The reproductive status of the host determines the tolerance and resistance to *Mycobacterium marinum* infection in *Drosophila melanogaster*

Marta Arch[1,2], Maria Vidal[1,4], Esther Fuentes[1-3], Anmaw Shite Abat[1,6], Pere Joan Cardona[1-5*]

[1] Tuberculosis Research Unit, Germans Trias i Pujol Research Institute (IGTP), Badalona, Catalonia, Spain.
[2] Comparative Medicine and Bioimage Centre of Catalonia (CMCiB), Germans Trias i Pujol Research Institute (IGTP), 08916 Badalona, Catalonia, Spain.
[3] Microbiology Department, Laboratori Clínic Metropolitana Nord, Germans Trias i Pujol University Hospital, Badalona, Catalonia, Spain
[4] Genetics and Microbiology Department, Universitat Autònoma de Barcelona, Bellaterra, Catalonia, Spain
[5] Centro de Investigación Biomédica en Red en Enfermedades Respiratorias (CIBERES), Instituto de Salud Carlos III (ISCIII), Madrid, Spain
[6] Department of Veterinary Paraclinical Studies, University of Gondar, Gondar, Ethiopia.

*** Correspondence:**
Pere-Joan Cardona
pj.cardona@gmail.com

Keywords: *Drosophila melanogaster*, immunity, sexual dimorphism, reproductive status, mycobacterial infections, *Mycobacterium marinum*

Abstract

Both the sex and the reproductive status of the host have a major impact on the regulation of the immune response against infection. Due that *Drosophila melanogaster* has become a powerful model for study such interactions, we wanted to understand whether the sex or the reproductive status has an impact on the tolerance or resistance of the host to the in the model of systemic *Mycobacterium marinum* infection. We measured gene expression by RT-qPCR of immune genes, *diptericin* and *drosomycin*, as well as host survival and the bacillary load at time of death. We also assessed the impact of metabolic (by expression of *upd3* and *impl2*) and hormonal (by *ecR* expression) regulation in the protection against infection. Data showed that resistance increased in actively mating males and females, and also in mated females while reducing the tolerance to infection. The data also suggest the Toll and IMD pathways determine tolerance and resistance, respectively, while basal levels of *ecR* favors the stimulation of the IMD pathway. A time related role has been found for *upd3* expression, linked to the increase or decrease in the mycobacterial load at the beginning and at the end of the infection, respectively. Finally, *impl2* expression has been related to the increase of resistance only in non-actively mating males. The results obtained allows further interpretation of the results when assessing the differences between sexes and highlights the role of the reproductive status in *D. melanogaster* to face infections, since our data demonstrated their importance to determine resistance and tolerance against *M. marinum* infection.

1. Introduction

Reproduction and immunity are resource-intensive traits in their deployment and maintenance, thus, a trade-off exists between immunity and reproduction when the resources are limited [1]. This trade-off has been documented in both mammals and non-mammals [2–5]. Its study is important to shed light on the impact of directional selection and evolution in the maintenance or the variation of these traits, thus helping to better characterize the relationship between these two physiological mechanisms [6].

In this regard, *Drosophila melanogaster* is a powerful model organism to study the interaction between reproduction and immunity due to its already well-studied courtship ritual and its well-characterized immune system [7]. Immunity in *D. melanogaster* relies solely on innate immune responses, nevertheless, there are a lot of aspects of this innate immunity that are conserved between flies and mammals, such as the organization of the immune response into humoral and cellular arms, and the presence of NF-κB family transcription factors and signal transduction pathways [8]. The humoral response is mainly based on the synthesis of antimicrobial peptides (AMPs) in the fat body of the fly. This process is regulated by two signalling pathways: the Toll and the immune deficiency (Imd) pathways, which activate different NF-κB-like factors and induce the production of Drosomycin and Diptericin (as the main AMP of each pathway, respectively) [9,10]. Flies also have a simpler but complete JAK/STAT pathway, which regulates a wide range of biological processes in the fruit fly, including immunity [11,12]. Regarding cellular immunity in *Drosophila,* it is mediated by hemocytes. When a septic injury, tissue damage, or exposure to lipopolysaccharides (LPS) occurs, hemocytes trigger the expression of Unpaired (Upd) 3, which activates the JAK/STAT pathway in the fat body, which in turn contributes to the humoral response development and leads to upregulation of immune genes [11,13].

It is well known that hormones and metabolism, as well as circadian cycles, have central roles in the regulation of systemic physiology, including immunity, in both insects and mammals [14–16]. The metabolic homeostasis in insects is mainly regulated by the insulin/insulin-like signalling (IIS) pathway. In *D. melanogaster* the activation of this pathway leads to the storage of energy in form of triglycerides in the fat body. Interestingly, after an immune stimulus, the immune pathways interact with the IIS pathway in the fat body resulting in loss of energy storage and suppression of host growth [17–19]. This fact has been recently described as the "selfish immune system theory", which is 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, thus inhibiting insulin signalling during infection [20–23]. Authors propose the insulin/IGF antagonist Imaginal morphogenesis protein late 2 (Impl2) – which has been identified as a cancer-derived cachectic factor in flies –, and Upd3 as potential SIFs in *Drosophila* [24–26].

Hormones also play an important role in the regulation of immunity [14]. Both in mammals and insects exists a sexual dimorphism affecting immunity (survival, pathology, bacterial load and activity in response to infections) at basal conditions, after a pathogenic challenge, and even upon ageing [27,28]. Few studies about the topic 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*, *Staphylococcus aureus* and *Serratia* infections [29–31]. 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 [32]. In mammals, steroid hormones have been linked with these sex differences, and interactions between these type of hormones and the innate immune system of *Drosophila* have also

been described [33,34]. The steroid hormone Ecdysone is the main regulator of the insect life cycle [35]. In adult flies, Ecdysone is produced in the ovaries after mating, thus showing higher levels in females than in males [36–38]. 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 [39] and the cellular immunity [34,40]. At the same time, Ecdysone levels are regulated by stress signals and it has been related to age-related immune changes, as its depletion increases *Drosophila's* lifespan [41,42].

Nevertheless, it is not only sex that affects immunity, but also reproductive status. In *Drosophila*, mated females showed decreased survival, higher pathogen loads and reduced AMPs production after pathogenic infections [43–45], although mating does not affect the clearance of non-pathogenic *E.coli* [46,47], thus making the trade-off between mating and immunity evident only when the infection is pathogenic [4]. On the contrary, increased resistance to infections correlates with a reduction in egg viability [48], but female flies unable to generate eggs do not have reduced immunity after mating [45] and females fed with yeast *ad libitum* improved both fecundity and resistance to infections proving that reproduction and immunity also compete for energy resources [49]. Along the same lines, males exposed to a higher number of females also showed increased susceptibility to bacterial infections [50]. Conversely, it has been also demonstrated that, despite the significant metabolic cost of infection, flies don't change and even increase the ability to survive infection and clear it [7,51]. Likewise, mated males have shown better survival curves against *Pseudomonas entomophila* infection as well as a better bacterial clearance ability against *Providencia rettgeri* than virgin ones, showing absence of reproduction-immunity trade-off [6].

This sexual dimorphism has been also observed in the pathogenesis of some infectious diseases in humans, including tuberculosis [52] which has been the leading cause of death due to a single pathogen (*Mycobacterium tuberculosis*, Mtb), until being surpassed by the COVID-19 pandemic on 2020 [53]. *Mycobacterium marinum* is the most common pathogenic mycobacteria used for infections in non-mammal animal models because it is a natural pathogen of ectotherms causing a granulomatous infection that highly resembles TB in humans [54–56]. The infection of this mycobacterium in the *D. melanogaster* model has also been well characterized. The microorganism is able to kill flies even with very low initial doses, at the early stages of the infection, as it is able to replicate inside *Drosophila* hemocytes [57]. It has also been described that the formation of lipid droplets inside phagocytic cells derived from the production of Upd3 in these cells benefits the intracellular growth of the pathogen, thus resembling the formation of foamy macrophages in humans. As the infection evolves, *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 IIS pathway [58].

In this study, we have assessed the role of sexual dimorphism and reproductive status (virgin and mated kept alone or together) on the response to the infection by *M. marinum* in *D. melanogaster*. We have also assessed the differences that these features have on the innate immune response triggered by the pathogen by measuring the expression of Toll- and Imd-dependent AMPs, *drosomycin* and *diptericin* respectively. In addition, due to the importance that metabolism has on the *M. marinum* infection in flies [58], we have assessed the expression levels after the infection of *upd3* and *impl2* based on previous studies that described the role that these molecules play in the metabolic regulation of the host during infections [21,59].

2. Materials and methods

2.1. Fly stocks and husbandry

Oregon-R-C wildtype flies were obtained from the Bloomington Drosophila Stock Centre (BDSC, Indiana University) and they belong to stock number #5. Flies were raised on a standard cornmeal medium at 25ºC, 65-70% humidity with a 12h light/dark cycle. Male and female flies were aged for 3-5 days before experimentation.

2.2. Mycobacterial stock preparation and infection

Mycobacterium marinum E11 strain resistant to kanamycin (a kind gift from Wilbert Bitter, Vrije Universiteit Amsterdam) was used [60]. The mycobacterial strain was cultured in 7H9 complete medium complemented with 20µg/ml of antibiotic and placed at 30ºC with constant agitation (170rpm) for 10 days (until an OD_{600nm} of 1.5). The cultures were centrifuged for 5min at 4000g, resuspended in phosphate buffered saline (PBS) with 0.2% Tween 80 and centrifuged 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. Mycobacterial cultures were then aliquoted and frozen at -80ºC. Each stock was tittered after being frozen at least overnight.

For systemic infections, an aliquot was defrosted and centrifuged at 15000g for 5min. Pellet was rinsed twice with sterile PBS and diluted to the proper concentration. Fourteen nanolitres of the diluted mycobacterial solution were injected systemically employing a nano-injector (Nanoject II, Drummond) into the abdomen of anaesthetized flies.

2.3. Experimental design

Flies were divided into three experimental groups: virgin flies, mated flies that were separated by sex after the infection and flies that were allowed to mate throughout the infection (actively mating). Virgin flies were collected at the 2-3h post-eclosion and kept in same-sex groups before and after experimentation. In all experiments, male and female flies were kept in groups of 30 individuals, either only one sex or equally distributed between males and females.

Overall, the experiment consisted of 30 male and 30 female flies per experimental group and 3 biological replicates were performed, with a total of 90 males and 90 females per group. Survival was checked daily and the bacterial load upon death (BLUD) was measured. Dead flies were collected every day, washed with 70% ethanol and rinsed twice with PBS. Each fly was then mechanically homogenized into 200µl of sterile PBS, diluted and plated into 7H10 plates complemented with kanamycin, and incubated for 10 days at 30ºC.

2.4. Tolerance and resistance

Previous studies have defined three defensive strategies against infections: qualitative resistance, as the ability to remain uninfected upon pathogen exposure; quantitative resistance, as the ability the reduce the pathogen load; and tolerance, as the ability of the host to maintain the fitness given a certain pathogen load [61,62]. Given our model of study in which the minimum lethal dose of the pathogen is 1, meaning that a single mycobacterium is able to kill the host, we did not assess qualitative resistance but focused on tolerance and quantitative resistance.

170 Tolerance was defined by the slope of the regression between the host fitness against the pathogen
171 burden [62–67], with more tolerant groups presenting less steep slopes. We used flies' survival rates
172 as an indicator of host fitness. Uninfected flies were included in the analysis to account for the fitness
173 of each group in the absence of infection, commonly referred to as general vigour [62]. On the other
174 hand, resistance was measured as the Y-intercept of the regression line between the bacterial load upon
175 death (BLUD) and the inoculation dose. When slopes are equal, the lower the Y-intercept the more
176 resistant the group is [62,64,65].

177 Although the best way to measure these traits would be by evaluating the specific range of bacterial
178 load in the host during the infection [65], in our model is not possible to recover the bacillary load of
179 live flies. Thus, we used the inoculation dose as a controlled measure of the pathogen burden because
180 it highly correlates with both survival times and bacterial load upon death.

181 **2.5. Gene expression by quantitative real-time PCR**

182 The differential gene expression was assessed at 24 hours, 5 days and 10 days post-infection. Overall,
183 a total of 9 pools of 3 flies each were analysed per sex, group and time point, obtained from the three
184 independent experiments. All flies were preserved in RNA later (Fisher Scientific, S.L.) and stored at
185 -80ºC before RNA extraction. Total RNA was extracted from flies using the MasterPure™ Complete
186 DNA and RNA Purification Kit (Lucigen) and converted to cDNA with the PrimeScript RT Master
187 Mix (Takara), following the instructions of the kit's manufacturers for both procedures. cDNA was
188 stored at -20ºC until used.

189 Quantitative real-time PCR (rt-qPCR) was carried out using the KAPA SYBR® FAST Mix (Sigma)
190 on a LightCycler 480 (Roche Diagnostics) system. RT-qPCR conditions were 95ºC for 5min followed
191 by 40 cycles of 95ºC for 10s and 60ºC or 62ºC for 20s. The specificity of each pair of primers was
192 checked by melting curve analysis (95ºC for 5s, 65ºC for 1min and a continuous rise in temperature to
193 97ºC at 2.5ºC/s ramp rate followed by 97ºC for 30s). To check reproducibility, each assay was
194 performed with technical triplicates for each biological sample. The relative transcripts levels of target
195 genes were calculated using the $2^{-\Delta\Delta CT}$ method [68] with r*pl32* used as the reference gene for
196 normalization of target gene abundance.

197 **2.6. Statistical analysis**

198 Tolerance and resistance analyses were carried out in GraphPad Prism (version 9.0.0). Survival curves
199 were analysed using Log-rank (Mantel-Cox) test. CFU counts and gene expression data were checked
200 for normality with the Shapiro-Wilk normality test and analysed using one-way ANOVA for multiple
201 comparisons, t-test for single comparisons with normally distributed groups and Mann-Whitney test
202 for non-normally distributed groups. The principal component analysis (PCA) was performed with the
203 Factoextra package in R (version 4.0.1). A linear regression model was used to analyse the tolerance
204 and resistance. Slopes and Y-intercepts were analysed for statistically significant differences with the
205 Extra sum-of-squares F test. Significant differences are as follow: *$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$ and
206 ****$p \leq 0.0001$ (same for #).

207

208

209

3. Results

3.1. The effect of reproduction in tolerance and resistance to *M. marinum* infections is sex-dependent

Our study revealed no significant differences in the general vigour of males, independently of their reproductive status. Instead, results showed that the main difference was due to males being reproductively active, rather than having previously mated or not. Thus, males kept together with females were less tolerant but more resistant to the infection by *M. marinum* compared to males kept alone and virgin males (Figure 1a – Table 2).

On the other hand, the general vigour was significantly lower in virgin females, when compared with both mated groups. In addition, results suggested that females' response to infection was more conditioned by whether they have mated or not, regardless of whether they were reproductively active at the time of infection or not. Thus, virgin females were more tolerant but less resistant to the infection, compared to both mated groups (Figure 1b – Table 2).

When we compared tolerance and resistance of males and females for each reproductive status independently, data showed that virgin flies differed neither in general vigour nor in overall tolerance to the infection, but males were significantly more resistant (Figure 1c – Table 3). The same pattern was observed in flies that were kept together in equal proportions throughout the procedure: both males and females had the same general vigour and the same tolerance levels, but males showed up to be more resistant (Figure 1e – Table 3). On the contrary, when flies had mated but were kept separated by sex after the infection, females showed increased general vigour and resistance, but reduced tolerance to the infection with *M. marinum* (Figure 1d – Table 3).

3.2. The humoral innate immune response against *M. marinum* infection depends on the reproductive status of the host

The differences in the innate immune response triggered by the infection in each reproductive status were measuring the expression of the antimicrobial peptides Diptericin and Drosomycin in flies infected with 500 CFU of *M. marinum* at different time points (Figure 2a). These AMPs are regulated by the Imd and the Toll pathways, respectively. In males, only those kept together with females showed immediate significant production of Diptericin, while the other groups showed a delayed production of this AMP. Moreover, virgin males presented significantly increased production of Drosomycin immediately after the infection, while in males alone this production was delayed, and no significant changes were observed in males kept together with females. In females, we observed similar expression profiles as males for each reproductive status: females kept together with males had immediate production of Diptericin, but not Drosomycin; virgin females had delayed production of Diptericin, but early production of Drosomycin; females alone showed delayed or null production of both AMPs.

These results for the expression levels showed correlation with the phenotypic results obtained previously. We found that the activation of the Toll pathway (production of Drosomycin) correlated with more tolerant but less resistant phenotypes in both males (virgins and alone) and females (virgins). In addition, the more sustained was this activation (males alone) the lower resistance was observed. On the other hand, we also observed that while early activation of the Imd pathway correlated with the more resistant groups (males and females together), late or null activation of this pathway seemed to be linked with reduced resistance with the exception of females alone. These correlations were also observed when comparing the expression levels between males and females for each reproductive status (Supp. Figure 1a). We found that the less resistant groups (virgin females, females kept together

253 with males, and males alone) showed significantly increased production of both AMPs late in the
254 infection, while the more tolerant group (males alone) had increased production of Drosomycin.
255 Finally, results also suggest that females kept alone after mating may acquire their resistance through
256 a mechanism that does not involve the humoral innate immunity, as they did not show any activation
257 of the Imd pathway throughout the infection.

258 **3.3. The metabolic regulation during infection is altered by the reproductive status of the host**

259 The link with the metabolism, especially with the insulin/insulin-like signalling pathway (IIS), together
260 with the suggested role for Upd3 and Impl2 as "selfish immune factors" (SIFs), prompted us to
261 investigate whether a differential expression of these molecules within the different reproductive status
262 of the host might also influence the tolerance and resistance levels to the infection by *M. marinum*
263 (Figure 2b).

264 In males, both virgins and those kept alone showed significant early overexpression of *upd3*, which
265 was not sustained later in the infection. On the other hand, those males that were kept together with
266 females during the whole procedure presented a significant overexpression of this gene later in the
267 infection. In females, virgins showed a significant early but not sustained overexpression of *upd3*,
268 while females that have mated presented a significantly increased expression of this gene later in the
269 infection whether they were in the presence or absence of males. Overall, these results suggest a dual
270 role for *upd3*, as its early expression correlated with more tolerant but less resistant groups while its
271 expression later in the infection correlated with more resistant and less tolerant groups.

272 Finally, assessing the expression of *impl2* during the *M. marinum* infection we observed no significant
273 differences among females depending on their reproductive status. On the other hand, both virgin males
274 and males alone showed a significant increase in the expression of this gene later in the infection, but
275 not sustained. These results suggested that, in males, the punctual expression of *impl2* might increase
276 tolerance to the infection with *M. marinum*, but not in females.

277 We also compared the expression levels of both genes between males and females for each
278 reproductive status independently (Supp. Figure 1b). We found that females have an overall increased
279 expression of *upd3* compared to males, while *impl2* seemed to only play a role in males with its late
280 production correlating with increased tolerance (males alone).

281 **3.4. Induction of the ecdysone receptor correlated with basal levels prior to the infection in males,**
282 **but not in females**

283 We also evaluated the expression levels of the ecdysone receptor (EcR) due to the tight relationship of
284 the Ecdysone pathway with immunity and reproduction. We measured the relative expression of this
285 gene after the infection and the basal expression levels when injected with PBS for each reproductive
286 status (Figure 3c).

287 We found that virgin males significantly induced the expression of the receptor later in the infection,
288 while males alone did it immediately after. On the other hand, the infection triggered a significant
289 repression of the receptor in those males kept together with females (Figure 3a). We also found that
290 males presented a significant correlation between the basal level of EcR and the induction of this
291 receptor post-infection, in which lower basal levels translated into higher activation of this gene post-
292 infection (Figure 3b and 3c). In females, virgins showed no changes neither in the basal levels nor in
293 the induction of EcR after the infection. Females that had mated showed similar patterns as their males
294 counterparts, while females kept alone showed decreasing induction of EcR when infected, but no

295 significant changes on the basal levels (Figure 3a and 3b). However, the correlation between basal
296 levels and induction of EcR after the infection was not statistically significant in females (Figure 3c).

297 The results also showed a link between the basal expression levels of EcR and the resistance to the
298 infection. Those groups with higher basal expression levels (males and females kept together and
299 females alone) were more resistant to the infection, while those groups with lower basal levels (virgin
300 flies and males alone) were less resistant.

301 When comparing both basal and induction levels between males and females (Supp. Figure 2a), we
302 observed that virgin males presented lower basal levels but increased production of EcR after the
303 infection, while flies separated after the infection showed no significant different basal levels but
304 females increased its production after the infection. Finally, when flies were kept together throughout
305 the procedure, no differences at basal levels among sexes were found, but a late induction in females.

306 **3.5. Gene expression of *D. melanogaster* upon infection shows a marked sexual dimorphism**
307 **marked by Impl2 and Upd3 in males and females, respectively**

308 Finally, we assessed the overall correlation between all the genes studied. The principal component
309 analysis (PCA) (Figure 4a) performed with the relative expression levels of infected flies including all
310 time-points for each condition revealed that, in males, all three groups presented significantly different
311 gene expression profiles, which linked with all three groups presenting different tolerance and
312 resistance profiles as well (Figure 4b). In females, only virgins presented a significantly different gene
313 expression profile (Figure 4b), also linking with the phenotypic results. Finally, the analysis of the
314 variable contribution (Figure 4b) for dimension 1 (PC1), revealed that, in males, this difference was
315 mainly driven by the differential expression of Impl2, together with Drosomycin and Upd2, while in
316 females the main differences were driven by Upd3, together with EcR and Impl2 (Figure 4c).

317 **4. Discussion and conclusions**

318 Typically, laboratory studies have revealed a significant "cost of mating" to *Drosophila* females in the
319 form of reduced longevity. However, here we present that virgin females show a significant decrease
320 in general vigour compared to mated females when uninfected, which is not observed in virgin males.
321 This phenomenon has only been described previously for wild-caught flies [69]. Previous studies
322 described that both males and females reduce their resistance to some infections after mating
323 [45,50,70]. In contrast, data presented herein shows that the opposite happens in both sexes during
324 infection with *M. marinum*. Males increase their resistance to the infection after mating when they are
325 together with females, although at the expense of reducing their fitness, while females increase their
326 resistance to *M. marinum* infection after mating, independently of the presence or absence of males.
327 Overall, phenotypic results from this study show that the sexual dimorphism observed in *D.*
328 *melanogaster* in the outcome of *M. marinum* infection is highly related to the reproductive status of
329 the host. This probes that when assessing the differences in the tolerance and/or resistance of *D.*
330 *melanogaster* to other infections, the hosts' reproductive situation needs to be characterised.

331 Regarding the hormonal levels and their relation with the immune response, our data show that certain
332 EcR levels are required for the flies after the infection, as flies with higher basal levels presented lower
333 induction or repression of the gene post-infection. However, this correlation was only statistically
334 significant in males. Previous studies showed that ecdysone induces the expression of the receptor
335 PGRP-LC and, thus, modulates the Imd pathway [39]. Other studies had related the Imd pathway in
336 the control of resistance to infections [71], while its negative regulation mediates tolerance to infections
337 [72], although any of these were performed in mycobacterial infections. Our data support these

338 findings, as those flies with higher basal levels of EcR show higher expression levels of Diptericin
339 when infected and correlate with the more resistant phenotypes, while flies that did not show early
340 production of this AMP were more tolerant to the infection.

341 Several studies performed with a wide range of pathogens have described the Toll pathway as key in
342 determining resistance to infections [32,73,74], and even to be essential for resistance in males, but not
343 in females [29]. However, no role in increasing tolerance to infection has been described for this
344 pathway previously. In our study, we showed that production of the Toll-dependant AMP, Drosomycin,
345 early in the infection was linked with increased tolerance but reduced resistance to *M. marinum*
346 infection in flies, while their activation in later stages of the infection correlated with increased
347 resistance. Altogether, these data suggest a role for the Toll pathway in determining both tolerance and
348 resistance to *M. marinum* infections. Recent studies have described a Toll-dependent metabolic switch
349 that directs fatty acids from neutral cellular storage toward phospholipid biosynthesis [75], which might
350 reduce the lipid droplet accumulation inside phagocytic cells and, thus, hindered the intracellular
351 replication of *M. marinum*.

352 This study also suggests a dual role for Upd3 in *M. marinum* infections. Our data correlate with
353 previous studies that linked Upd3-deficient flies with a reduction of lipid droplet accumulation within
354 cells and reduced bacillary loads [59], showing that flies with increased expression of Upd3 early in
355 the infection presented higher bacillary loads. Yet, this study also suggests a second role for Upd3, as
356 its production late in the infection correlate with increased resistance, probably due to its role in
357 increasing the metabolism of phagocytic cells [23]. In addition, our data also suggest that the increased
358 resistance in females alone might be driven by Upd3 rather than by innate immune pathways.

359 Finally, the expression of Impl2 during *M. marinum* infections seems to be only relevant in males that
360 are not actively mating and to be related to increased bacillary loads, although further studies should
361 be performed to determine its role during mycobacterial infections in *D. melanogaster*.

362 **Concluding remarks**

363 It has been extensively studied that males and females respond differently to the same infections. Many
364 of these studies have focused on establishing the physiological basis for these differences, but very few
365 have studied how the reproductive status of the host affects males and females and the role it plays in
366 the response to infection. This study aimed to show that the intrinsic differences observed between
367 males and females during the infection by *M. marinum* were tightly related to the reproductive status
368 of the host and that reproduction affects males and females differently and that when assessing the
369 sexual dimorphism of *D. melanogaster,* the hosts' reproductive situation need to be characterised.

370 Here we show that being actively mating (kept together) increased the resistance but reduced the
371 tolerance to the infection in males and females. The same phenotype was found in mated females
372 regardless if they were kept alone or together after mating.

373 The results obtained in this study also suggest a possible role for the Toll pathway in determining
374 tolerance to infection, while it appears that the IMD pathway is associated with increased resistance.
375 Results also showed a correlation between basal levels of EcR and its induction after the infection,
376 suggesting that a certain amount of this receptor is required upon infection. This phenomenon was
377 observed in both males and females in all reproductive statuses, although only in males was statistically
378 significant. In addition, basal levels of EcR also correlated with higher expression levels of Diptericin.

379 Finally, we have also proposed a dual role for Upd3 upon infection with *M. marinum*. Our results show
380 that an early production of this molecule correlate with higher bacillary loads. This might be linked
381 with the Upd3-mediated lipid droplets accumulation inside phagocytic cells, which favours
382 mycobacterial replication. However, the late production of this molecule is associated with lower
383 bacillary loads, most likely due to its role in increasing phagocytic cells metabolism. Finally, we could
384 only validate the role for Impl2 in increasing the tolerance in non-virgin non-actively mating males.

385 **5. Acknowledgements**

386

387 This research has been funded by "La Caixa" Foundation (ID 100010434), under agreement
388 LCF/PR/GN16/10290002, by Spanish Government-FEDER Funds through PI20/01431 grant, and by
389 the "CIBER Enfermedades Respiratorias" Network (CIBERES).

390

391 **Bibliography**

392 1 Stearns SC. Trade-Offs in Life-History Evolution. *Funct Ecol* 1989;**3**:259. doi:10.2307/2389364

393 2 Mills SC, Grapputo A, Jokinen I, *et al.* FITNESS TRADE-OFFS MEDIATED BY IMMUNOSUPPRESSION
394 COSTS IN A SMALL MAMMAL. *Evolution (N Y)* 2010;**64**:166–79. doi:10.1111/J.1558-5646.2009.00820.X

395 3 Abrams ET, Miller EM. The roles of the immune system in Women's reproduction: Evolutionary constraints and
396 life history trade-offs. *Am J Phys Anthropol* 2011;**146**:134–54. doi:10.1002/AJPA.21621

397 4 Schwenke RA, Lazzaro BP, Wolfner MF. Reproduction–Immunity Trade-Offs in Insects.
398 *http://dx.doi.org/101146/annurev-ento-010715-023924* 2016;**61**:239–56. doi:10.1146/ANNUREV-ENTO-
399 010715-023924

400 5 Albery GF, Watt KA, Keith R, *et al.* Reproduction has different costs for immunity and parasitism in a wild
401 mammal. *Funct Ecol* 2020;**34**:229–39. doi:10.1111/1365-2435.13475/SUPPINFO

402 6 Syed ZA, Gupta V, Arun MG, *et al.* Absence of reproduction-immunity trade-off in male Drosophila
403 melanogaster evolving under differential sexual selection. *BMC Evol Biol* 2020;**20**:1–10. doi:10.1186/S12862-
404 019-1574-1/FIGURES/3

405 7 Rose S, Beckwith EJ, Burmester C, *et al.* Pre-copulatory reproductive behaviours are preserved in Drosophila
406 melanogaster infected with bacteria. *Proc R Soc B* 2022;**289**. doi:10.1098/RSPB.2022.0492

407 8 Buchon N, Silverman N, Cherry S. Immunity in Drosophila melanogaster — from microbial recognition to
408 whole- organism physiology. *Nat Rev Immunol* 2014;**14**:796. doi:10.1038/NRI3763

409 9 De Gregorio E, Spellman P, Rubin G, *et al.* Genome-wide analysis of the Drosophila immune response by using
410 oligonucleotide microarrays. *Proc Natl Acad Sci U S A* 2001;**98**:12590–5. doi:10.1073/PNAS.221458698

411 10 Tanji T, Hu X, Weber ANR, *et al.* Toll and IMD Pathways Synergistically Activate an Innate Immune Response
412 in Drosophila melanogaster . *Mol Cell Biol* 2007;**27**:4578–88. doi:10.1128/mcb.01814-06

413 11 Agaisse H, Petersen U, Boutros M, *et al.* Signaling role of hemocytes in Drosophila JAK/STAT-dependent
414 response to septic injury. *Dev Cell* 2003;**5**:441–50. doi:10.1016/S1534-5807(03)00244-2

415 12 Arbouzova N, Zeidler M. JAK/STAT signalling in Drosophila: insights into conserved regulatory and cellular
416 functions. *Development* 2006;**133**:2605–16. doi:10.1242/DEV.02411

417 13 Lagueux M, Perrodou E, Levashina E, *et al.* Constitutive expression of a complement-like protein in toll and JAK
418 gain-of-function mutants of Drosophila. *Proc Natl Acad Sci U S A* 2000;**97**:11427–32.
419 doi:10.1073/PNAS.97.21.11427

420 14 Sternberg E. Neural regulation of innate immunity: a coordinated nonspecific host response to pathogens. *Nat Rev*
421 *Immunol* 2006;**6**:318–28. doi:10.1038/NRI1810

422 15 Lee J, Edery I. Circadian regulation in the ability of Drosophila to combat pathogenic infections. *Curr Biol*
423 2008;**18**:195–9. doi:10.1016/J.CUB.2007.12.054

424 16 Hotamisligil G, Erbay E. Nutrient sensing and inflammation in metabolic diseases. *Nat Rev Immunol*
425 2008;**8**:923–34. doi:10.1038/NRI2449

426 17 DiAngelo J, Bland M, Bambina S, *et al.* The immune response attenuates growth and nutrient storage in
427 Drosophila by reducing insulin signaling. *Proc Natl Acad Sci U S A* 2009;**106**:20853–8.
428 doi:10.1073/PNAS.0906749106

429 18 Suzawa M, Muhammad N, Joseph B, *et al.* The Toll Signaling Pathway Targets the Insulin-like Peptide Dilp6 to
430 Inhibit Growth in Drosophila. *Cell Rep* 2019;**28**:1439-1446.e5. doi:10.1016/J.CELREP.2019.07.015

431 19 Agrawal N, Delanoue R, Mauri A, *et al.* The Drosophila TNF Eiger Is an Adipokine that Acts on Insulin-
432 Producing Cells to Mediate Nutrient Response. *Cell Metab* 2016;**23**:675–84. doi:10.1016/J.CMET.2016.03.003

433 20 Bajgar A, Kucerova K, Jonatova L, *et al.* Extracellular adenosine mediates a systemic metabolic switch during
434 immune response. *PLoS Biol* 2015;**13**. doi:10.1371/JOURNAL.PBIO.1002135

435 21 Almajwal A, Alam I, Zeb F, *et al.* Energy Metabolism and Allocation in Selfish Immune System and Brain: A
436 Beneficial Role of Insulin Resistance in Aging. *Food Nutr Sci* 2018;**10**:64–80. doi:10.4236/FNS.2019.101006

437 22 Dolezal T, Krejcova G, Bajgar A, *et al.* Molecular regulations of metabolism during immune response in insects.

438 *Insect Biochem Mol Biol* 2019;**109**:31–42. doi:10.1016/J.IBMB.2019.04.005

439 23 Bajgar A, Krejčová G, Doležal T. Polarization of Macrophages in Insects: Opening Gates for Immuno-Metabolic
440 Research. *Front Cell Dev Biol* 2021;**0**:103. doi:10.3389/FCELL.2021.629238

441 24 Krejčová G, Bajgar A, Nedbalová P, *et al.* Macrophage-derived insulin/IGF antagonist ImpL2 regulates systemic
442 metabolism for mounting an effective acute immune response in Drosophila. *bioRxiv* 2021;:2020.09.24.311670.
443 doi:10.1101/2020.09.24.311670

444 25 Chakrabarti S, Dudzic J, Li X, *et al.* Remote Control of Intestinal Stem Cell Activity by Haemocytes in
445 Drosophila. *PLoS Genet* 2016;**12**. doi:10.1371/JOURNAL.PGEN.1006089

446 26 Shin M, Cha N, Koranteng F, *et al.* Subpopulation of Macrophage-Like Plasmatocytes Attenuates Systemic
447 Growth via JAK/STAT in the Drosophila Fat Body. *Front Immunol* 2020;**0**:63. doi:10.3389/FIMMU.2020.00063

448 27 Kopp A, Duncan I, Carroll SB. Genetic control and evolution of sexually dimorphic characters in Drosophila. *Nat*
449 *2000 4086812* 2000;**408**:553–9. doi:10.1038/35046017

450 28 Millington JW, Rideout EJ. Sex differences in Drosophila development and physiology. *Curr Opin Physiol*
451 2018;**6**:46–56. doi:10.1016/J.COPHYS.2018.04.002

452 29 Duneau DF, Kondolf HC, Im JH, *et al.* The Toll pathway underlies host sexual dimorphism in resistance to both
453 Gram-negative and Gram-positive bacteria in mated Drosophila. *BMC Biol 2017 151* 2017;**15**:1–17.
454 doi:10.1186/S12915-017-0466-3

455 30 Kutch IC, Fedorka KM. A test for Y-linked additive and epistatic effects on surviving bacterial infections in
456 Drosophila melanogaster. *J Evol Biol* 2017;**30**:1400–8. doi:10.1111/JEB.13118

457 31 Leech T, Evison SEF, Armitage SAO, *et al.* Interactive effects of social environment, age and sex on immune
458 responses in Drosophila melanogaster. *J Evol Biol* 2019;**32**:1082–92. doi:10.1111/JEB.13509

459 32 Chowdhury M, Li C-F, He Z, *et al.* Toll family members bind multiple Spätzle proteins and activate
460 antimicrobial peptide gene expression in Drosophila. *J Biol Chem* 2019;**294**:10172–81.
461 doi:10.1074/JBC.RA118.006804

462 33 Flatt T, Heyland A, Rus F, *et al.* Hormonal regulation of the humoral innate immune response in Drosophila
463 melanogaster. *J Exp Biol* 2008;**211**:2712–24. doi:10.1242/JEB.014878

464 34 Regan JC, Brandão AS, Leitão AB, *et al.* Steroid Hormone Signaling Is Essential to Regulate Innate Immune
465 Cells and Fight Bacterial Infection in Drosophila. *PLOS Pathog* 2013;**9**:e1003720.
466 doi:10.1371/JOURNAL.PPAT.1003720

467 35 Di Cara F, King-Jones K. How clocks and hormones act in concert to control the timing of insect development.
468 *Curr Top Dev Biol* 2013;**105**:1–36. doi:10.1016/B978-0-12-396968-2.00001-4

469 36 Schwedes CC, Carney GE. Ecdysone signaling in adult Drosophila melanogaster. *J Insect Physiol* 2012;**58**:293–
470 302. doi:10.1016/J.JINSPHYS.2012.01.013

471 37 Sieber MH, Spradling AC. Steroid Signaling Establishes a Female Metabolic State and Regulates SREBP to
472 Control Oocyte Lipid Accumulation. *Curr Biol* 2015;**25**:993–1004. doi:10.1016/J.CUB.2015.02.019

473 38 Ameku T, Niwa R. Mating-Induced Increase in Germline Stem Cells via the Neuroendocrine System in Female
474 Drosophila. *PLOS Genet* 2016;**12**:e1006123. doi:10.1371/JOURNAL.PGEN.1006123

475 39 Rus F, Flatt T, Tong M, *et al.* Ecdysone triggered PGRP-LC expression controls Drosophila innate immunity.
476 *EMBO J* 2013;**32**:1626–38. doi:10.1038/EMBOJ.2013.100

477 40 Sorrentino RP, Carton Y, Govind S. Cellular immune response to parasite infection in the Drosophila lymph
478 gland is developmentally regulated. *Dev Biol* 2002;**243**:65–80. doi:10.1006/DBIO.2001.0542

479 41 Hirashima A, Rauschenbach Iy, Sukhanova Mj. Ecdysteroids in stress responsive and nonresponsive Drosophila
480 virilis lines under stress conditions. *Biosci Biotechnol Biochem* 2000;**64**:2657–62. doi:10.1271/BBB.64.2657

481 42 Simon A, Shih C, Mack A, *et al.* Steroid control of longevity in Drosophila melanogaster. *Science (80-)*
482 2003;**299**:1407–10. doi:10.1126/SCIENCE.1080539

483 43 Fedorka K, Linder J, Winterhalter W, *et al.* Post-mating disparity between potential and realized immune
484 response in Drosophila melanogaster. *Proceedings Biol Sci* 2007;**274**:1211–7. doi:10.1098/RSPB.2006.0394

485 44 Short S, Lazzaro B. Female and male genetic contributions to post-mating immune defence in female Drosophila
486 melanogaster. *Proceedings Biol Sci* 2010;**277**:3649–57. doi:10.1098/RSPB.2010.0937

487 45 Short SM, Wolfner MF, Lazzaro BP. Female Drosophila melanogaster suffer reduced defense against infection
488 due to seminal fluid components. *J Insect Physiol* 2012;**58**:1192. doi:10.1016/J.JINSPHYS.2012.06.002

489 46 McKean KA, Nunney L. Bateman's Principle And Immunity: Phenotypically Plastic Reproductive Strategies
490 Predict Changes In Immunological Sex Differences. *Evolution (N Y)* 2005;**59**:1510. doi:10.1554/04-657

491 47 Barnes A, Wigby S, Boone J, *et al.* Feeding, fecundity and lifespan in female Drosophila melanogaster.
492 *Proceedings Biol Sci* 2008;**275**:1675–83. doi:10.1098/RSPB.2008.0139

493 48 Ye Y, Chenoweth S, McGraw E. Effective but costly, evolved mechanisms of defense against a virulent
494 opportunistic pathogen in Drosophila melanogaster. *PLoS Pathog* 2009;**5**:e1000385.
495 doi:10.1371/JOURNAL.PPAT.1000385

496 49 McKean K, Yourth C, Lazzaro B, *et al.* The evolutionary costs of immunological maintenance and deployment.
497 *BMC Evol Biol* 2008;**8**. doi:10.1186/1471-2148-8-76

498 50 McKean KA, Nunney L. Increased sexual activity reduces male immune function in Drosophila melanogaster.
499 *Proc Natl Acad Sci U S A* 2001;**98**:7904. doi:10.1073/PNAS.131216398

500 51 Gupta V, Ali ZS, Prasad NG. Sexual activity increases resistance against Pseudomonas entomophila in male
501 Drosophila melanogaster. *BMC Evol Biol* 2013;**13**:1–11. doi:10.1186/1471-2148-13-185/FIGURES/7

502 52 Neyrolles O, Quintana-Murci L. Sexual Inequality in Tuberculosis. *PLoS Med* 2009;**6**.
503 doi:10.1371/JOURNAL.PMED.1000199

504 53 Global Tuberculosis Report 2021. https://www.who.int/teams/global-tuberculosis-programme/tb-reports/global-
505 tuberculosis-report-2021 (accessed 9 Aug 2022).

506 54 Prouty MG, Correa NE, Barker LP, *et al.* Zebrafish-Mycobacterium marinum model for mycobacterial
507 pathogenesis. *FEMS Microbiol Lett* 2003;**225**:177–82. doi:10.1016/S0378-1097(03)00446-4

508 55 Bouley DM, Ghori N, Mercer KL, *et al.* Dynamic nature of host-pathogen interactions in Mycobacterium
509 marinum granulomas. *Infect Immun* 2001;**69**:7820–31. doi:10.1128/IAI.69.12.7820-7831.2001

510 56 Tobin DM, Ramakrishnan L. Comparative pathogenesis of Mycobacterium marinum and Mycobacterium
511 tuberculosis. *Cell Microbiol* 2008;**10**:1027–39. doi:10.1111/J.1462-5822.2008.01133.X

512 57 Dionne MS, Ghori N, Schneider DS. Drosophila melanogaster is a genetically tractable model host for
513 Mycobacterium marinum. *Infect Immun* 2003;**71**:3540–50. doi:10.1128/IAI.71.6.3540-3550.2003

514 58 Dionne M, Pham L, Shirasu-Hiza M, *et al.* Akt and FOXO dysregulation contribute to infection-induced wasting
515 in Drosophila. *Curr Biol* 2006;**16**:1977–85. doi:10.1016/J.CUB.2006.08.052

516 59 Péan CB, Schiebler M, Tan SWS, *et al.* Regulation of phagocyte triglyceride by a STAT-ATG2 pathway controls
517 mycobacterial infection. *Nat Commun 2017 81* 2017;**8**:1–11. doi:10.1038/ncomms14642

518 60 Weerdenburg EM, Abdallah AM, Rangkuti F, *et al.* Genome-Wide Transposon Mutagenesis Indicates that
519 Mycobacterium marinum Customizes Its Virulence Mechanisms for Survival and Replication in Different Hosts.
520 *Infect Immun* 2015;**83**:1778. doi:10.1128/IAI.03050-14

521 61 Gandon S, Michalakis Y. Evolution of parasite virulence against qualitative or quantitative host resistance. *Proc*
522 *R Soc London Ser B Biol Sci* 2000;**267**:985–90. doi:10.1098/RSPB.2000.1100

523 62 Lefèvre T, Williams AJ, De Roode JC. Genetic variation in resistance, but not tolerance, to a protozoan parasite
524 in the monarch butterfly. *Proc R Soc B Biol Sci* 2011;**278**:751. doi:10.1098/RSPB.2010.1479

525 63 Simms EL, Triplett J. Costs and Benefits of Plant Responses to Disease: Resistance and Tolerance. *Evolution (N*
526 *Y)* 1994;**48**:1973. doi:10.2307/2410521

527 64 Kover PX, Schaal BA. Genetic variation for disease resistance and tolerance among Arabidopsis thaliana
528 accessions. *Proc Natl Acad Sci U S A* 2002;**99**:11270–4. doi:10.1073/PNAS.102288999/ASSET/27071C60-
529 6AAC-4D25-9BBD-B784E2456EA1/ASSETS/GRAPHIC/PQ1422889003.JPEG

530 65 Råberg L, Sim D, Read AF. Disentangling genetic variation for resistance and tolerance to infectious diseases in
531 animals. *Science* 2007;**318**:812–4. doi:10.1126/SCIENCE.1148526

532 66 Blanchet S, Rey O, Loot G. Evidence for host variation in parasite tolerance in a wild fish population. *Evol Ecol*
533 2010;**24**:1129–39. doi:10.1007/S10682-010-9353-X/FIGURES/2

534 67 Howick VM, Lazzaro BP. The genetic architecture of defence as resistance to and tolerance of bacterial infection
535 in Drosophila melanogaster. *Mol Ecol* 2017;**26**:1533–46. doi:10.1111/MEC.14017

536 68 Livak KJ, Schmittgen TD. Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and
537 the 2−ΔΔCT Method. *Methods* 2001;**25**:402–8. doi:10.1006/METH.2001.1262

538 69 Markow TA. "Cost" of virginity in wild Drosophila melanogaster females. *Ecol Evol* 2011;**1**:596.
539 doi:10.1002/ECE3.54

540 70 Fan Y, Rafaeli A, Moshitzky P, *et al.* Common functional elements of Drosophila melanogaster seminal peptides
541 involved in reproduction of Drosophila melanogaster and Helicoverpa armigera females. *Insect Biochem Mol Biol*
542 2000;**30**:805–12. doi:10.1016/S0965-1748(00)00052-7

543 71 Nelson B, Freisinger T, Ishii K, *et al.* Activation of Imd pathway in hemocyte confers infection resistance through
544 humoral response in Drosophila. *Biochem Biophys Res Commun* 2013;**430**:1120–5.
545 doi:10.1016/J.BBRC.2012.12.027

546 72 Prakash A, Monteith KM, Vale PF. Negative regulation of IMD contributes to disease tolerance during systemic
547 bacterial infection in Drosophila. *bioRxiv* 2021;:2021.09.23.461574. doi:10.1101/2021.09.23.461574

548 73 Lau GW, Goumnerov BC, Walendziewicz CL, *et al.* The Drosophila melanogaster Toll Pathway Participates in
549 Resistance to Infection by the Gram-Negative Human Pathogen Pseudomonas aeruginosa. *Infect Immun*
550 2003;**71**:4059. doi:10.1128/IAI.71.7.4059-4066.2003

551 74 Ferreira ÁG, Naylor H, Esteves SS, *et al.* The Toll-Dorsal Pathway Is Required for Resistance to Viral Oral
552 Infection in Drosophila. *PLOS Pathog* 2014;**10**:e1004507. doi:10.1371/JOURNAL.PPAT.1004507

553 75 Martínez BA, Hoyle RG, Yeudall S, *et al.* Innate immune signaling in Drosophila shifts anabolic lipid
554 metabolism from triglyceride storage to phospholipid synthesis to support immune function. *PLOS Genet*
555 2020;**16**:e1009192. doi:10.1371/JOURNAL.PGEN.1009192

556

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

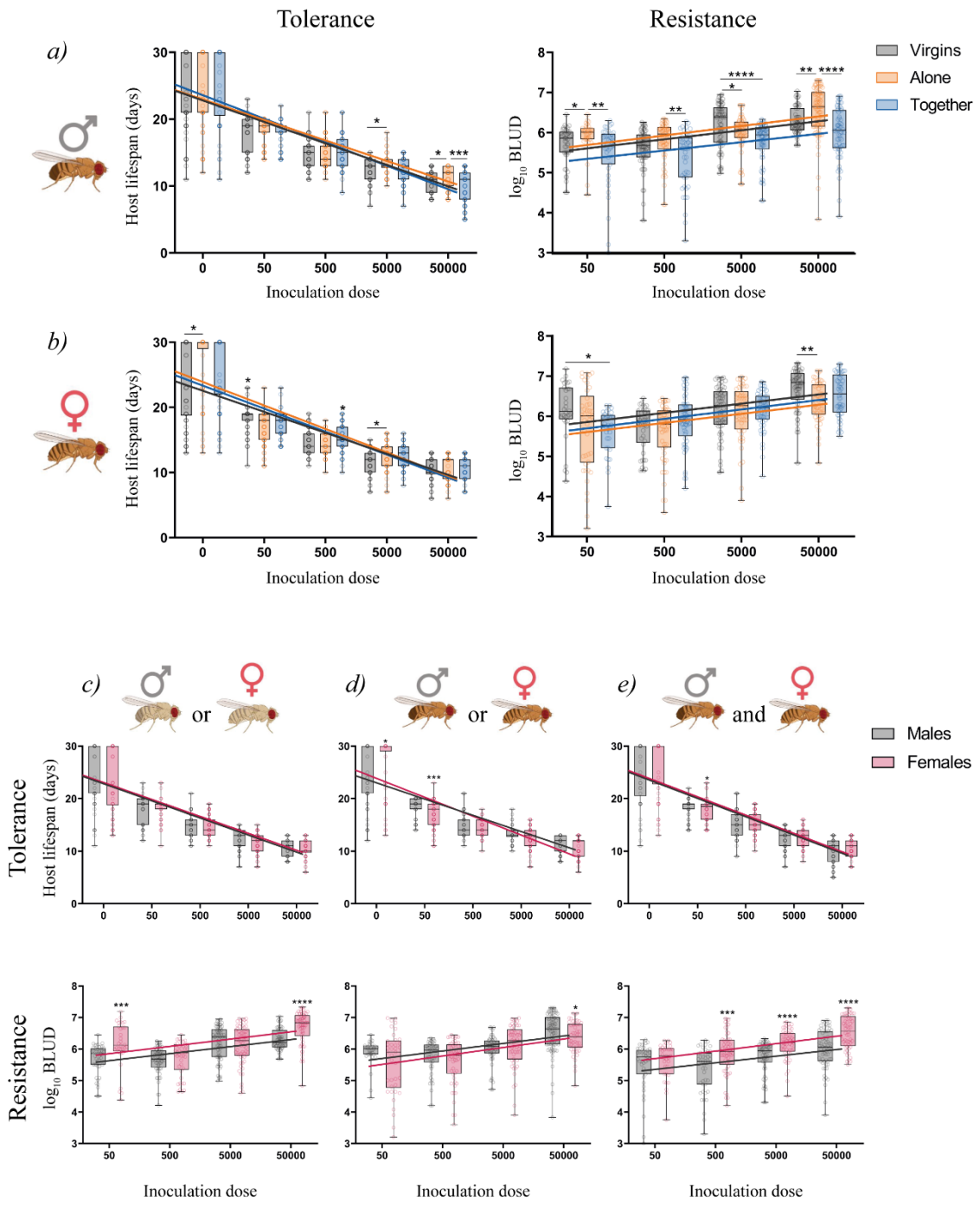

Figure 1| Tolerance and resistance of *D. melanogaster* to *M. marinum* infections depending on the reproductive status or the sex of the flies. (*a*) Males that were not in presence of females (virgins and alone) were more tolerant but less resistant than those kept together with females. (*b*) In females, virgins were more tolerant but less resistant than mated females, independently of the presence or absence of males. When comparing both sexes for each reproductive status, we observed that males were more resistant when virgins or together with females, although no differences in tolerance were found in these groups (*c* and *e*). When flies were kept alone after infection, females showed higher general vigour and resistance, but lower tolerance to infection (*d*). 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).

Table 2| Analysis of tolerance and resistance to *M. marinum* infections depending on the reproductive status. Significance (p<0.05) is indicated by asterisks. Slopes for resistance were not statistically significant

Males			
Tolerance			
Group	Slope	Comparison	p value
Virgins	-3.149	Alone	0.3492
Alone	-3.014	Together	0.0026**
Together	-3.447	Virgins	0.0447*
Resistance			
Group	Y-inter	Comparison	p value
Virgins	5.138	Alone	0.0325*
Alone	5.196	Together	<0.0001****
Together	4.913	Virgins	<0.0001****
Females			
Tolerance			
Group	Slope	Comparison	p value
Virgins	-3.165	Alone	0.0175*
Alone	-3.536	Together	0.6273
Together	-3.465	Virgins	0.0484*
Resistance			
Group	Y-inter	Comparison	p value
Virgins	5.366	Alone	<0.0001****
Alone	4.947	Together	0.2354
Together	5.100	Virgins	0.0067*

Table 3| Analysis of tolerance and resistance to *M. marinum* infections depending on the sex. Significance (p<0.05) is indicated by asterisks. Slopes for resistance were not statistically significant.

Tolerance			
Group	Slope males	Slope females	p value
Virgins	-3.171	-3.142	>0.9999
Alone	-3.014	-3.505	0.0008***
Together	-3.447	-3.351	0.5099
Resistance			
Group	Y-inter. males	Y-inter. females	p value
Virgins	5.138	5.366	<0.0001****
Alone	5.196	4.947	0.0288*
Together	4.913	5.196	<0.0001****

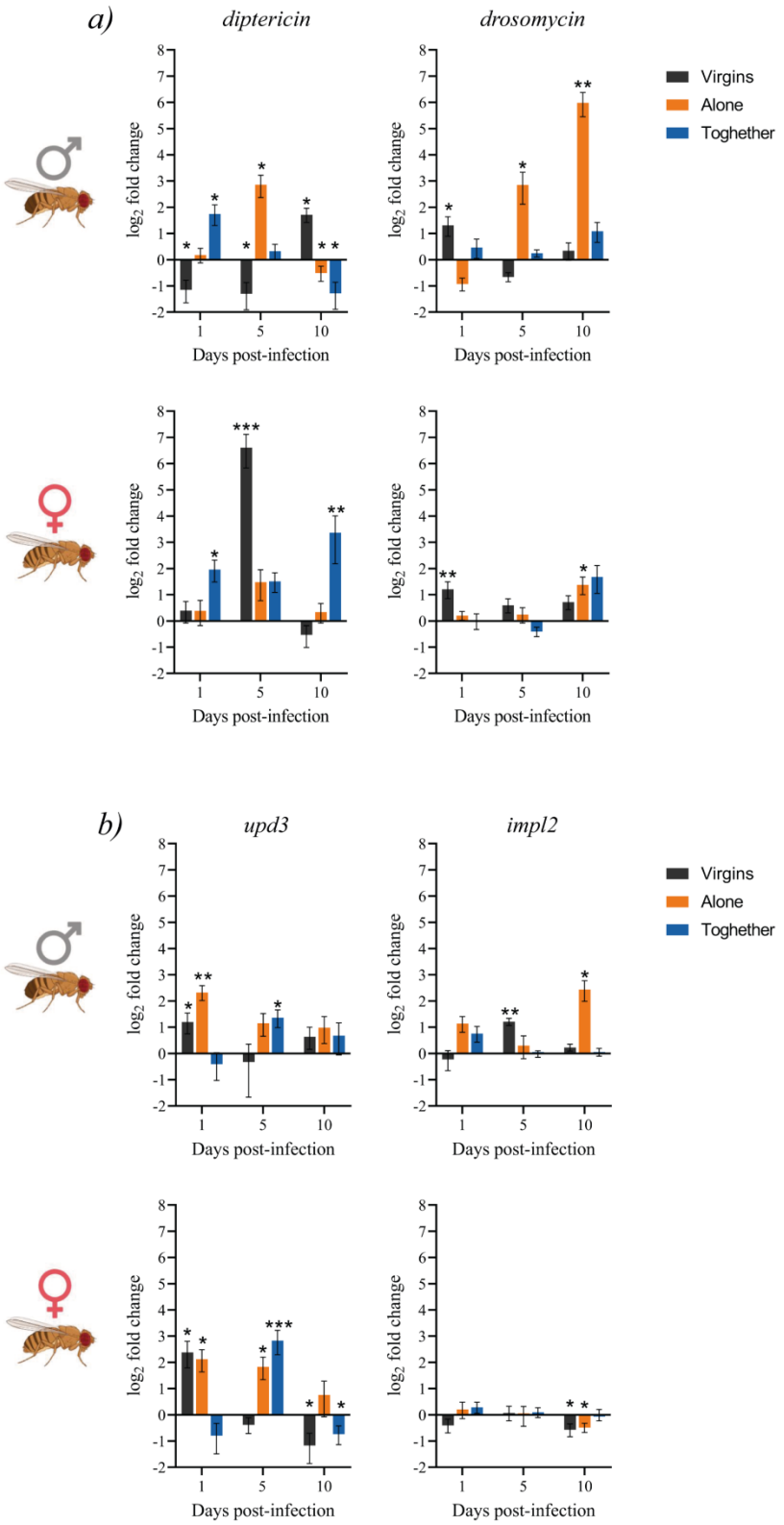

Figure 2| Expression of innate immunity (*a*) and metabolic (*b*) genes during the infection in males and females depending on their reproductive status. Gene expression relative to the internal control gene *rpl32* was quantified in 9 replicate pools of 3 males or females exposed to the infection with *M. marinum* relative to their expression in uninfected controls. Each group at each time-point was compared to its relative control independently (the line in each graph represents the controls' relative expression with a $\log_2$ fold change of 0). Data was analysed for normality and significant differences were represented as follow: *$p \leq 0.05$, **$p \leq 0.01$ (Welch's corrections for normally distributed data and Mann-Whitney test for not normally distributed data).

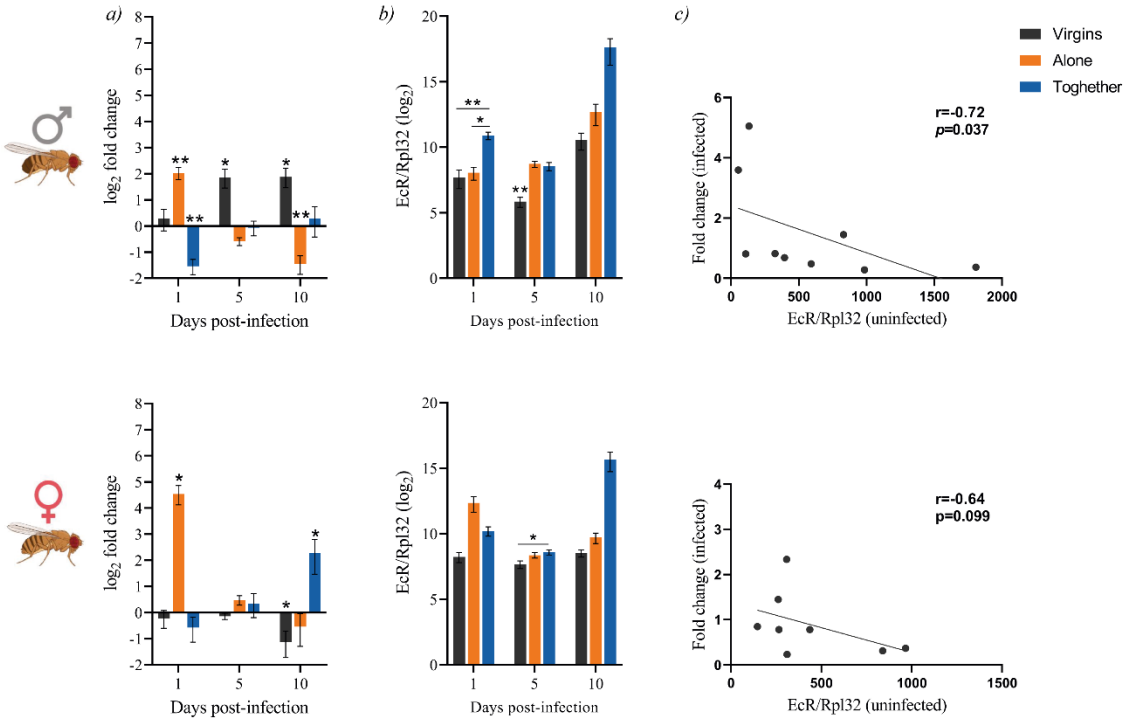

Figure 3| Expression of the ecdysone receptor (EcR) in males (up) and females (down) depending on their reproductive status after the infection (*a*). Gene expression relative to the internal control gene *rpl32* was quantified in 9 replicate pools of 3 males and 3 females each exposed to the infection with *M. marinum* relative to their expression in uninfected controls. Each group at each time-point was compared to its relative control independently (the line in each graph represents the controls' relative expression with a $\log_2$ fold change of 0). 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). Basal expression levels of the EcR gene in uninfected flies (*b*) 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. Data was analysed for normality and significant differences were represented as follow: *p≤0.05, **p≤0.01 (Kruskal-Wallis test). Finally, the correlation between the basal levels and the fold change after infection was performed using the nonparametric spearman correlation test (*c*).

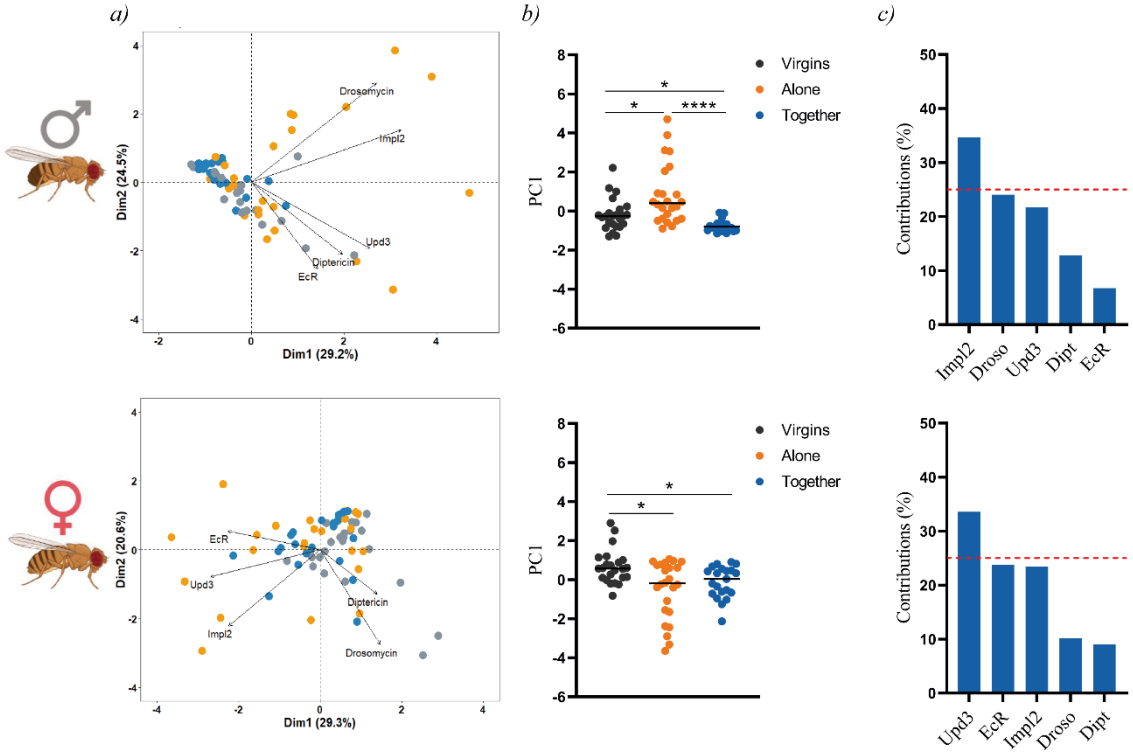

Figure 4| Heterogeneity of gene expression among flies with different reproductive status. Principal component analysis (PCA) based on expression of genes of interest in males and females infected with M. marinum at all time-points (*a*), PC1 scores (*b*) and variable contribution to PC1 (*c*). Each circle represents an individual and lines are means. Statistically significant differences were represented as follow: *p≤0.05, ****p≤0.0001 (Kruskal-Wallis test).