114	0.001
	Vha68-1	-1.180	0.003
	btv	-2.329	0.001
	Tsp	-1.260	0.002
Phosphatydylinositol	Eip63F-1	-1.574	0.001
	CG34384	-2.605	0.002
TGF-β signalling pathway	Ugt37D1	-1.080	0.006
	dpp	-1.399	0.006
	vis	2.462	0.001
	Actbeta	-2.409	0.004
Wnt signalling pathway	RhoL	-1.187	0.002
	CanB	-1.661	0.001
	wg	-2.696	0.001
	Notum	-2.402	0.001
	drl	-1.079	0.022

Following the same pattern, at 10d p.i. both sexes presented the majority of the top significant DEGs repressed. These genes were mainly related with the metabolic pathways: arginine metabolism, glutathione metabolism, glycolysis and retinol metabolism. These flies also presented a significant repression of the genes related

with endocytosis, lysosome and peroxisom (Figure 54C and Table 32). However, these flies did not showed significant differences on the regulation of Toll-relate genes, which were up-regulated in the group from experiment 1, although females showed high repression of the Imd signalling pathway. These results reinforced the idea that the Toll signalling pathway might be involved in the increased tolerance to the infection with *M. marinum*.

Table 32| Top DEGs for generation 5 of coevolved flies 10d post-infection

10d Males			
Pathway	Gene	Log2FC	Adj.Pval
Arginine metabolism	arg	-1.631	0.001
	CG6726	-1.484	<0.001
	bb8	-2.091	<0.001
	CG5144	-1.264	<0.001
Autohagy	Tango5	-1.084	<0.001
Endocytosis	Hsc70-2	-1.043	<0.001
	Snx1	-1.019	<0.001
Fatty acid degradation	CG4592	-1.347	<0.001
Foxo signalling pathway	Ilp2	1.550	<0.001
Glutathione metabolism	GstE10	-2.224	<0.001
	CG17636	-1.661	<0.001
Glycolysis	CG7069	-2.136	<0.001
	CG9961	-1.249	<0.001
	CG9010	-1.807	0.001
	Hex-t2	-1.590	0.002
	CG7024	-1.084	0.002
Hormone biosynthesis	Eo	-3.660	<0.001
Lysosome	Acph-1	-1.785	<0.001
	CG30160	10.723	<0.001
	Tsp42Eb	-1.827	0.002
mRNA	CG32568	-3.707	<0.001
	Rbp4	-1.208	<0.001
	CG7907	-1.378	0.001
mTOR	mnd	-1.356	<0.001
	CG14812	1.005	<0.001
Oxidative phosphorilation	CG14077	-2.027	0.001
	mt:ATPase6	1.741	0.001
	mt:ND3	1.994	0.001
	cype	1.458	0.001
Peroxisome	CG10097	-2.100	<0.001
	CG17562	-1.372	0.001
	pth	1.547	0.001
	CG5265	-1.337	0.001
Phagosome	Vha36-3	1.135	0.001
	Calr	-1.209	<0.001
Phosphatydylinositol	azot	-4.193	<0.001
	CG17770	-3.834	<0.001
Retinol metabolism	Ugt37A1	-3.497	0.001

10d Females			
Pathway	Gene	Log2FC	Adj.Pval
Apoptosis	Egfr	-1.275	0.004
Arginine metabolism	Oat	-1.938	<0.001
	PH4alphaEFB	-1.897	<0.001
	arg	-1.592	<0.001
Glutathione metabolism	CG17636	-1.998	<0.001
	CG4829	-1.286	0.001
Hormone metabolism	CG10352	-3.068	<0.001
	CG2680	-1.972	<0.001
	Jhe	-1.286	0.010
Lysosome	Tsp42Ea	-1.351	<0.001
	Lip4	-1.824	<0.001
	DNaseII	-2.976	<0.001
	CG18278	1.152	0.001
Notch signalling pathway	fng	-1.149	<0.001
	Dl	1.363	0.001
Peroxisome	Cat	-1.377	<0.001
	CG10097	-3.189	<0.001
	CG17562	-1.999	<0.001
	wat	-1.932	<0.001
	Sgp	-3.723	<0.001
	CG10096	-2.276	<0.001
Phagosome	Itgbn	-2.501	0.001
Retinol metabolism	Ugt49C1	-2.396	<0.001
	Ugt317A1	-2.512	<0.001
	mdy	-1.323	<0.001
	Ugt49B1	-1.986	<0.001
	Ugt36D1	-1.270	<0.001
	Ugt36F1	-2.064	<0.001
	Ugt301D1	-1.405	0.001
TGF-β signalling pathway	dpp	-1.153	0.007
Toll and Imd signalling pathways	SPE	-3.190	<0.001
	GNBP3	-1.710	<0.001
	Dif	-1.381	0.001
	BomS1	-3.706	0.002
	BomS2	-2.416	0.003
	imd	-1.030	0.007
	DptB	-4.383	0.007
	BomS3	-2.111	0.008
	DptA	-4.388	0.012
	PGRP-SD	-2.051	0.031
	PGRP-LF	-1.301	0.032
Wnt signalling pathway	ida	1.004	<0.001
	ImgA	1.147	<0.001
	vih	1.047	0.001

6.2.2.4| Differential expression analysis of *D. melanogaster* that coevolved with *M. marinum* and orally treated with hkMm

Finally, we also evaluated the differential expression profile of those flies that had coevolved together with *M. marinum* at the same time that were being orally treated with hkMm. Results revealed that these flies that had coevolved together with the

pathogen (Figure 55A). The GSEA analysis showed that, as happened with coevolved but untreated flies, these seemed to have almost all pathways repressed in comparison with flies from experiment 1, with the same exceptions in the oxidative phosphorylation and the ubiquitin-mediated proteolysis modules (Figure 55B).

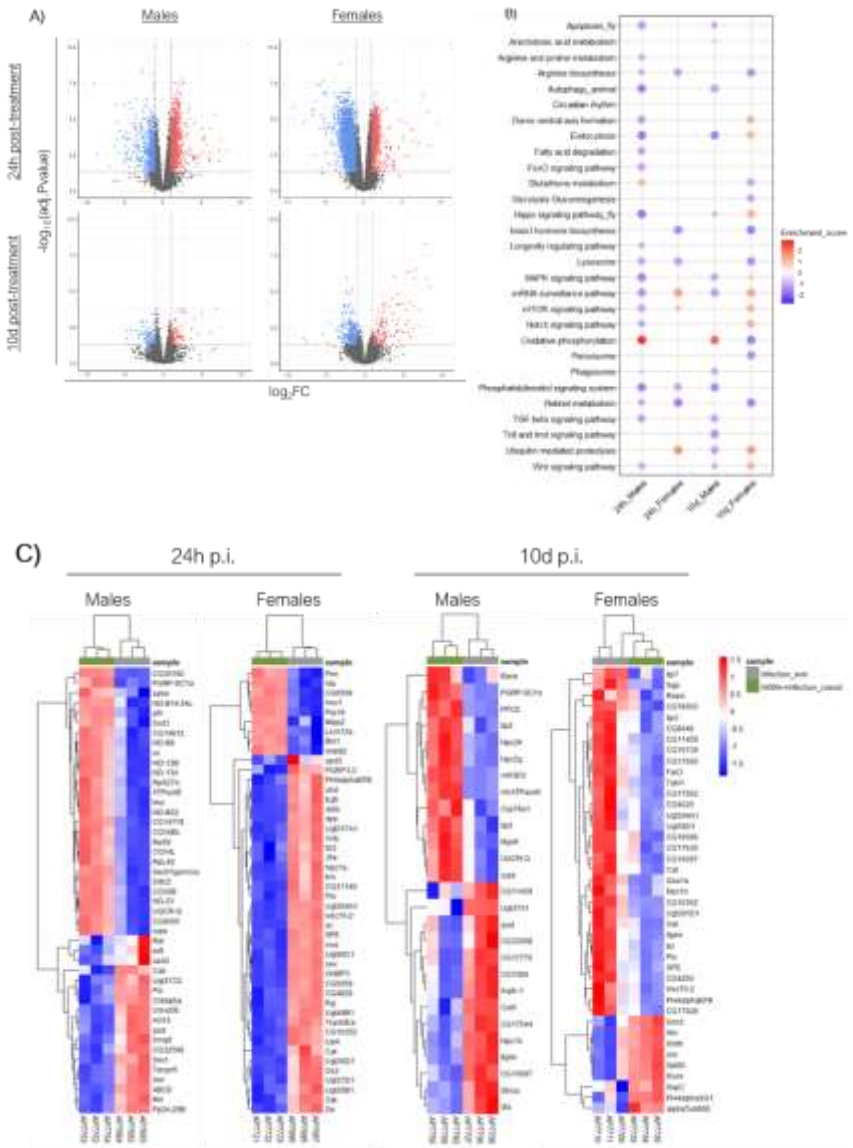


Figure 55| Differential expression analysis of infected flies that coevolved with both *M. marinum* and hkMm. (A) Volcano plots of DEGs (genes with adjusted Pvalue <0.05 were considered significantly down-regulated when the $\log_2$ fold change was <-1 (blue) and up-regulated when the $\log_2$ fold change was >1 (red)). **(B)** Modular transcriptional analysis. Red and blue indicate modules over- or under-abundant compared with control; size of dots represents the reverse of the nominal Pvalue (lower values are represented as bigger dots). Only modules with FDR <25% and nominal Pvalue <0.05 were considered significant. **(C)** Heatmap of the top DEGs. Only genes with adjusted Pvalue <0.05 and absolute $\log_2$ fold change >1 were considered and were then selected based on decreasing p value.

The analysis of the top significant DEGs at 24h p.i. on those flies (Table 33) that coevolved with *M. marinum* and were also orally treated with hkMm showed that both males and females presented down-regulation of endocytosis-, retinol- and innate immune-related genes. On the other hand, males presented high up-regulation of many genes form the oxidative related pathway, while females showed repression of the glutathione metabolism and hormone biosynthesis. Same results as those observed in flies that coevolved only with *M. marinum*.

In both sexes, we also observed a significant repression of Upd3, which it is produced by infected hemocytes to induce tissue repair by the JAK/STAT pathway, as well as mediates the metabolic switch of these phagocytic cells.

Table 33| Top DEGs for generation 5 of flies that coevolved with both *M. marinum* and hkMm 24h post-infection

24h Males			
Pathway	Gene	Log2FC	Adj.Pval
Autophagy	Tango5	-1.074	<0.001
Endocytosis	Snx1	-1.331	<0.001
Foxo signalling pathway	InR	-1.714	0.002
Hippo signalling pathway	Pp2A-29B	-1.108	<0.001
Lysosome	CG30160	12.362	<0.001
	Mvl	-1.306	<0.001
mRNA surveillance	CG32568	-3.538	<0.001
	Smg6	-1.117	<0.001
Oxidative phosphorilation	COX4L	1.603	<0.001
	COX5BL	1.729	<0.001
	cype	2.061	<0.001
	COX6B	1.468	<0.001
	ND-B8	2.386	<0.001
	levy	1.766	<0.001
	ATPsynE	1.555	<0.001
	ND-23	1.502	<0.001
	ND-13B	1.716	<0.001
	ND-B14.5AL	4.113	<0.001
	UQCR-Q	1.954	<0.001
	ND-13A	1.581	<0.001
	ox	2.139	<0.001
	CG9065	1.728	<0.001
	ND-B22	1.644	<0.001
Peroxisome	Sod1	1.074	<0.001
	CG14778	1.552	<0.001
	pih	2.013	<0.001
	ABCD	-1.038	<0.001
	AOX3	-1.336	<0.001
Phagosome	Sec61gamma	1.399	<0.001
	Sdic2	2.827	<0.001
	Calr	-1.144	<0.001
Phosphatydylinositol	azot	-2.589	<0.001
	Pis	-1.441	<0.001
Retinol metabolsim	Ugt37C2	-2.888	<0.001
Toll and Imd signalling pathways	Rel	-1.087	0.041
	PGRP-SC1a	3.118	0.002
	Ank	-1.271	<0.001
	upd3	-2.490	0.023
	sphe	1.017	0.005
mTOR signalling pathway	RpS6	1.193	<0.001
	CG14812	1.245	<0.001
Ubiquitin mediated proteolysis	RpL40	1.618	<0.001
	Ubi-p5E	-1.165	<0.001
	RpS27A	1.419	<0.001
Wnt	Cklalpha	-1.531	<0.001

24h Females			
Pathway	Gene	Log2FC	Adj.Pval
Arginine biosynthesis	PH4alphaEFB	-1.968	<0.001
	Oat	-1.987	<0.001
	Gs2	-1.858	<0.001
Circadian rhythm	vri	-2.148	<0.001
	tim	-2.379	<0.001
	Lsm12a	1.035	<0.001
Endocytosis	Hsc70-2	-2.948	<0.001
Foxo signalling pathway	Cat	-1.564	<0.001
	Egfr	-1.748	<0.001
	CG5059	-1.691	<0.001
Glutathione metabolism	Zw	-1.267	<0.001
	CG4829	-1.563	<0.001
	Mipp2	1.029	<0.001
Hippo signalling pathway	dally	-2.674	<0.001
Hormone biosynthesis	shd	-2.423	<0.001
	CG10352	-2.871	<0.001
	Jhe	-1.701	<0.001
Lysosome	CG6508	7.791	<0.001
	Lip4	-2.195	<0.001
	Tsp42Ea	-1.358	<0.001
	Npc1a	-1.707	<0.001
MAPK signalling pathway	sev	-2.808	<0.001
	dpp	-1.737	<0.001
mRNA surveillance	Wdr82	1.109	<0.001
	Bin1	1.399	<0.001
	Pnn	1.249	<0.001
Notch signalling pathway	fng	-1.454	<0.001
Phosphatydylinositol	CG31140	-5.591	<0.001
	Pis	-1.403	<0.001
Retinol metabolism	Ugt49C1	-2.510	<0.001
	Ugt36D1	-1.567	<0.001
	Ugt37D1	-1.716	<0.001
	Ugt304A1	-3.439	<0.001
	Ugt49B1	-2.239	<0.001
	Ugt35B1	-1.928	<0.001
	Ugt317A1	-2.311	<0.001
	rdy	-1.334	<0.001
Toll and Imd signalling pathways	upd3	-2.496	0.017
	imd	-1.313	<0.001
	SPE	-2.176	0.001
	GNBP3	-1.390	0.001
	PGRP-LC	-1.164	0.013
Ubiquitin mediated proteolysis	fzr2	-2.477	<0.001
	Aos1	1.370	<0.001
	ida	1.105	<0.001
	Prp19	1.032	<0.001

The analysis of the top significant DEGs at 10d p.i. (Table 34) revealed that both sexes still showed repression of the Toll but not the Imd signalling pathway when compared with not coevolved flies, again suggesting a role for the Imd pathway in resistance to *M. marinum* infections.

In addition, both sexes presented several lysosome-related genes highly repressed at this time point, as well as up-regulation of ROS production-related genes. Again, we observed a high up-regulation of the oxidative phosphorylation genes in males and a high down-regulation of the metabolic pathways in females.

Table 34| Top DEGs for generation 5 of flies that coevolved with both *M. marinum* and hkMm 10d post-infection

10d Males			
Pathway	Gene	Log2FC	Adj.Pval
Apoptosis	Strica	-1.084	0.033
Autophagy	Bace	1.736	0.022
Fatty acid degradation	CG17544	-1.083	0.026
Foxo signalling pathway	Ilp5	1.725	0.009
	Ilp2	1.164	0.024
Glutathione metabolism	Mgstl	1.006	0.024
Glycolysis	CG7069	-1.339	0.014
Hippo signalling pathway	dia	-1.056	0.014
Hormone biosynthesis	Cyp18a1	1.138	0.029
Lysosome	Acph-1	-1.310	0.006
	Npc2g	1.141	0.012
	Npc1b	-2.137	0.020
	Npc2h	1.044	0.033
mRNA surveillance	CG32568	-2.207	0.037
Oxidative phosphorylation	mt:ATPase6	1.719	0.004
	mt:ND3	1.703	0.014
	UQCR-Q	1.158	0.020
	cype	1.240	0.020
Peroxisome	CG10097	-1.463	0.034
Phagosome	CG11459	-2.040	0.029
	Itgbn	-1.878	0.009
Phosphatysylinositol	CG17770	-1.739	0.007
	azot	-1.938	0.008
Retinol matebolism	Ugt37A1	-3.520	0.005
ROS production	PPO2	1.830	0.012
Toll signalling pathway	PGRP-SC1a	4.978	<0.001
Ubiquitin proteolysis	Cul6	-1.158	0.018

10d Females			
Pathway	Gene	Log2FC	Adj.Pval
Arginine metabolism	Oat	-1.595	0.002
	PH4alphaSG1	4.473	0.005
	PH4alphaEFB	-1.148	0.030
Autophagy	CG11459	-2.333	0.014
	Gcn2	1.186	0.035
	Spt20	1.183	0.046
Foxo signalling pathway	Cat	-1.615	0.000
	Ilp3	-1.511	0.046
Glutathione metabolism	CG17636	-1.141	0.041
Hormone biosynthesis	CG10352	-2.629	<0.001
	CG15739	-1.260	0.019
Longevity regulation	Ilp7	-1.967	0.002
	Hsc70-2	-1.880	0.006
Lysosome	Gba1a	-2.637	<0.001
	Npc1b	-2.518	<0.001
	CG8646	-1.774	0.005
	CG4250	-1.340	0.029
MAPK signalling pathway	tsl	-1.050	0.016
	cnk	1.055	0.042
Notch signalling pathway	Rbp4	-1.160	0.038
Peroxisome	H	1.154	0.033
	CG17560	-2.429	<0.001
	CG17562	-2.032	<0.001
	CG10097	-2.699	<0.001
	CG4020	-1.320	0.005
	FarO	-1.581	0.012
	Sgp	-2.975	0.016
	CG18003	-1.358	0.020
	CG10096	-1.502	0.036
Phagosome	alphaTub85E	2.453	<0.001
	Itgbn	-1.855	0.033
Phosphatydylinositol	Takl1	-2.371	0.009
	Pis	-1.108	0.002
	CG17028	-1.412	0.009
Retinol metabolism	Ugt301D1	-1.418	0.002
	Ugt36D1	-1.059	0.004
	Ugt304A1	-2.057	0.025
ROS production	Duox	1.338	0.045
Toll signalling pathway	SPE	-1.784	0.027
mTOR signalling pathway	Wnt5	1.183	0.025
Wnt signalling pathway	Axn	1.156	0.013
	SkpC	2.413	0.021

6.3| Discussion

The results from this study provide a new perspective on the possibilities that the *D. melanogaster* model offers in the study of host-pathogen interactions. The knowledge on the evolutive adaptation that a host experiments against pathogenic microbes is of high relevance to understand host-pathogen interactions and, thus, try to develop new host directed therapies. In this study we aimed to determine which genetic changes did *D. melanogaster* experienced throughout several generations of exposure not only to pathogenic mycobacteria, but also to the heat-killed *M. manresensis* (hkMm).

The first aim of this study was to characterize the effect that the oral administration of hkMm have on *D. melanogaster* and how this treatment modifies the innate immune response of the host against mycobacterial infection. Thus, first we showed that the oral administration of hkMm in absence of infection induced a short-time lasting response in *D. melanogaster* mediated by an increase in the oxidative phosphorylation-related genes and up-regulation of Foxo and the signalling pathways involved in tissue repair, cell proliferation and Toll signalling pathways in both males and females. Surprisingly, a similar response was induced by the infection with *M. marinum*.

However, the oral administration of hkMm at the moment of the infection increased the resistance to *M. marinum* in flies. Although both sexes showed common traits due to the treatment, such as significant increased expression of the epidermal growth factor receptor (Egfr), required for stem cell proliferation and gut remodeling upon infection (393), and endocytosis- and phagosome-related genes, some differences were observed between them.

Males show an anti-inflammatory response to the infection mediated by (1) repression of the ecdysone receptor (EcR) which, as explained in study 1, redirects the energy of the host towards immunity over reproduction (297,302,303), (2) reduction on the expression of Foxo, (3) repression of Impl2 and oxidative phosphorylation-related genes, which mainly encode for mitochondrial respiratory chain component. The stimulation of mitochondrial respiration has been linked with increased immunity (i.e. resistance) as direct the metabolic switch in hemocytes that is required for mounting a proper immune response and mitochondrial metabolites act as pro-inflammatory signals and immune cell activators (391). The repression of this process, as well as the down-regulation of Impl2, together with up-regulation of genes related with apoptosis, phagosome and lysosome activity might indicate more efficiency killing of intracellular bacteria without the induction of a pro-inflammatory environment. Finally, treated flies also showed stimulation of the Toll signalling pathway, which as explained in study 1, has been link with impaired metabolic switch in hemocytes (394). Later in

the infection, we could still see this anti-inflammatory response in treated flies, mainly represented by the increased expression of several Turandots, which are induced by the JAK/STAT pathway in response to stress in the fat body, and the repression of Sting. Sting has been linked with the induction of a pro-inflammatory response and also, recently, it has been described a role in lipid metabolism in the fat body (395).

Females also showed repression on the genes encoding for mitochondrial respiration chain machinery and an up-regulation of the Imd and Toll pathway mediated by the down-reglation of the repressors of these pathways, PGRP-SB1 and PGRP-LF. However, females did not show changes in the EcR levels neither a repression of the "selfish immune factors", Upd3 and Impl2, but repression of the Lactate dehydrogenase (Ldh), wich is required hemocytes activation (396,397). However, on the other hand, females presented an up-regulation of the phosphatydylinositol signalling system, which net effect can be either pro- or anti-inflammatory depending of the cellular environment (398). As seen in Study 1, the induction of the Toll signalling pathway directs fatty acids from neutral cellular storage toward phospholipid biosynthesis (374). The reduction of lipid content inside cells might help reduce the intracellular pathogen replication (281). In addition, untreated females have highly repressed several genes encoding for important cellular immune-effectors (Eater, Hml and PPO2) and ROS-production molecules late in the infection, repression that we did not find in treated females.

The second aim of this study was to characterize, phenotypically and genotypically, the evolutive adaptation of the host to further infections with *M. marinum* due to the exposure to the oral treatment with hkMm, to the pathogen itself, and to the combination of both stimuli. *Drosophila* has been commonly used by evolutionary biologist as a model organism to test evolutionary theories often related to adaptation to environmental stresses (399,400), but very few studies have focused on host-pathogen adaptation (401,402) and none in mycobacteria.

In this study we show that *D. melanogaster* increases its tolerance but not its resistance to further infection with *M. marinum* due to the exposure of both the pathogen and the hkMm, while the combination of both stimuli increases both traits only in males. We also show that this adaptation often implies a decrease in the general vigor of the flies. Overall, all tolerant groups presented increased levels of endocytosis and reduced levels of oxidative phosphorylation, peroxisome and glutathione metabolisme, thus, suggesting that these flies generated less oxidative stress in response to infection.

We also show sexual dimorphism in the evolutionary adaptation, as we observed in the analysis of the generation 0. Males exposed either to the infection or the oral treatment show up-regulation of the Toll signalling pathway, although in hkMm

exposed males this induction is immediate after the infection and in males exposed to infection we observe this up-regulateion at 10d p.i.. In both cases we also see increased expression of several genes related with reduction of insulin resistance (InR, Ilp5, Ilp3, Gadd45) and, again, repressed production of Ldh. On the other hand, females exposed to the same two stimuli showed up-regulation of the cellular immune response, with up-regulation of the gene encoding for Yantar, an arginine-rich protein involved in hemocyte differentiation (403), but suppression of the humoral innate immunity. However, pathways related with oxidative stress (oxidative phosphorilation, glutathione metabolism, peroxisome) are repressed.

Those flies exposed over time to the combination of both stimuli also present a clear sexual dimorphism. Males showed not only tolerance, but also resistance to subsequent infection with *M. marinum*. The differential expression analysis showed increased levels of autophagy, endocytosis, phagosome, as in tolerant groups, but also increased expression of genes related with hemocytes activation (ytr, Ldh, Impl2, Upd3). Late in the infection they present increased levels of the Toll signalling pathways, as in tolerant groups, but also increase in the Imd signalling pathways. On the other hand, females presented over-expression of several Toll receptors as well as several genes related with regulation of lifespan, which links with their tolerant phentype, but not induction of the Imd pathway. These results suggest a possible role for the Imd pathway in regulating resistance against *M. marinum* infections xpecially at the extracellular phase of the pathogen, as it has been described other bacteria (375).

Finally, we also wanted to validate the coevolution of the host and the pathogen together. Phenotypic results of the infection of those flies that did not coevolved together with the pathogen suggest that the mycobacteria reduces its virulence without diminishing its ability to replicate within the host, as same levels of resistance for coevolved and stock mycobacteria were observed in both groups at generation 5. However, at generation 10 we see a reduction in the bacillary load of coevolve mycobacteria only in those flies that have not been exposed to any stimuli.

As expected, results from coevolved flies infected with their own isolate compared with infection with a fresh stock of *M. marinum* show that flies become less resistant to mycobacteria from the stock, proving that both the pathogen and the host adapt to each other over the course of generations. Interestantly, flies that coevolved with the pathogen at the same time that were orally treated with hkMm present a tolerance increase, which might indicate that the exposure to hkMm benefits more the host.

The differential expression analysis of these flies compared with flies exposed to *M. marinum* from experiment 1 (infected with fresh stocked *M. marinum*) coincides with the phenotypic results and presented major repression of almost all signalling

pathways. However, males showed up-regulation of the oxidative stress, while females have higher endocytic activity and arginine metabolism. This links with the increase of resistance in both sexes, although they use different metabolic strategies. However, both sexes showed reduced insulin resistance which correlate with their tolerant phenotype.

The comparison among flies that have been exposed to infection and hkMm from experiments 1 and 2 show a similar profile. However, some relevant genes pop-up in co-evolved flies that might explain the increased fitness we observed. Both males and females present repression of Upd3 and both humoral immune pathways. This translates reduced cachexia (272) and reduced acumulation of LD and lower bacillary loads (281). In addition, females present reduced expression of the juvenile hormone, that promotes oogenesis and inhibits immunity (290). These results link with their tolerant and resistant phenotype.

Overall, data suggest that the Toll signalling pathway might play a dual role in the tolerance and resistance of *D. melanogaster* to *M. marinum* infection by reducing the oxidative stress in hemocytes, althoug at expenses of reducing their bactericidal activity, while also reducing the LD accumulation inside the cell and, thus, imparing the replication of the bacilli. In addition, we have also shown the link and the importance of a good balance between metabolism and immunity in the evolutionary adaptation to infections, as well as the role of the sex of the host. Although both males and females had similar traits, they also showed several differences in the adaptation mechanisms. However, it has become clear that an intermediate time point in the infection might be crucial to draw a clearer picture of the mechanisms of adaptation of *D. melanogaster*.

7| Final remarks

The fruit fly *Drosophila melanogaster* is a whole-animal model system that has been highly used for the understanding of fundamental principles of genetics and regenerative biology as well as for human diseases and drug discovery for over a century. The adult fly is not so different from higher organisms and has conserved structures that perform the equivalent function of the mammalian organs and also shares several key pathways and transcriptional regulators that are crucial for development, metabolism and immunity. Lately, with the increasing interest on finding new animal models that might help reduce, refine and replace the current mammal models, *Drosophila melanogaster* has begun to be considered an essential tool for the study of several human infections, including mycobacterial infections.

Classically, studies on infectious diseases and the mechanism of action of possible new prophylactic and therapeutic compounds, have been focused on the adaptive branch of immunity. However, the innate immune response plays a crucial role in the fight against invading microbes and, thus, might be a good target in the development of new Host Directed Therapies (HDT).

The use of *Drosophila melanogaster* as a model has provided a huge insight on the mechanisms of action of the innate immunity, as insects rely solely on this type of response thus avoiding the variability that adaptive mechanisms imply. Also due to the high homology of the genes that determine it with humans; approximately 75% of human disease genes have homologs in *D. melanogaster*. As in vertebrates, *Drosophila*'s immune system is also divided into humoral and cellular responses.

In this regard, we have stablished a standardized *in vivo* model with the pathogenic mycobacteria *M. marinum* in which we characterise the survival rate, the bacillary load and the levels of gene expression. We have also shown the importance of the impl2/upd3 axis in mycobacterial infection, showing that controlled production of these factors is important for building an appropriate immune response, but an excessive and permanent production is ultimately detrimental to the host and appears to favour intracellular replication of the mycobacterium and, thus, proving the link and the importance of a good balance between metabolism and immunity in the fight against mycobacterial infections.

We have also shown the importance not only of the sex of the host but also of its reproductive status. We have shown that the possibility of reproduction has a different effect in males than in females on the pattern of antimicrobial peptide secretion, on the regulation of the Impl2/Upd3 axis and on hormone levels measured with the ecdysone receptor (EcR), which regulates the host's energy to be used for immunity rather than reproduction.

In this study we have also shown that *Drosophila melanogaster* can be a good model for the study of new treatments that might help reduce and refine the use of animal

models in this field. In our case, we have focused on the oral administration of hkMm at different final doses, demonstrating that this treatment induces a change in the innate immune response of flies and that acts in an unspecific manner, protecting the host not only from infection by a different mycobacterium, but also influencing the impact of other gram-positive and gram-negative bacteria. Moreover, we have also proven that the importance of sex also extends to the effect of oral administration of hkMm and is therefore a very relevant factor to consider when designing future *in vivo* experiments for the evaluation of new treatments.

Finally, we have focused on the importance of the innate immune response in the evolutionary adaptation of the host against mycobacterial infections. We have probed that *D. melanogaster*, an organism that only relies on innate immunity, is able to adapt and to become more tolerant to mycobacterial infections by modifying its immune and metabolic responses to infections. Again, we demonstrated the importance that the sex of the host plays in this evolutionary adaptation as males and females followed different strategies. In addition, we have also assessed the importance that coevolution has both in the host and in the pathogen. Our results probe that when both evolve together over generations, the pathogen become less virulent while the host improve its ability to kill the pathogen without losing fitness. However, when we add the oral treatment with hkMm to the equation, this probes to be beneficial for the host, as they increase its ability to reduce the bacillary load at the same time that it increases their fitness.

In conclusion, this thesis is of relevance due to the characterization of mycobacterial infections in the *D. melanogaster* model and for pointing out the importance of sex in the infectious diseases field. Moreover, this work opens a window to continue using this animal model to study the importance of sex and metabolism in other infectious diseases, as well as in the field of developing new therapeutic strategies and new host directed therapies against infectious diseases.

8| Conclusions

1. *Drosophila melanogaster* is a suitable model for the study of the innate immune response against mycobacterial infections.

2. The systemic infection with *Mycobacterium marinum* kills flies in a dose-dependent manner, while the infections with this mycobacterium it is not stablished by natural ingestion.

3. The innate immune response of *D. melanogaster* is no-species-specific and the ability to replicate within the host has a key role in the virulence of mycobacteria.

4. The reproductive status of the host directly affects the tolerance and resistance to mycobacterial infections in a different manner depending on the sex of the host.

5. The basal levels of ecdysone receptor determine its induction upon infection. In general, flies with higher basal levels of EcR induce higher production of Diptericin after infection.

6. Variance in the expression of Upd3 is key in the development of resistance to *M. marinum* infections in *D. melanogaster*.

7. Increased levels of Impl2 upon infection increases resistance to *M. marinum* infections at expenses of tolerant reduction.

8. The Toll pathway seems to be a dual player increasing both the tolerance and resistance to *M. marinum* infections in *D. melanogaster*.

9. The oral administration of heat killed *Mycolicibacterium manresensis* (hkMm) induces an unspecific innate immune response that might protect the host against a wide range of bacterial pathogens.

10. The protection given by the oral administration of hkMm is sex-dependant, with females needing a higher temporary dosage to induce the same level of protection as males.

11. The oral administration of hkMm induces a Nox-dependant production of ROS, resembling the activity triggered by normal gut microbiota.

12. The oral administration of hkMm induces resistance to mycobacterial infections in both males and females flies via different mechanisms. Males enhance the metabolic activation in hemocytes, while females reduce the formation of lipid droplets.

13. Evolutionary adaptation increased phagocytosis and endocytosis and reduces oxidative stress in response to infections with *M. marinum*.

14. A sexual dimorphism was also observed in evolutionary adaptation. Males favoured the humoral innate immunity and reduced insulin resistance, while females prioritized the cellular immune response and increased expression of genes related with longevity regulation.

15. An Imd-dependent immune response correlates with increased resistance to infections with *M. marinum*.

16. The *D. melanogaster-M. marinum* coevolution reduces the virulence of the pathogen.

17. The oral administration of hkMm benefit the tolerance of the host in the coevolution with the pathogen.

9| Bibliography

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