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

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Abstract

Both the sex and the reproductive status of the host have a major impact on the regulation of the immune response against infection. The fruit fly *Drosophila melanogaster* has become a powerful model for study such interaction due to its well-characterized hormonal, metabolic and immune traits. In order to further characterize this in mycobacterial infections, we tested the systemic *Mycobacterium marinum* infection of *D. melanogaster*. We wanted to understand whether the sex or the reproductive status has an impact on the tolerance or resistance of the host to the infection. We measured gene expression by RT-qPCR of immune genes (*diptericin* and *drosomycin*) as well as host survival and bacillary load at time of death. We 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 the effect of reproductive status (virgin and mated kept alone or together) in tolerance and resistance to infection is sex dependent. We found differences in protection according to the levels of expression in immune, metabolic and hormone-related genes which also showed differences between sex and reproductive status, highlighting the expression of *impl2* in males and *upd3* in females to face the infection. The results obtained allows further interpretation of the results when assessing the differences between sexes in *D. melanogaster* for other infections, since data demonstrated the importance of the reproductive status in 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 along time 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 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 potentials SIFs in *Drosophila* [24–26].

Hormones also play an important role in the regulation of immunity [14]. Both in mammals and insects it 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* lifespan [41,42].

Nevertheless, it is not only sex 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 [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]. In 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 to 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 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 benefit the intracellular growth of the pathogen, thus resembling to 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) has 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].

128 ## 2. Materials and methods

129 ### 2.1. Fly stocks and husbandry

130 Oregon-R-C wildtype flies were obtained from the Bloomington Drosophila Stock Centre (BDSC,
131 Indiana University) and they belong to stock number #5. Flies were raised on a standard cornmeal at
132 25ºC, 65-70% humidity with a 12h light/dark cycle. Male and female flies were aged for 3-5 days
133 before experimentation.

134 ### 2.2. Mycobacterial stock preparation and infection

135 The mycobacterial strain used in this study is *Mycobacterium marinum* E11 strain resistant to
136 kanamycin (a kind gift from Wilbert Bitter, Vrije Universiteit Amsterdam) [60]. The mycobacterial
137 strain was cultured in 7H9 complete medium complemented with 20µg/ml of antibiotic and placed at
138 30ºC with constant agitation (170rpm) for 10 days (until an OD_{600nm} of 1.5). The cultures were
139 centrifuged for 5min at 4000g, resuspended in phosphate buffered saline (PBS) with 0.2% Tween 80
140 and centrifuge again for 5min at 500g to remove clumps. Supernatants were transferred to a new tub,
141 centrifuged for 5min at 4000g and resuspended in 1ml of 7H9 with 15% glycerol. Bacterial cultures
142 were then aliquoted and frozen at -80ºC. Each stock was tittered after being frozen at least overnight.

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

147 ### 2.3. Experimental design

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

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

159 ### 2.4. Tolerance and resistance

160 Previous studies have defined three defensive strategies against infections: qualitative resistance, as
161 the ability to remain uninfected upon pathogen exposure; quantitative resistance, as the ability the
162 reduce the pathogen load; and tolerance, as the ability of the host to maintain the fitness given a certain
163 pathogen load [61,62]. Given our model of study in which the minimum lethal dose of the pathogen is
164 1, meaning that a single mycobacterium is able to kill the host, we did not assess qualitative resistance
165 but focused on tolerance and quantitative resistance.

166 Tolerance was defined by the slope of a regression between the host fitness against the pathogen burden
167 [62–67], with more tolerant groups presenting less sleep slopes. We used flies' survival rates as
168 indicator of host fitness. Uninfected flies were included in the analysis to account for the fitness of
169 each group in the absence of infection, commonly referred as general vigour [62]. On the other hand,
170 resistance was measured as the Y-intercept of the regression line between the bacterial load upon death
171 (BLUD) and the inoculation dose. When slopes are equal, the lower the Y-intercept more resistant is
172 the group [62,64,65].

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

177 **2.5. Gene expression by quantitative real-time PCR**

178 The differential gene expression was assessed at 24 hours, 5 days and 10 days post-infection. Overall,
179 a total of 9 pools of 3 flies each were analysed per sex, group and time point, obtained from the three
180 independent experiments. All flies were preserved in RNA later (Fisher Scientific, S.L.) and stored at
181 -80ºC prior to RNA extraction. Total RNA was extracted flies using the MasterPure™ Complete DNA
182 and RNA Purification Kit (Lucigen) and converted to cDNA with the PrimeScript RT Master Mix
183 (Takara), following the instructions of the kit's manufacturers for both procedures. cDNA was stored
184 at -20ºC until used.

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

193 **2.6. Statistical analysis**

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

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204

205

206 **3. Results**

207 **3.1. The effect of reproduction in tolerance and resistance to *M. marinum* infections is sex-**
208 **dependent**

209 Our study revealed no differences in the general vigour when male flies were not infected, neither in
210 the overall survival at low doses of infection (50 and 500 CFU), depending on the reproductive status.
211 At increasing initial doses (5000 and 50000 CFU), males that had mated but were kept alone after the
212 infections showed slower mortality although no changes in the tolerance levels compared to virgin
213 males were found. On the other hand, males that were kept together with females at all times showed
214 significantly lower tolerance to *M. marinum* infections (Figure 1a - Table 2). When looking into the
215 resistance we found, again, that males that are not actively mating (virgin and alone males) show
216 similar profiles. Both groups tend to have higher bacillary loads and were significantly less resistant to
217 *M. marinum* infections compared to those males that are in constant contact with females, although
218 those that have previously mated show even lower resistance (Figure 1b – Table2).

219 In females, the general vigour was significantly lower in virgin flies, although they also showed a better
220 survival to low inoculation doses and an overall higher tolerance when compared with both mated
221 groups. Females that had mated but were kept alone after infection showed increased survival at higher
222 doses (5000 and 50000 CFU) while females kept together with males showed better survival at the
223 intermediate dose (500 CFU). However, no significant differences were observed between their general
224 tolerance levels (Figure 1a – Table 2). As observed in males, the more tolerant group (virgins) showed
225 lower resistance to the infection when compared with mated females (Figure 1b – Table 2).

226 Taken together, this data revealed that the tolerance and resistance against *M. marinum* infection in
227 males is more affected when they are actively mating, being less tolerant but more resistant those that
228 are in contact with females. On the other hand, once females have mated, they become less tolerant but
229 more resistant to *M. marinum* infections independently of the presence or absence of males.

230 When we compared tolerance and resistance of males and females for each reproductive status
231 independently (Figure 1c and 1d – Table 3), data showed that virgin flies did not differ neither in
232 general vigour nor in overall tolerance to the infection, but males were significantly more resistant.
233 The same pattern was observed in flies that were kept together in equal proportions throughout all the
234 procedure: both males and females had the same general vigour and the same tolerance levels, but
235 males showed up to be more resistant to the infection with *M. marinum*. On the other hand, when flies
236 had mated but were kept separated by sex after the infection, females showed increased general vigour
237 but an overall reduced tolerance to the infection with *M. marinum*. In addition, females showed to be
238 significantly more resistant to the infection compared to males when they are alone after mating.

239 These results indicate that those studies conducted only with males or females should consider their
240 sexual status when interpreting the results, since we have seen that the reproductive status affects
241 immunity differently in each sex.

242 **3.2. The humoral innate immune response against *M. marinum* infection depends on the**
243 **reproductive status of the host**

244 Given the differences we found among flies depending on their reproductive status, we wanted to
245 investigate if they were due to differences in the immune response triggered by the infection. Thus, we
246 measured the expression of the antimicrobial peptides Diptericin and Drosomycin, which are regulated
247 by the Imd and the Toll pathways, respectively, in flies infected with 500 CFU of *M. marinum* at

248 different days post-infection (Figure 2a). Virgin males showed a significant immediate overexpression
249 of *drosomycin*, while males together with females presented a significant increased expression of
250 *diptericin*. Males alone showed increased expression of both AMPs, although later in the infection. In
251 females, virgins and those together with males showed similar results as males. On the other hand,
252 females alone only presented significant overexpression of the Toll-related AMP late in the infection
253 (Figure 2a).

254 Comparing these expression levels with the phenotypic results obtained previously, we found that
255 activation of the Toll pathway might correlate with more tolerant but less resistant phenotypes in both
256 males (virgins and alone) and females (virgins). On the other hand, we also observed that while early
257 activation of the Imd pathway correlated with the more resistant groups (males and females together),
258 late or null activation of this pathway seemed to be linked with reduced resistance. These correlations
259 have been also observed when comparing the expression levels between males and females for each
260 reproductive status (Supp. Figure 1a). We found that the less resistant females (virgins and together)
261 showed significant increased expression of *diptericin* later in the infection, while the more tolerant
262 males (alone) showed increased expression of *drosomycin.* Finally, results also suggest that females
263 kept alone after mating may acquire their resistance through a mechanism that do not involve the
264 humoral innate immunity, as they are more resistant compared to both males and females kept together
265 but did not showed any activation of the Imd pathway throughout the infection.

266 **3.3. The metabolic regulation during infection it is altered by the reproductive status of the host**

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

272 In males, both virgins and those kept alone after the infection showed a significant early overexpression
273 of *upd3*, which was not sustained later in the infection. On the other hand, those males that were kept
274 together with females during the whole procedure presented a significant overexpression of this gene
275 later in the infection. In females, again virgins showed a significant early but not sustained
276 overexpression of *upd3*, while females that have mated presented a significant increased expression of
277 these gene later in the infection whether they were in presence or absence of males. Overall, these
278 results suggest a dual role for *upd3*, as its early expression correlated with tolerant groups while its
279 expression later in the infection correlated with resistant groups.

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

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

289

290 **3.4. Induction of the ecdysone receptor correlated with basal levels prior to the infection in males,**
291 **but not in females**

292 We also evaluated the expression levels of the ecdysone receptor (EcR) due to the tight relationship
293 that Ecdysone has with immunity and reproduction. In this case, we measured the relative expression
294 of this gene after the infection, but also the expression levels when injected with PBS to evaluate the
295 differences in the basal expression depending on the reproductive status (Figure 3c).

296 Virgin males showed increased production of EcR throughout the infection while males alone
297 presented the opposite pattern and males that were kept together did not show any increased production.
298 However, when observing the basal expression levels when flies were not infected, we observed
299 significant differences among groups. Virgin males and males kept alone showed increasing basal
300 levels of EcR over time, while males kept together with females presented no significant changes on
301 the basal expression levels of EcR over time. Thus, we found that males presented a significant
302 correlation between basal level of EcR and the induction of this receptor post-infection, in which lower
303 basal levels translated into higher activation of this gene post-infection (Figure 3).

304 In females, virgins showed no changes neither in the basal levels nor in the induction of EcR after the
305 infection. However, females that had mated showed similar patterns as their males' counterparts, while
306 females kept alone showed decreasing induction of EcR when infected, but no significant changes on
307 the basal levels. The correlation between basal levels and induction of EcR after the infection was not
308 statistically significant (Figure 3).

309 When comparing both basal levels and induction levels between males and females (Supp. Figure 2a),
310 we observed that virgin males presented lower basal levels but increased production of EcR after the
311 infection, while flies separated after the infection showed no significant different basal levels but
312 females increased its production after the infection. In both cases, the increased production of EcR
313 linked with increased resistance. Finally, when flies were kept together throughout all the procedure,
314 no differences at basal levels among sexes were found, but a late induction in females.

315 **3.5. Gene expression of *D. melanogaster* upon infection shows a marked sexual dimorphism**
316 **marked by Impl2 and Upd3 in males and females, respectively**

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

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327

328

329 **4. Discussion**

330 Typically, laboratory studies have revealed a significant "cost of mating" to *Drosophila* females in the
331 form of reduced longevity. However, here we present that virgin females show a significant decrease
332 in general vigour compared to mated females when uninfected, which is not observed in virgin males.
333 This phenomenon has only been described previously for wild-caught flies [69]. Previous studies
334 described that females reduce their resistance to some infection after mating, while males exposed to
335 females are more susceptible [45,50,70]. However, data presented herein suggest that, once mated,
336 males will only prioritise the infection clearance when reproduction is assured, whereas when kept
337 apart from females, they tend to prioritise fitness. In contrast, females seem to devote more effort to
338 the clearance of the infection once they have mated than when they are virgins. Still, in general, males
339 tend to have a higher ability to control infection compared to females, except for those mated kept
340 alone. In this case males increase their fitness over infection clearance. Overall, phenotypic results
341 from this study shows that the sexual dimorphism observed in *D. melanogaster* in the outcome of *M.*
342 *marinum* infection is highly related to the reproductive status of the host. Thus, suggesting that when
343 assessing the differences between sexes in *D. melanogaster* for other infections the characteristics of
344 the hosts' reproductive situation need to be characterised.

345 Regarding the hormonal levels and its relation with the immune response, our data show that certain
346 EcR levels are required for the flies after the infection, as when flies have higher basal levels the
347 infection does not induce and even represses its production. Consequently, flies with lower basal levels
348 of EcR trigger its expression after the infection. However, this correlation was only statistically
349 significant in males. Previous studies showed that ecdysone induces the expression of the receptor
350 PGRP-LC and, thus, modulates the Imd pathway [39]. Other studies had related the Imd pathway in
351 the control of resistance to infections [71] while its negative regulation mediates tolerance to infections
352 [72], although any of these were performed in mycobacterial infections. Our data support these
353 findings, as those flies with higher basal levels of EcR show higher expression levels of Diptericin
354 when infected and correlate with the more resistant phenotypes, while flies that did not show early
355 production of this AMP were more tolerant to the infection.

356 On the other hand, we also saw that production of the Toll-dependant AMP, Drosomycin, early in the
357 infection linked with increased tolerance but reduced resistance to *M. marinum* infection in flies, while
358 their activation in later stages of the infection correlated with increased resistance. Altogether, these
359 data suggest a role for the Toll pathway in determining both tolerance and resistance to *M. marinum*
360 infections. Several studies performed with a wide range of pathogens have described the Toll pathway
361 as key in determining resistance to infections [32,73,74], and even to be essential for resistance in
362 males, but not in females [29]. In addition, other recent studies have described a Toll-dependant
363 metabolic switch that directs fatty acids from neutral cellular storage toward phospholipid biosynthesis
364 [75], which might reduce the lipid droplet accumulation and, thus, the bacillary load of *M. marinum*.
365 However, no role in incresing tolerance to an infection has been described for this pathway previously.

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

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

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377

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547

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

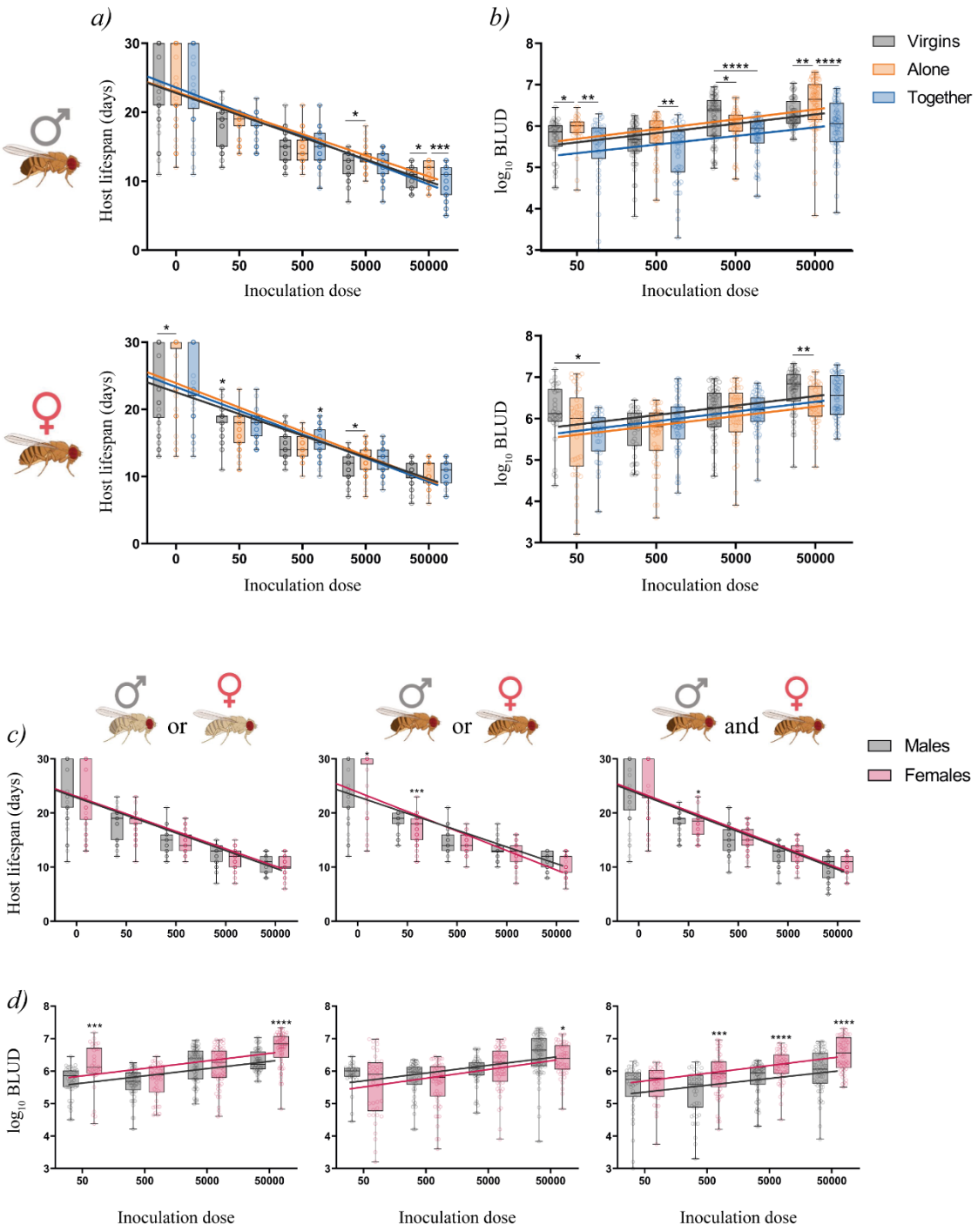

Figure 1| Tolerance (a and c) and resistance (b and d) of *D. melanogaster* to *M. marinum* infections depending on the reproductive status or the sex of the flies. Virgin males and females are more tolerant (*a*), but less resistant *(b)*. After mating, males alone behave as virgins, while males together with females showed increased resistance to the infection at expenses of their lifespan. On the other hand, females after mating behave the same in the presence or absence of males, with increased resistance (*b*) but reduced tolerance (*a*). When comparing males and females for each reproductive status, we observed that males were more tolerant to the infection only when they have mated but are kept alone during the infection (*c*), while females are more resistant to the infection only in this same condition (*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****

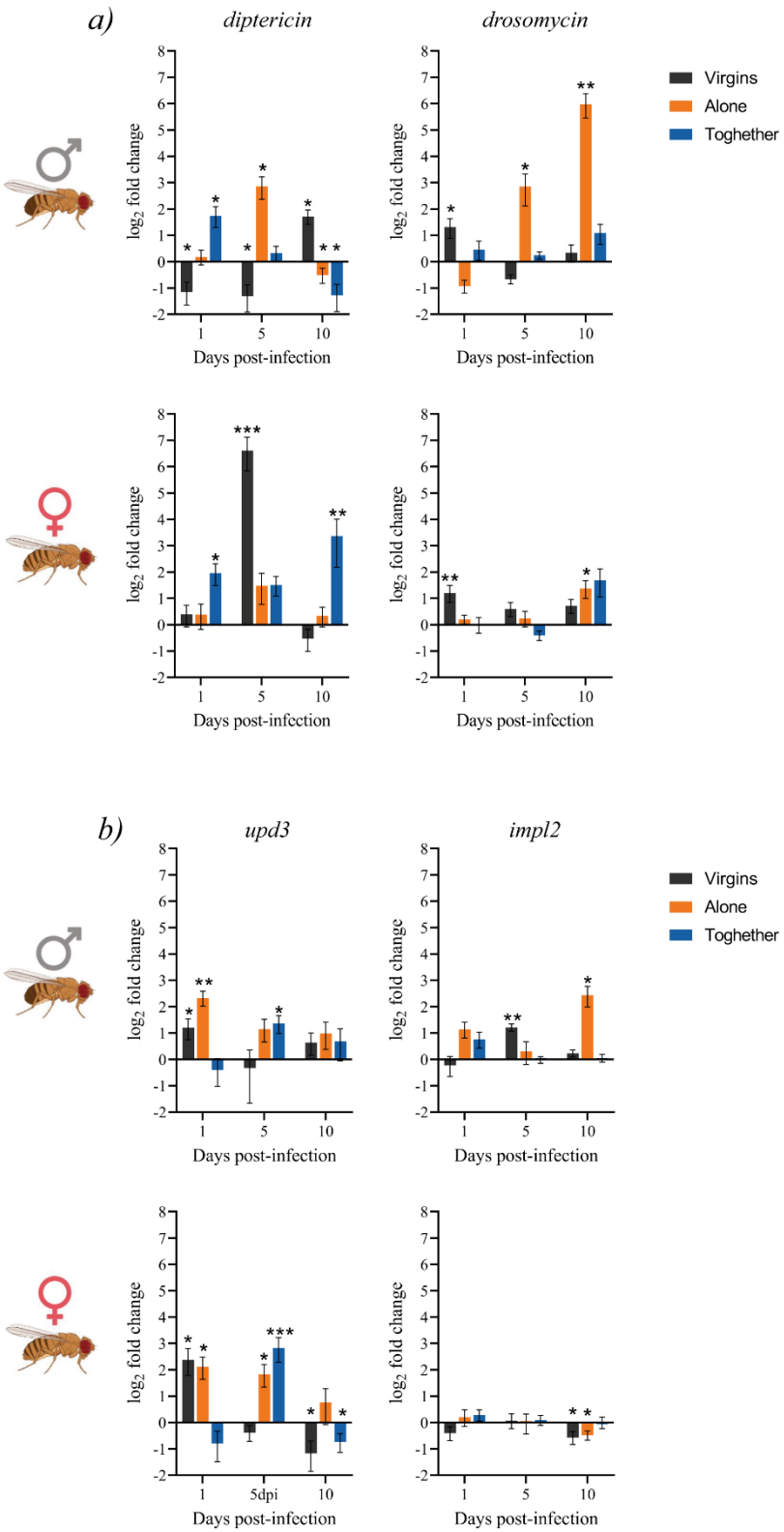

Figure 2| Expression of innate immune (*a*) and metabolic (*b*) genes during the infection in males and females depending on their reproductive status. Gene expression relative to the internal control gene *rpl32* was quantified in 9 replicate pools of 3 males or females exposed to the infection with *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).

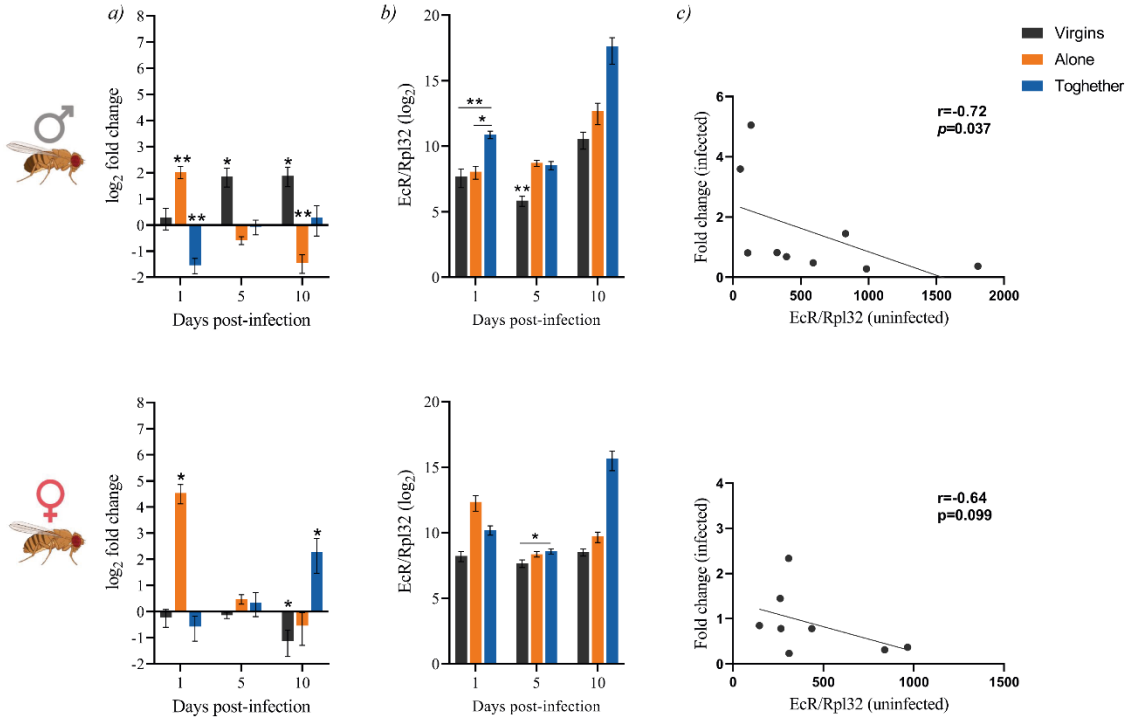

Figure 3| Expression of the ecdysone receptor (EcR) in males and females depending on their reproductive status after the infection **(*a*).** Gene expression relative to the internal control gene *rpl32* was quantified in 9 replicate pools of 3 males and 3 females each exposed to the infection with *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*)**.

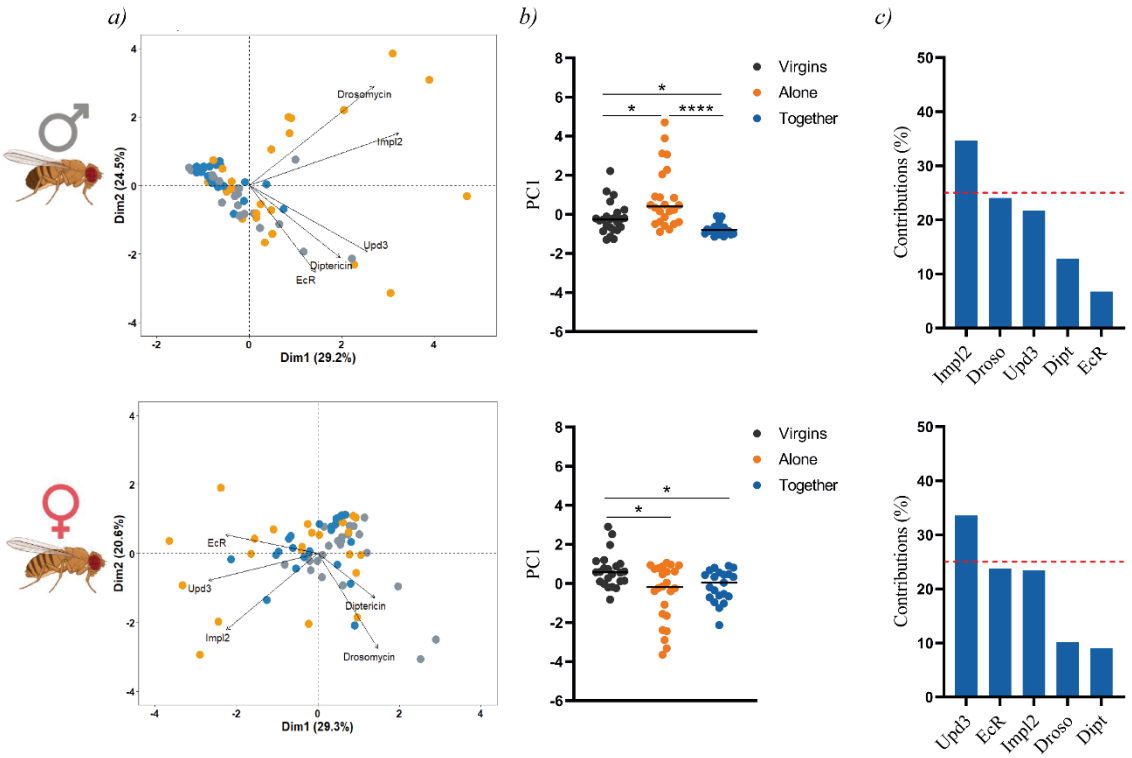

Figure 4| Heterogeneity of gene expression among flies with different reproductive status. Principal component analysis (PCA) based on expression of genes of interest in males and females infected with M. marinum at all time-points (*a*), PC1 scores (*b*) and variable contribution to PC1 (*c*). Each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