Sex Dimorphism in Expression of Immune Response Genes in Drosophila

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Abstract

Sex dimorphism in immunity is commonly observed in a wide variety of taxa and is thought to arise from fundamental life history differences between females and males. In *Drosophila melanogaster*, infection with different pathogens typically results in different modes of immune sex dimorphism, with male- or female-bias, likely due to specific disease-causing mechanisms of pathogens and host-pathogen interactions. Studies showed that some pathways, such as IMD and Toll, can explain these sex-dimorphic immune responses in Drosophila. However, it is unclear if sex differences in the immune response observed in *D. melanogaster* are conserved, even in closely related species. One window into identifying conserved and evolving sex differences in the immune response is to examine the sex-differential expression of immunity-related genes. Here, we aim to understand whether two closely related species, *D. melanogaster* and *D. simulans*, show conserved sex dimorphism in innate immunity, focusing on associated changes in gene expression in response to infection with a gram-negative bacterium, *Providencia rettgeri*. Survival, bacterial load, and bacterial load upon death (BLUD) were investigated to assess overall sex differences. *D. melanogaster* females and males differed significantly in survival, whereas *D. simulans* did not. Enrichment analyses revealed that both sexes and species upregulate genes involved in similar immune-related biological processes, but downregulated groups differed. We identified conserved sex differential gene expression of genes in the bacterial infection response pathways IMD, Toll, Jak/STAT, their regulators, and other immune-related gene classes (e.g., BOMs), as well as sex and species differences. In *D. melanogaster,* the effector antimicrobial peptides (AMPs) regulated by IMD were more highly upregulated relative to *D. simulans* in both sexes. Moreover, *D. melanogaster* females uniquely initiated high levels of gene expression that were involved in negative feedback mechanisms that controlled the overstimulation of IMD. Genes in the Toll pathway were also

sex-differentially expressed with a higher level of upregulation in *D. melanogaster*. Remarkably, comparing expression across species, we find that *D. simulans* likely employs both the conventional peptidoglycan recognition-driven PRR-SPE-Spz pathway and the microbial protease recognition-based Psh-dependent activation of Toll; in contrast, *D. melanogaster* appears to solely rely on the PRR-SPE-Spz pathway in this context. In summary, our findings indicate that sex differences are conserved in both species for the majority of upregulated genes, while downregulation patterns and specific gene subsets show notable differences between sexes or species.

Keywords: Sex dimorphism, sex-biased gene expression, innate immunity, *Drosophila melanogaster*, *Drosophila simulans*, Toll, IMD, BOMs, PGRPs, Bacterial infection.

1. Introduction

Sex dimorphism influences a diverse set of biological processes, affecting nearly all aspects of an organism's biology (Morrow 2015; Millington and Rideout 2018; Lopes-Ramos *et al.* 2020). Sex dimorphism of easily observable characteristics such as external coloration or anatomy has been widely described and studied, but dimorphism also extends to less readily apparent traits, including immune responses, infection outcomes, and disease prognosis (Carton et al. 1992; Fischer et al. 2015; Morrow 2015; McDonald et al. 2021; Wilkinson et al. 2022). Sex-biased immune responses have been observed at different stages of life history and may also be present in local tissue-level responses such as in the brain, intestinal layer, and hemolymph (Mackenzie et al. 2011; Deng and Jasper 2016; Millington and Rideout 2018; Belmonte et al. 2020; Khodursky et al. 2020; Wilkinson et al. 2022). The magnitude of sex differences may be mediated by the involvement of distinct immune compartments, such as hemocytes, melanization, epithelial immunity, and systemic immunity, depending on the pathogen and route of infection (Belmonte et al. 2020; Yu et al. 2022). At a mechanistic level, this may be explained by regulatory differences in key pathways, such as the Toll and IMD pathways, or more broadly by sex dimorphism of key tissues (Duneau et al. 2017b; Vincent and Dionne 2021). Even though immunological differences between sexes are observed frequently and impact fundamental mechanisms underlying immunity, these differences have received relatively little attention, including in model organisms and human studies (Morrow 2015; Klein and Flanagan 2016; Belmonte et al. 2020; Wilkinson et al. 2022).

Sex is a central axis of variation, explaining a significant amount of variation within species and differences between them (Bateman, 1948; Darwin, 1871; Lande, 1980). Sex differences have been observed in the intensity of the response against pathogens, specific outcomes of infections, and susceptibility, resistance, and tolerance to pathogens (Belmonte et al., 2020; Fischer et al., 2015; Klein & Flanagan, 2016). The evolution of differences in the immune response between males and females is most frequently attributed to differences in reproductive strategies and life history traits (Lande 1980; Zuk 2009; Morrow 2015). For instance, females typically invest more in offspring production and care, leading to the prediction that more robust immune responses will be associated with higher fitness in females (Lande 1980; Zuk 2009; Morrow 2015). Conversely, males may invest more in traits that enhance competitiveness, potentially at the expense of immune function (Lande 1980; Zuk 2009; Morrow 2015). Behavioral differences between males and females may also impact the frequency, intensity, and timing of exposure to various challenges such as viral, bacterial, fungal, and chemical, which could contribute to the evolution of sex differences or environmentally induced sex differences in immunity observed in natural contexts (Oertelt-Prigione 2012; Asahina 2018). In addition, other processes may intersect with sex dimorphism in immunity, contributing to variation within populations. For example, emerging work has detected sex differences in the aging of immune tissues, immune senescence, and inflammation (Mondal and Rai 1999; Metcalf et al. 2020; Abancens et al. 2020; Shepherd et al. 2021). Differences in food intake and diet are also observed between males and females and could modulate immunity in a sex-differential fashion (Oertelt-Prigione 2012; Asahina 2018; Rai et al. 2023).

Hormone signaling pathways, including sex hormones, have a role in the transcriptional regulation of the immune genes (Graze et al. 2018; Gal-Oz et al. 2019; Shepherd et al. 2021). Moreover, previous studies found that steroid hormones can directly regulate the function of the immune cells, such as the absence of ecdysone led to the inability of the macrophage-like cells to phagocytize bacteria in Drosophila (Regan *et al.* 2013). The peptide hormone IIS/TOR signaling disruption also reported the sex-differential immune gene expression in Drosophila (Graze *et al.* 2018). However, hormone signaling likely does not fully explain sex dimorphism in immune responses, as sex differences in immune response have been observed even at sexually immature life stages (Klein and Flanagan 2016; Shepherd et al. 2021). Sex chromosomes and dosage compensation mechanisms can also contribute to the differential expression of immune genes as well as the function of immune cells (Scofield et al. 2008; Libert et al. 2010). For instance, in mammals, the distinct composition of sex chromosomes can lead to female-specific mosaicism due to cell-specific X chromosome inactivation and may influence the

92 expression of immune genes (Klein and Flanagan 2016; Pinheiro and Heard 2017). In humans, significant
93 proportions of primary B lymphocytes, monocytes, and plasmacytoid dendritic cells express TLR7 on
94 both X chromosomes by escaping X inactivation of XX individuals (Libert et al. 2010; Souyris et al. 2018;
95 Heintze 2018). Overall, multiple aspects of sex determination and development impact sex dimorphism
96 in immunity and often interact in complex ways.

97 Drosophila is a crucial model in the study of development and physiology and is growing as a model for
98 sex differences in body size, aging, stress responses, and disease progression (Rideout et al. 2015;
99 Millington and Rideout 2018; Belmonte et al. 2020). As a model organism for innate immunity,
100 Drosophila showcases sex differences in both the cellular and humoral responses (Reviewed in
101 (Belmonte *et al.* 2020)). These two central components contribute to sex-specific immune responses
102 depending on the type of pathogen and the context of the infection (Hoffmann 2003; Lazzaro and Little
103 2009; Millington and Rideout 2018; Belmonte *et al.* 2020). Thus, *D. melanogaster* exhibits sexual
104 dimorphism in the outcome of infectious diseases (Vincent and Dionne 2021; Rai *et al.* 2023; Khan and
105 Graze 2024).

106 The Drosophila immune system comprises various cellular and humoral components that work together
107 to recognize, signal proliferation of, and eliminate pathogens (Hoffmann 2003; Vlisidou and Wood 2015;
108 Yu *et al.* 2022). One of the key cellular components of the *Drosophila* innate immune system is a
109 specialized cell type called the plasmatocyte, the functional equivalent of mammalian
110 macrophages/monocytes, which accounts for around 95% of the circulating hemocytes (Evans *et al.*
111 2003; Vlisidou and Wood 2015; Banerjee *et al.* 2019). Plasmatocytes can engulf and destroy invading
112 microorganisms and maintain communication with the humoral response, such as response to
113 antimicrobial peptides (AMPs) binding (Evans *et al.* 2003; Vlisidou and Wood 2015; Banerjee *et al.* 2019).
114 The rest of the hemocytes (5%) primarily consist of crystal cells that produce phenol oxidase (PO) for
115 melanization, primarily as an immune response involved in wound healing (Evans *et al.* 2003; Dudzic *et*
116 *al.* 2015). Finally, upon pathogenic attack, fruit flies generate lamellocytes, which are larger than
117 plasmatocytes and specialize in the encapsulation of large pathogens such as wasp eggs (Evans *et al.*
118 2003; Vlisidou and Wood 2015).

119 In the humoral response, the most critical aspect of the *Drosophila* innate immune response is the
120 consistent production of small peptides called antimicrobial peptides (AMPs) and their assistance in
121 recruiting additional immune cells such as plasmatocytes (Lai and Gallo 2009; Vlisidou and Wood 2015;
122 Yu *et al.* 2022). Upon immune signal activation, AMPs are produced in the fat body and released into the

123 hemolymph (Tzou *et al.* 2000; Hoffmann 2003). Due to their positive charge, AMPs are attracted to
124 microbes with negatively charged cell membranes, where they embed in the hydrophobic region and
125 cause membrane destabilization, resulting in cell death (Lai and Gallo 2009; Lin *et al.* 2020). Moreover,
126 certain tissues, such as the trachea, midgut, oviduct, spermatheca, ganglia, and a subpopulation of
127 hemocytes, produce AMPs in response to local infection (Tzou *et al.* 2000; Lai and Gallo 2009). In
128 general, the level of AMP expression directly reflects the strength of the immune response (Lai and Gallo
129 2009; Lin *et al.* 2020).

130 The core response is initiated by pattern recognition receptors (PRRs) that recognize PAMPs, for
131 example, specific molecular patterns on the surface of microorganisms, and initiate a signaling cascade
132 that leads to the production of AMPs via the IMD and Toll pathways (Tanji and Ip 2005). The IMD and
133 Toll pathways are exceptionally well studied in this system (Valanne *et al.* 2011; Myllymäki *et al.* 2014).
134 They are homologous to the vertebrate TNF and Toll-like pathways, respectively (Tanji and Ip 2005; Yu *et*
135 *al.* 2022; Valanne *et al.* 2022). In general, the IMD pathway is activated upon the detection of
136 peptidoglycan produced by gram-negative bacteria, whereas the Toll pathway responds to the
137 peptidoglycan of most gram-positive bacteria, and fungal cell wall components such as β-(1,3)-glucan
138 (Tanji and Ip 2005; Valanne *et al.* 2022). Moreover, the Drosophila STING pathway activates upon
139 detection of bacterial cyclic dinucleotides (CDNs) to initiate an innate immune response by triggering the
140 NF-κB transcription factor Relish of IMD pathway to produce AMPs (Martin *et al.* 2018). Of note, a
141 second cascade besides PRR-based activation, the danger signal cascade, can activate the Toll pathway
142 (Ligoxygakis *et al.* 2002; Gottar *et al.* 2006; Chamy *et al.* 2008). The danger cascade initiates upon the
143 detection of the abnormal activity of bacterial or fungal proteases (danger signal) and activates the Toll
144 pathway by using Clip-Serine Protease Persephone (Psh) via Psh-SPE-Spz or Psh-Protease-Spz (SPE
145 independent) cascades (Gottar *et al.* 2006; Yamamoto-Hino and Goto 2016; Issa *et al.* 2018; Dudzic *et al.*
146 2019). Upon activation, each pathway launches a rapid and efficient transcriptional response to
147 infection via nuclear translocation of NF-κB family transcription factors, Dif and Dorsal (Tanji and Ip
148 2005; Valanne *et al.* 2022).

149 Recent studies have reported that *Drosophila* males and females differ in response to localized and
150 systemic bacterial, fungal, viral, and parasitic infections (Reviewed in (Klein and Flanagan 2016;
151 Belmonte *et al.* 2020)). In many cases, for example, Kalithea (viral), *Candida albicans* (fungal),
152 *Tubulinosema ratisbonensis* (microsporidial), and *Serratia marcescens* (bacterial), there was a bias
153 toward females in the effectiveness of the immune response as measured by survival (Kutch and

154 Fedorka 2017; Palmer *et al.* 2018; Franchet *et al.* 2019; Chowdhury *et al.* 2019). At the same time, this
155 pattern is not consistently observed even among similar types of pathogens; male flies infected with
156 *Beauveria bassiana* (fungal), *Providencia alcalifaciens* (bacterial), and *Enterococcus faecalis* (bacterial)
157 have higher survival rates relative to females (Taylor and Kimbrell 2007; Le Bourg 2011; Duneau *et al.*
158 2017b; Shahrestani *et al.* 2018). Collectively, these studies, along with others, have also revealed
159 considerable context dependency. Sex dimorphism in immunity can be influenced by various factors
160 such as hormonal regulation, environmental conditions, dietary habits, and metabolic processes (Leech
161 *et al.* 2019; Shepherd *et al.* 2021; Rai *et al.* 2023). Conditional impacts are especially evident when the
162 same pathogen shows different directions of sex dimorphism under different experimental conditions,
163 either in different laboratories or in the same study with different treatments (Rai *et al.* 2023). For
164 example, two independent studies of *Pseudomonas aeruginosa* infections demonstrate opposite-sex
165 bias (Vincent and Sharp 2014; Chowdhury *et al.* 2019). Moreover, recent findings suggest that sex-
166 determination pathway genes such as *transformer (tra)* might influence immune sex dimorphism in
167 Drosophila (Khan and Graze 2024).

168 Several recent reviews have highlighted a significant gap in our understanding of sex differences in
169 immunity, particularly in adult animals and in the context of evolutionary biology (Beery and Zucker
170 2011; Belmonte *et al.* 2020). Further, there is a relative lack of comparative studies to understand which
171 aspects of sex dimorphism in the immune response are conserved. Comparative studies are also crucial
172 for understanding the evolutionary forces influencing sex differences across the species. For instance,
173 comparative studies of closely related species can help to expound whether observed sex differences
174 are lineage-specific and/or adaptive (Hill-Burns and Clark 2010; Khodursky *et al.* 2020; Nanni *et al.*
175 2023).

176 In *D. melanogaster*, both the IMD and Toll pathways play pivotal roles in immune dimorphism (Duneau
177 *et al.* 2017b; Vincent and Dionne 2021). The Toll pathway and its regulators may be responsible for a
178 significant portion of immune sex dimorphism in *D. melanogaster* (Duneau *et al.* 2017b). In addition,
179 regulation of the IMD pathway also plays a role in sex differences (Vincent and Dionne 2021). While this
180 is a growing area of study, there are many remaining questions regarding the complex molecular basis
181 of sex dimorphism in immune responses and the degree to which the role of the Toll and IMD pathways
182 in sex dimorphism is conserved. The intricate crosstalk among evolutionarily conserved immune
183 pathways, including Toll, IMD, Jak/STAT, and JNK, introduces further complexities to the investigation of
184 sex dimorphism (Dudzic *et al.* 2019; Tafesh-Edwards and Eleftherianos 2020; Alejandro *et al.* 2022).

185 Overall, a comprehensive understanding of sexual dimorphism in immunity can provide valuable insights
186 into the complex interplay among evolution, diversity, immunity, and sex, which also has important
187 implications for the treatment and prevention of disease.

188 To comprehensively characterize sex dimorphism in immunity in *Drosophila*, we conducted a
189 comparative study, profiling outcomes of infections and gene expression in unmated-adult females and
190 males in two species, *D. melanogaster* and *D. simulans*. This study allows a direct comparison of the
191 response to infection in *D. melanogaster*, where most of the immunological studies have been
192 completed, and *D. simulans*, a relatively well-studied and closely related species of the same sub-group
193 (Figure 1A) (Bhutkar *et al.* 2007; Paris *et al.* 2013). In addition, to the best of our knowledge, this is the
194 first time bacterial infection in *D. simulans* has been reported for both sexes, and it is the first
195 comparative immune response study with *D. melanogaster.* This work identifies sex differences that are
196 in common, as well as divergence of sex differences in immune gene expression, laying the groundwork
197 for an improved understanding of the evolutionary context of sex dimorphism in the immune response.

198 **2. Methods and Materials**

199 **2.1 Fruit fly stocks and sample collection**

200 *D. melanogaster* and *D. simulans* were reared on a modified version of the Bloomington Drosophila
201 Stock Center (BDSC) standard cornmeal medium recipe (33 L H_2O, 237 g agar, 825 g dried deactivated
202 yeast, 1560 g cornmeal, 3300 g dextrose, 52.5 g Tegosept in 270 mL 95% ethanol and 60 mL propionic
203 acid). Flies were reared at 25°C with a 12:12 light: dark cycle. Density-controlled crosses were set up for
204 each species, *D. melanogaster* (Canton S, Gift Michelle Arbeitman) and *D. simulans* (Dsim w[501],
205 14021-0251.195, National Drosophila Species Stock Center, Cornell University); newly emerged progeny
206 was collected as virgins and separated by sex. Female and male individuals were isolated in standard
207 vials, and all experimental flies were unmated, adult, and aged 5-9 days prior to infection.

208 **2.2 Experimental treatment**

209 A strain of the gram-negative *Providencia rettgeri* species isolated from wild-caught *D. melanogaster* (*P.*
210 *rettgeri* strain: Dmel, Gift Brian Lazzaro) was used as an infectious agent, which is known as moderately
211 virulent (Juneja and Lazzaro 2009; Galac and Lazzaro 2011; Duneau *et al.* 2017b). In this study,
212 stationary phase *P. rettgeri* bacterial culture (OD600nm = 1.2) was used to inject bacteria into the
213 thoracic region (soft spot near the wings) of the fly to introduce around 1200-1500 cells directly into the

214 hemolymph using the needle prick method (Figure 1B; following (Khalil *et al.* 2015)). For control flies,
215 sterile phosphate buffer solution (1x PBS) was injected as a wound-only control (Figure 1B).

216 **2.3 Bacterial culture preparation**

217 Glycerol stocks of *P. rettgeri* were stored at -80 °C prior to use. Stocks were used to streak LB-Agar
218 plates (Teknova, California) and incubated at 37°C for 12 hours to isolate single colonies. A single colony
219 was then transferred to 5 mL Lysogeny broth LB-Broth (Teknova, California) and incubated at 37°C at
220 220 rotation per minute (rpm) for 9-10 hours. Stationary phase bacteria were centrifuged at 4000 rpm
221 for 3 minutes to collect bacterial pellets. Pellets were washed with sterile 1x PBS using pipette aspiration
222 (5 times) to remove residual LB Broth. After five washes, the pellet was resuspended with sterile 1x PBS
223 to achieve OD600 nm of 1.2.

224 **2.4 Bacterial injection of flies**

225 The flies were anesthetized by CO_2 during the injection. The injections were made in the thoracic region
226 beside the wings in the soft spot using the needle pricking techniques previously described by Khalil et
227 al. 2015 to introduce approximately 1200-1500 viable bacteria into each fly. This approach introduces
228 bacteria directly into the hemolymph. The fly vials were kept horizontal to allow recovery until the flies
229 were completely active.

230 **2.5 Survival rate**

231 For each sample type, the survival assay was performed in 5 replicates, each of which contained 30 flies.
232 Survival was scored at 2, 6, 12, 18, 24, and 36 hours after bacterial injection. Flies were scored as dead if
233 the fly was on its back and was not moving or could not turn by itself (Duneau *et al.* 2017a). Scoring
234 ceased when all the flies were dead in any of the experimental groups or after the 36-hour time point.
235 Survival analysis was conducted in R using the package "survival" (Therneau 2023). Kaplan-Meier
236 survival curves were generated to visualize the survival proportion. The Cox regression was performed
237 to determine the statistical difference among experimental groups (Therneau and Grambsch 2000).

238 **2.6 Bacterial load time series**

239 Bacterial load was assessed for each sample in six replicates, with each replicate consisting of one fly at
240 each time point. The bacterial load was determined at 4, 8, 12, 16, 20, and 24 hours after injections,
241 with six replicate measurements following Duneau et al. (Duneau *et al.* 2017a). A single fly per replicate
242 measurement was homogenized in 200 µL 1X PBS using a sterile pestle. The homogenized sample (10
243 µL) was transferred to a 96-well plate and diluted with 90 µL 1X PBS. From the first dilution (1/10), 10 µL

244 of the solution was transferred successively, and serial dilution was carried out 8 times. Subsequently,
245 20 µL of the dilution $1:10^{2,}$ $1:10^{4}$, $1:10^{6}$, and $1:10^{8}$ were spread on LB agar plates to count CFU. Plates
246 were incubated at 37 °C for 12-14 hours to count the CFU before gut microbes typically form colonies
247 that occur at approximately 36 hours (Khalil *et al.* 2015). To test the differences between infected and
248 control animals for each time point and to get the differences across groups, the linear model was used
249 (Sparks 2017).

250 **2.7 Bacterial load upon death (BLUD)**

251 The bacterial load upon death was determined for each sample in four replicates, with each replicate
252 comprising three flies per pool. For the BLUD experiment, flies were determined to be dead flies if the
253 fly was on its side or back without any movement (Duneau *et al.* 2017a). A single fly was homogenized in
254 200 µl of 1x PBS within 30 minutes of determining death using a sterile pestle. Serial dilutions and CFU
255 counts were obtained, as described in the previous section. To find the differences in groups for each
256 time point, the linear model (CFU ~ Groups) was fit and pairwise differences tested for species and sex
257 comparisons.

258 **2.8 RNA extraction and prep**

259 Four replicate pools of 5–9 day old flies with 20 flies per pool were collected for each sex and each
260 group. Only live flies were used for RNA isolation. Replicate pools of wound control and bacteria-
261 infected flies were flash-frozen in liquid nitrogen 16 hrs post-injection (hpi). The frozen samples were
262 stored at -80°C prior to total RNA isolation. The 20 flies (per pooled sample) were homogenized entirely
263 in 700 µL of TRI-Reagent using a pellet pestle motor and RNase-Free pellet pestles in a 2 mL
264 microcentrifuge tube. Total RNA was extracted using the Direct-zol RNA Miniprep Plus Kit (Zymo
265 Research) according to the manufacturer's instructions. The TURBO DNase-free kit (Thermo Fisher
266 Scientific) was used to remove genomic DNA contamination following the manufacturer's instructions.
267 The concentration was determined using Nanodrop 2.0 (Thermo Fisher Scientific), and RNA integrity
268 Number (RIN) was measured by using a TapeStation system with an RNA ScreenTape Analysis kit
269 (Agilent Technologies).

270 **2.9 TagSeq library prep**

271 3' TagSeq was performed at the University of Texas Genomic Sequencing and Analysis Facility, Austin
272 (UT-GSAF), based on the protocols from Lohman et al. (2016) and Meyer et al. (2011) (Lohman et al.,
273 2016; Meyer et al., 2011). Libraries were quantified using the Quant-it PicoGreen dsDNA assay

274 (ThermoFisher) and pooled equally for subsequent size selection at 350-550 bp on a 2% gel using the
275 Blue Pippin (Sage Science). The final pools were checked for size and quality with the Bioanalyzer High
276 Sensitivity DNA Kit (Agilent Technologies), and their concentrations were measured using the KAPA SYBR
277 Fast qPCR kit (Roche). Libraries were sequenced on a NovaSeq 6000 (Illumina), 100 bp single-end reads,
278 with 5-10 million reads per replicate (File S1).

279 **2.10 Preprocessing and alignment**

280 Single-end 100 bp reads were generated using 3′ Tag RNA Seq for each experimental group with 4
281 replicates each. The total raw reads generated by 3′ Tag RNA Seq was between 5.7 and 8.4 million (M)
282 reads per sample, with an average of 6.99 M reads for *D. melanogaster* and 7.16 M reads for *D. simulans*
283 (File S1). The quality assessment of raw data was performed by FastQC (Babraham Institute, University
284 of Cambridge) (Anders 2010). The FastQC analysis showed that all 32 samples had high quality
285 throughout, and no trimming was performed except the removal of the adapter by the sequencing
286 facility (Trimmomatic; Bolger et al., 2014).

287 The reads were aligned against the respective *Drosophila* genomic references (FlyBase Releases:
288 dmel_r6.47 for *D. melanogaster* and dsim_r2.02 for *D. simulans*) using the read alignment tool STAR
289 (Gelbart *et al.* 1996; Gramates *et al.* 2022). The alignments were used to generate raw read counts for
290 each gene in the reference genome annotation by using the "genomeDir", "readFilesIn", and
291 "outSAMtype" options in STAR (Dobin *et al.* 2013). Gene expression levels were then quantified using
292 the "quantMode GeneCounts" option of STAR (Dobin *et al.* 2013).

293 The mean uniquely mapped reads percentage for *D. melanogaster* samples was 74.48%, and for *D.*
294 *simulans* samples, it was 76.81%, which is typical for 7 M reads of 3′ Tag RNA Seq (File S1) (Ma *et al.*
295 2019). Upon mapping, raw read counts of 17,633 genes in *D. melanogaster* and 15,266 genes in *D.*
296 *simulans* were generated by STAR. Of 17,633 genes of *D. melanogaster* and 15,266 genes of *D. simulans*,
297 13,588 and 13,010 genes passed the detection cutoff, respectively (File S2-S5). Each experimental
298 sample passed the quality control analysis prior to normalization and DEG analysis.

299 An edgeR-limma-voom workflow in R, was used to normalize raw counts (Log2CPM) and fit a cell means
300 model, with defined contrasts used to determine differential expression between experimental groups,
301 focusing on sex and species differences in gene expression resulting from infection (Robinson *et al.*
302 2010; Ritchie *et al.* 2015). Differences were classified as up or downregulated relative to wound control.
303 The interaction of treatment by sex was tested to identify significant differences between the sexes,
304 within each species (Law *et al.* 2014; Ritchie *et al.* 2015). A significant treatment by sex interaction term

305 means that the sexes differ in the way that the gene expression for a gene changes in response to the
306 infection. Additional tests for interactions were included to investigate species differences. In between-
307 species comparisons, significant interaction terms for a gene indicate differences between species in the
308 changes in gene expression observed in comparisons for a single-sex (treatment by species) or in
309 comparisons between sexes (treatment by sex by species).

310 **2.11 Differential gene expression analysis**

311 Gene expression differences were first evaluated for each species individually to allow analysis of all
312 expressed annotated genes in a species. Genes with fewer than 2 read counts (summed across samples
313 within a group) were excluded from the analysis. Expression was estimated as the Log2CPM normalized
314 counts per gene for *D. melanogaster* (mel) or *D. simulans* (sim), females (F) or males (M), with infection
315 by *P. rettgeri* (pret) or PBS-only injected controls (PBS). Voom variance modeling was implemented in
316 limma, with a means model and comparisons between groups tested using contrasts (Law *et al.* 2014;
317 Ritchie *et al.* 2015). In *D. melanogaster,* the comparisons tested were mel_f_pret = mel_f_pbs and
318 mel_m_pret = mel_m_pbs, the effect of treatment for each sex, and the interaction of treatment by sex
319 mel_f_pret - mel_f_pbs = mel_m_pret - mel_m_pbs. Likewise, for the *D. simulans*, the corresponding
320 tests were conducted. For the enrichment, gene class heatmaps, and pathway analysis in *D. simulans,*
321 the annotated *D. melanogaster* orthologs were used (dmel_orthologs_in_dsim_Flybase_2017_05; File
322 S13). An FDR-adjusted *p*-value (*p*-adj) cutoff of 0.05 was used for all tests of differential gene expression.

323 For comparisons between species, unique ortholog pairs were identified and included in the analysis. If
324 two putative orthologs were found for one gene, then only one representative was kept for the analysis,
325 meaning a single representative pair for each set of ambiguous homologs, for which orthology has not
326 been clearly defined, was included in the analysis. As mentioned above, raw counts per gene were
327 normalized using edgeR-limma-voom. For the comparison between species, normalization also included
328 TMM density normalization of Log2CPM transformed counts. The contrast used to test the interaction
329 of treatment by species in females is dmel_f_pret - dmel_f_pbs = dsim_f_pret - dsim_f_pbs, and the
330 effect of treatment by species in males is dmel_m_pret - dmel_m_pbs = dsim_m_pret - dsim_m_pbs
331 (Law *et al.* 2014). Additionally, contrasts were used to test the three-way interaction of treatment by sex
332 by species ((dmel_f_pret - dmel_f_pbs) - (dmel_m_pret - dmel_m_pbs)) = ((dsim_f_pret - dsim_f_pbs) -
333 (dsim_m_pret - dsim_m_pbs)) (Law *et al.* 2014; Ritchie *et al.* 2015). An FDR-adjusted *P*-value (*p*-adj)
334 cutoff of 0.05 was used for all tests conducted. Unless otherwise mentioned, all the reported
335 log2FoldChanges (log2FC) and interactions are significant at p-adj < 0.05.

336 **2.12 Enrichment and pathways**

337 The "clusterProfiler" R program package was used for gene ontology (GO) based gene set enrichment
338 analysis (Wu *et al.* 2021). GO-Biological Process terms with adjusted *p*-value (FDR) less than 0.05 were
339 considered significantly enriched. GO-BP annotations were performed separately for each contrast. The
340 enrichment of DEGs in the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways was also tested
341 using the "clusterProfiler" package in the R (FDR < 0.05). Expression differences of DEGs for selected
342 pathways were visualized using "Pathvisio" and the wiki pathways plugin. The wiki pathway WP3830
343 was used to generate the Toll-IMD-Jak/STAT gene list and pathways.

344 **2.13 Functional groups**

345 Flybase was used to identify the functional groups/class gene list using key terms for Bomanin (BOMs),
346 Drosophila immune-induced molecules (DIMs), Turandot (Tots), Thioester-containing protein (Teps),
347 Insulin, Antimicrobial peptides (AMPs), Jun N-terminal kinase (JNK)/TNF-alpha-Eiger Pathway and
348 Regulator, Positive and Negative Regulators of Toll, Positive and Negative Regulators of immune
349 deficiency (IMD), and Spätzle (Spz) Activating Pathway with Regulators (Gelbart *et al.* 1996; Gramates *et*
350 *al.* 2022). The Jak/STAT and melanization gene list was created manually from earlier studies and review
351 articles (Tang 2009; Myllymäki and Rämet 2014; Yu *et al.* 2022). Heat maps of functional groups/classes
352 were generated by "ggplot2" in the R program.

353 **3. Results**

354 Sex dimorphism is commonly observed in immune responses but is variable based on the pathogen,
355 host, and other environmental factors (Fischer *et al.* 2015; Klein and Flanagan 2016). There is clear
356 evidence of sex differences in immunity in *Drosophila melanogaster*, both at the physiological and
357 molecular level, with a few genes identified as sex-differentially induced or repressed in response to
358 infection (Taylor and Kimbrell 2007; Duneau *et al.* 2017b; Shahrestani *et al.* 2018). However, the range
359 and causes of variation in sex dimorphism in immune responses, how and why these differences have
360 evolved, and the underlying molecular mechanisms are unknown. This study aimed to understand if sex
361 differences in the immune response are conserved between closely related species of *Drosophila* (Figure
362 1A). The study characterized the sex differences between unmated males and females associated with
363 the immune response against a gram-negative extracellular bacterium, *P. rettgeri*, in *D. melanogaster*
364 and its close relative *D. simulans*, by combining survival assays with RNA-seq-based gene expression
365 estimation (Figure 1B). DEGs were analyzed within species, for all annotated genes, and between species
366 for orthologs.

367 **3.1 Sex dimorphism in survival**

368 Fruit flies initiate rapid and efficient innate immune responses in response to pathogens that are crucial
369 for survival, which influence the eventual outcome of bacterial infections. As one metric of relative
370 effectiveness of the immune response, survival assays were conducted for females and males of *D.*
371 *melanogaster* and *D. simulans* upon *P. rettgeri* infection over 36 hours at 6 to 12-hour intervals in five
372 replicates where each replicate contained 30 flies (Figure 2). The survival assay demonstrated significant
373 sex dimorphism in *D. melanogaster* (p-value < 0.001), but no significant differences in survival between
374 the sexes were identified in *D. simulans.* This demonstrates that there are species differences in sex
375 dimorphism in response to bacterial infection. Primarily, the initial stages (0-12hpi) showed no
376 significant difference in survival between sexes for either species (Figure 2A). Nonetheless, survival
377 started to differ by sex around 12hpi when the infection was expected to be established. At 18hpi, there
378 were significant differences between males and females, where females exhibited higher survival than
379 males in *D. melanogaster*. This difference in survival rate was persistent between sexes from 18hpi to
380 36hpi. Overall, the Kaplan Meier survival curve and Cox-regression model for *D. melanogaster* showed
381 significant differences between males and females for *P. rettgeri* infection (Figure 2A).

382 For *D. simulans*, a sister species and member of the *D. melanogaster* subgroup, comparable results were
383 observed in the initial stages of the infection (up to 12hpi) (Figure 2B). However, survival data began to
384 diverge at 18hpi relative to *D. melanogaster.* In *D. simulans,* females showed no survival differences
385 compared to males. While males and females continued to have no survival differences for 18hpi to
386 36hpi, the *D. simulans* sexes did not differ significantly in overall survival. Unlike *D. melanogaster* flies,
387 all the *D. simulans* infected flies had died at 36hpi for both sexes (Figure 2B).

388 Comparing between species, female *D. melanogaster* had significantly higher survival after the
389 establishment of the infection relative to *D. simulans* (p-value < 0.001). In all data points after infection
390 initiation, *D. melanogaster* females consistently showed significantly higher survival rates than *D.*
391 *simulans* females. Males of both species had similar survival during the initial stages, but around 24- and
392 36hpi, the survival of *D. simulans* males is significantly lower (p-value < 0.001). Overall, *D. melanogaster*
393 females had markedly higher survival than *D. melanogaster* males and both males and females of *D.*
394 *simulans* (Figure 2).

395 **3.2 Bacterial load and BLUD**

396 Bacterial load determination is a measure of the ability of the immune response to ease bacterial
397 growth rates. The bacterial load data showed no statistical differences among experimental groups

398 when comparing the samples across sexes by each interval for either species (Figure 3, File S14).
399 Previous studies in the females of these two species also detected no significant bacterial load
400 differences upon gram-negative *Serratia marcescens* infection at 12hpi and 25hpi (Hill-Burns and Clark
401 2010). The data showed that the loads were similar for most individuals in the early stages, at 4- and
402 12hpi. Overall, the load increased in all the experimental groups for *D. melanogaster* and *D. simulans* at
403 16- and 20hpi; however, a subset of flies in each group showed a higher load than other individual flies.
404 At 20hpi, both the females and males showed a binary outcome of the infection clustering into two
405 groups. This can be explained if some individuals are able to suppress the bacterial infection progression
406 and keep the bacterial count lower than average, whereas others are less able to inhibit the infection
407 (Duneau *et al.* 2017a).

408 The results of the BLUD assay showed no sexually dimorphic pattern in bacterial burden at the time of
409 death upon *P. rettgeri* infection in *D. melanogaster* and *D. simulans*; moreover, there was no significant
410 difference in the species comparison (p-value > 0.05) (Figure 4). Overall, the bacterial load was unable to
411 explain the sex differences observed in survival.

412 **3.3 Identification of DEGs in each sex and species**

413 Within species in *D. melanogaster*, after normalization, 13,588 genes were tested. In response to
414 infection, 2269 genes were downregulated in males compared to 977 in females; with 629 genes
415 downregulated in both sexes (Figure 5A, Table S1). A higher number of genes (1640) were uniquely
416 downregulated in males, compared to females (348), which might indicate higher stress levels in males
417 upon infection. The number of upregulated genes was more comparable between males and females.
418 The number of genes that were upregulated in males was 530, and in females, 599, and 264 upregulated
419 genes were in common (Figure 5A).

420 After normalization, 13,010 genes were tested in *D. simulans,* which also showed a higher number of
421 genes downregulated in males (1871 genes) compared to females (1683 genes), but the difference is
422 less than in *D. melanogaster*, where males had roughly 2-fold higher numbers of downregulated genes
423 relative to females (Figure 5B, Table S1). In females, 633 genes were upregulated, whereas 503 genes
424 increased expression under infection in males. Both sexes downregulated 850 genes and upregulated
425 270 genes in common in *D. simulans*. The treatment by sex interaction, was tested separately for *D.*
426 *melanogaster* and *D. simulans* (comparison within species). There were 994 genes (*D. melanogaster*)
427 and 1041 genes (*D. simulans*) that showed sex differences in expression in response to infection with
428 FDR < 0.05 (Figure 5B, Table S1).

429 To compare across species for each sex, the interaction for the effect of treatment by species was tested
430 separately for females and males (File S6-S7). There were 11,849 genes for which the species could be
431 compared directly. In females, 569 genes were differentially regulated upon infection in the comparison
432 between *D. melanogaster* and *D. simulans* at FDR < 0.05 (File S8). In males, 477 genes have differential
433 regulation in the comparison between these two species (FDR < 0.05) (File S8). Additionally, 159 of the
434 differentially regulated genes are shared between females and males; thus, females showed unique
435 differential regulation for 410 genes and males for 318 genes (File S8).

436 **3.4 Comparison of gene expression profiles in female and male adults**

437 The analysis of the DEGs in the experimental groups indicated a complex pattern of sex dimorphism for
438 both species (Figure 6, File S4-S5). However, all experimental groups expressed immune response genes
439 such as AMPs at significantly higher levels than wound control, as expected upon infection. The volcano
440 plots of the four experimental groups showed that *D. melanogaster* females broadly expressed key
441 systemic immune and stress response genes at higher levels than the rest of the three experimental
442 groups. In *D. melanogaster*, both males and females upregulated AMPs, PAMP recognition proteins,
443 regulatory proteins, and stress response genes, but females expressed them at higher levels relative to
444 males. The *D. simulans* females also upregulated immune genes at higher levels than males, but
445 generally, the increase in expression was much lower than that for *D. melanogaster* females (Figure 6).

446 The comparison between species showed that females of the closely related species expressed quite
447 different sets of genes (Figure 6). Comparing across males and females of the species, males suppress
448 different genes than females do in both species. Downregulated genes in females of both species
449 included several female reproductive genes, for example, *Yp1* and *Yp2*. This pattern is explored further
450 in the enrichment analysis. Moreover, as mentioned earlier, the sex differences are visible in the case of
451 the up, and down-regulated gene numbers for females and males in both species; however, the sex
452 differences are more prominent when the data was separated based on the fold changes (FC) (Figure S1,
453 File S4-S5).

454 **3.5 Gene set enrichment analysis**

455 Gene set enrichment analysis (GSEA) was conducted using the Gene Ontology Biological Process (GO-BP)
456 annotation. The enriched GO-BP terms showed that males and females expressed defense and stress
457 response gene sets, but they used distinct strategies to different degrees in response to the bacterial
458 infection (File S9).

459 In *D. melanogaster*, 50 GO-BP terms were significantly enriched in females; among them, 49 were
460 upregulated, and only 1 was downregulated (Figure 7, Table S2, File S9). In males, a total of 101
461 significantly enriched GO-BP terms were identified, with 84 corresponding to upregulation and 17 to
462 downregulation (Figure 7, Table S2, File S9). Interestingly, 46 out of 49 GO-BP terms for upregulated
463 genes in females are also significant for upregulated genes in males, with 1 out of 1 GO-BP sets of genes
464 commonly suppressed in both sexes.

465 In *D. melanogaster*, the top 20 enriched GO-BP terms for upregulated genes in females showed immune
466 response terms such as defense response and response to gram-negative and -positive bacterium,
467 response to fungus, and antimicrobial humoral response (Figure 8, File S9). *D. melanogaster* males also
468 upregulated gene sets related to similar immune responses, such as response to gram-positive
469 bacterium, antibacterial humoral response, and response to fungus. Females (5 GO-BP terms) and males
470 (7 GO-BP terms) upregulated genes related to negative regulation of the immune and defense responses
471 (Figure 8, File S9). This could correspond to the modulation of immune responses through feedback
472 mechanisms (Tanji and Ip 2005; Vincent and Dionne 2021). The enriched terms corresponding to
473 downregulated genes showed that *D. melanogaster* females downregulate only one GO-BP term, lipid
474 metabolic process; however, in males, 17 GO-BP were identified where almost all of the terms were
475 related to metabolism (14 metabolic processes, 2 catabolic processes, 1 homeostasis) (Figure 8, File S9).

476 In *D. simulans*, 68 GO-BPs were significantly enriched in females; among them, 47 corresponded to
477 upregulated genes, and 21 downregulated genes (Figure 7, Table S2, File S9). In males, 72 total
478 significantly enriched GO-BP terms were identified, with 57 for upregulated genes and 15 for those that
479 were downregulated (Figure 7, Table S2, File S9). In *D. simulans*, the enriched groups among
480 upregulated genes of the males and females showed 42 commonly activated gene sets, whereas no
481 common GO terms were identified as enriched among downregulated genes (Figure 7, File S9). In *D.*
482 *simulans*, the top 20 GO-BP terms for the males and females corresponded to activation of the immune
483 response, such as response against gram-negative and positive bacteria, antimicrobial humoral
484 response, innate immune response, etc. (Figure 9, File S9). In addition, both males (6) and females (6)
485 had positively regulated immune-related terms among the top 20 most enriched categories. However,
486 females expressed less terms related to negative regulation of the immune response (3) than males (6)
487 in *D. simulans*. The males of *D. simulans* showed suppression of metabolism-related genes (11 metabolic
488 processes, 4 catabolic processes), which was also observed in *D. melanogaster* males (Figure 8-9). On
489 the other hand, females of *D. simulans* downregulated predominantly reproduction-related gene sets

490 such as eggshell formation, developmental processes involved in reproduction, etc. Thus, although
491 females of both species downregulated the iconic yolk protein genes, widespread downregulation of
492 reproductive genes occurred only in *D. simulans* females (Figure 8-9, File S9).

493 In comparisons between species, for females in *D. simulans*, there were 3 upregulated and 21 uniquely
494 downregulated gene sets, whereas for *D. melanogaster* females, there were 5 upregulated terms and 1
495 downregulated term (File S9). In addition, 44 upregulated terms showed overlap between females of *D.*
496 *melanogaster* and *D. simulans*, but there is no overlap among the downregulated terms. However,
497 males in both species had 56 upregulated and 12 downregulated GO-BP terms in common. There were
498 28 uniquely upregulated terms in *D. melanogaster* males and 5 downregulated terms, whereas in *D.*
499 *simulans* only 1 upregulated term and 3 downregulated terms were unique. Overall, the enrichment
500 analysis showed distinct sex dimorphism with respect to up- or downregulated functional categories of
501 genes in *D. melanogaster* and *D. simulans*. The analysis revealed differences between males and females
502 not only in upregulated biological processes but also in downregulated biological processes, which might
503 reflect different infection levels and control strategies for the sexes (File S9).

504 **3.6 Infection-induced differences in the expression of IMD pathway genes**

505 In the IMD pathway, the signal receptors and cellular response showed sex dimorphism in expression in
506 response to infection (Figure 10-11, File S10). This could be caused by differences in the regulation of
507 the IMD pathway between sexes and may be associated with sex differences in the effectiveness of the
508 immune response. In *D. melanogaster*, peptidoglycan recognition proteins (PGRP) of the IMD pathway
509 showed significantly higher expression in females for PGRP-LB and PGRP-LC than in males upon infection
510 (p-adj < 0.05) (Figure 10, File S10). The signal transducers for the IMD pathways were not significantly
511 different between sexes; however, the key IMD transcription factor Relish was significantly upregulated
512 more in males (log2FC = 2.16) than in females (log2FC = 1.54) in *D. melanogaster* (p-adj < 0.05). The
513 effector genes of IMD showed broad upregulation in both sexes, which includes AMPs such as *Attacins*
514 (*Att*), *Cecropins* (*Cec*), and *Diptericin-B* (*DptB*). In both sexes, *AttD* was the most highly expressed AMP; it
515 was upregulated around 20-fold in females, as compared to 15-fold in males (p-adj < 0.05) (File S10). In
516 addition, *CecB*, *CecA1*, *AttB*, *AttA*, and *DptB* were upregulated at significantly higher levels in females
517 relative to males (p-adj < 0.05) (File S10). Moreover, females exhibited upregulation in *Dro* (log2FC =
518 2.89), *DptA* (log2FC = 3.66), *CecC* (log2FC = 6.22), *CecA2* (log2FC = 6.08), and *AttC* (log2FC = 3.78), and
519 these genes are also strongly upregulated in males, *Dro* (log2FC = 2.63), *DptA* (log2FC = 3.38), *CecC*
520 (log2FC = 4.34), *CecA2* (log2FC = 4.46), and *AttC* (log2FC = 3.29) (File S10).

521 In *D. simulans*, the IMD pathway also showed female-biased upregulation in response to bacterial
522 infection, but not all sex differences in specific genes are conserved between species (Figure 11, Figure
523 S2, File S10). The focal signal-transducing innate immune signal receptor PGRP-LC is expressed
524 significantly higher in females relative to males in *D. simulans* when each group is compared with its
525 wound control (*p*-adj < 0.05) (Choe *et al.* 2005). Interestingly, PGRP-LF, which inhibits the activation of
526 IMD pathways by capturing circulating peptidoglycan, is highly upregulated in *D. simulans* females
527 (log2FC = 3.08) compared to the other three experimental groups, and also showed significant sex
528 differences with *D. simulans* males (log2FC = 1.34) (*p*-adj < 0.05) (Figure 11, File S10) (Maillet *et al.*
529 2008). The key IMD transcription factor Relish was upregulated in females (log2FC = 2.02) and males
530 (log2FC = 1.84) in *D. simulans*. The effector genes of IMD showed broad upregulation in both sexes,
531 which includes AMPs such as *Attacins* (*Att*), *Cecropins* (*Cec*), and *Diptericin-B* (*DptB*). In both sexes, *AttD*
532 was the most highly expressed AMP; it was upregulated around 14.6-fold in females, as compared to
533 13.3-fold in males (*p*-adj < 0.05) (File S10). In addition, *AttA*, *AttB*, *AttC*, *CecA1*, *CecA2*, *CecB*, *CecC*, *Dro*,
534 *DptA*, and *DptB* were upregulated at significantly higher levels in females relative to males (*p*-adj < 0.05)
535 (File S10). Overall, the IMD cellular effector molecules of *D. simulans* showed higher expression in
536 females than males. However, the degree of upregulation of these AMPs in *D. simulans* females is lower
537 on average than in *D. melanogaster* females (File S10).

538 **3.7 Regulators of IMD pathway genes**

539 The positive and negative regulators of the IMD pathway were analyzed for each sex in both species. In
540 the case of the IMD positive regulators (FBgg0001197), PGRP-SD, which can significantly contribute as a
541 'positive regulator' of the IMD pathway, showed significant differential expression between females
542 (7.64-Fold) and males (5.56-Fold) of *D. melanogaster* (*p*-adj < 0.05) (Figure S3, File S11) (Gelbart *et al.*
543 1996). Expression patterns of this gene also differed between females and males in *D. simulans* (*p*-adj <
544 0.05); however, both sexes of *D. simulans* showed lower levels of upregulation than their *D.*
545 *melanogaster* counterparts. In addition, expression of the gene encoding Charon, which can directly
546 activate Rel/NF-kB, was upregulated in males (log2FC = 1.19) to a higher degree than in females (log2FC
547 = 0.36) in only *D. melanogaster* (*p*-adj < 0.05). Differences in the 'negative regulators' of IMD
548 (FBgg0001196) also included significantly higher expression of PGRP-SC2, a down-regulator of the IMD
549 pathway, and PGRP-LB in *D. melanogaster* females relative to males (*p*-adj < 0.05; Figure S3, File S11)
550 (Bischoff et al. 2006; Guo et al. 2014). While PGRP-LB is one of the prominent receptors involved in the
551 recognition and activation of the IMD pathway, researchers have found that it enables females to

552 reduce IMD activity upon a decrease in bacterial numbers (Zaidman-Rémy et al. 2006). Thus, the
553 significant upregulation of two negative regulators of IMD, PGRP-LB and PGRP-SC2, in *D. melanogaster*
554 females tempts us to speculate that in females, there is more controlled IMD expression relative to
555 males (Bischoff et al. 2006; Zaidman-Rémy et al. 2006; Guo et al. 2014). Additionally, the negative
556 regulator of IMD, PGRP-LB and PGRP-SC2, were not highly upregulated in *D. simulans* compared to *D.*
557 *melanogaster* sexes (*p*-adj < 0.05). However, another negative regulator, PGRP-LF, is highly upregulated
558 in *D. simulans* females (log2FC = 3.08) compared to males (log2FC = 1.34) (*p*-adj < 0.05) (Figure S3)
559 (Maillet et al. 2008). Overall, the IMD pathway is involved in the response to *P. rettgeri* via effector
560 molecules for females and males in a sex differential manner in *D. melanogaster* and *D. simulans*;
561 however, higher upregulation of the negative regulators such as PGRP-LB, PGRP-SC2, and PGRP-LF in
562 females might indicate that these flies activated IMD pathways at a level where suppression was
563 initiated, whereas other experimental groups did not reach that elevated level.

564 **3.8 Infection-induced differences in the expression of Toll pathway genes**

565 The Toll pathway was originally identified as an effector pathway activated upon gram-positive bacterial
566 and fungal infection, but recent studies show it also plays a crucial role in the responses to gram-
567 negative bacterial infections and viral infections (Valanne et al. 2022). In *D. melanogaster*, signal
568 receptor PGRP-SD was more elevated in females than males (*p*-adj < 0.05) (Figure 10, Figure S2, File
569 S10). In addition, the extracellular molecule *Serine Protease Immune Response Integrator* (*spirit*) showed
570 significant differences between females (log2FC = 3.81) and males (log2FC = 1.73) (*p*-adj < 0.05). The
571 males significantly upregulated *spz;* Spz is a core ligand of Toll receptor (Tl)*,* relative to females in *D.*
572 *melanogaster* (*p*-adj <0.05) (Valanne et al. 2011). The 'intracellular signal transducers' of Toll had no
573 significant changes in regulation between sexes, including the key NF-κB-related transcription factors
574 Dorsal-related immunity factor (Dif) and Dorsal (dl) (Valanne et al. 2011). In contrast, the effector
575 molecules showed higher upregulation of *Drosomycin* (*Drs*), *Defensin* (*Def*), and *Metchnikowin-like*
576 (*Mtkl*) in females relative to males in *D. melanogaster* (*p*-adj < 0.05). *Mtkl* was the most highly expressed
577 Toll-AMP for both sexes in *D. melanogaster*, which was upregulated by approximately 10-fold in females
578 and 7-fold in males (*p*-adj < 0.05). Interestingly, the gene encoding focal antifungal peptide-producing
579 AMP, *Drosomycin* (*Drs*), was also upregulated in both sexes, but other antifungal AMP genes such as
580 *Daishos* (*Dso1* and *Dso2*) and *Drosomycin-like* (*Drs1-6*) showed no significant increase in expression
581 upon bacterial infection, instead some of them including *Dso1* and *Dso2* showed significant

582 downregulation, where these genes were downregulated in males to a higher degree than in females (*p*-
583 adj < 0.05) (Figure 10, File S10).

584 *D. simulans* showed analogous expression patterns to *D. melanogaster* in the extracellular portion of the
585 Toll Pathway. PGRP-SA and PGRP-SD were significantly upregulated at higher levels in females relative to
586 males (*p*-adj < 0.05), but the degree of upregulation is lower than in *D. melanogaster* (Figure 10-11, File
587 S10). This also holds true for the expression of *spirit*, which exhibits significant sex differences (*p*-adj <
588 0.05), although the expression level is lower compared to *D. melanogaster*. The gene *spz* was
589 significantly overexpressed in males relative to females (*p*-adj < 0.05), as also identified in *D.*
590 *melanogaster* (Figure 10-11). In *D. simulans*, the key NF-κB-related transcription factors *Dorsal-related*
591 *immunity factor* (*Dif*) and *Dorsal* (*dl*) showed no significant differences between sexes (Valanne et al.
592 2011). The effector molecules showed higher upregulation of *Drosomycin* (*Drs*), *Defensin* (*Def*), and
593 *Metchnikowin* (*Mtk*) in females relative to males In *D. simulans* (*p*-adj < 0.05). Interestingly, *Drs* was also
594 upregulated in both sexes, but other antifungal AMPs such as *Drosomycin-like* (*Drsl*) *2*, *Drsl 4*, and *Drsl 5*
595 showed significant downregulation upon bacterial infection, where females showed higher levels of
596 downregulation than males did (*p*-adj < 0.05) (Figure 10, File S10).

597 In between species comparisons, *D. simulans* showed significant downregulation of *SPE*, unlike *D.*
598 *melanogaster*, with females (log2FC = -1.3) exhibiting more downregulation than males (log2FC = -0.44)
599 (*p*-adj < 0.05). Another key difference was observed in the expression of Clip-SP *Persephone (psh)*, *D.*
600 *simulans* females (7-Fold) and males (4-Fold) upregulated *psh*, where the reverse direction is observed
601 in females (log2FC = -0.81) and males (log2FC = -0.45) of *D. melanogaster*. Overall, these findings
602 suggest that *D. simulans* might be using a different route to stimulate Toll pathways other than PRR base
603 activation.

604 **3.9 Regulators of Toll pathway genes**

605 The regulation of the Toll pathway is much more complex than the regulation of IMD, as there are
606 diverse functions performed by the Toll pathway in addition to immunity. Drosophila possesses six
607 distinct Toll receptors, each contributing individually or in concert to accomplish complex
608 multidimensional functions; the data showed none of the Toll receptors were sex differentially
609 expressed in either species (Valanne et al. 2011) (File S11). However, the *spz* activation pathway, which
610 is critical to the regulation of the Toll pathway as Spz can be activated in multiple ways, such as PRR-
611 based, Psh-SPE-Spz, and Psh-Protease-Spz, shows apparent sex differences. Multiple genes in this
612 pathway are expressed in a sex differential manner, such as *spirit, psh*, *Hayan*, and *PGRP-SC2* (Figure S4,

613 File S11). The sex differential regulation of *spirit* and *psh* has previously been discussed with respect to
614 the overall Toll pathways (Duneau et al. 2017b). There were also female-biased sex differences for
615 wound-related serine protease Hayan in *D. simulans* (*p*-adj < 0.05), which is involved in the activation of
616 the melanization cascade as well as Spz based Toll pathway activation (Yamamoto-Hino and Goto 2016;
617 Dudzic et al. 2019). *Hayan* was also upregulated in females (log2FC = 3.22) and in males (log2FC = 2.45)
618 in *D. melanogaster*. The gene encoding PGRP-SC2, which acts as a negative regulator of the IMD
619 pathway, is expressed at higher levels upon infection in females (log2FC = 3.59) than males (log2FC =
620 3.15) in *D. melanogaster* (*p*-adj < 0.05) (File S12) (Bischoff et al. 2006). *PGRP-SC2* was also upregulated in
621 both females (log2FC = 1.60) and males (log2FC = 1.56) in *D. simulans*. (File S12). Aside from the *spz*
622 activating pathway, positive (FBgg0001057) and negative (FBgg0001053) regulators of the Toll pathway
623 showed no significant sex differences in either species (Figure S4, File S11).

624 Overall, considering the Toll pathway and its regulators, suggests there is female-biased sex differential
625 upregulation of Toll-AMPs for both species; however, the degree of upregulation is higher in *D.*
626 *melanogaster* than in *D. simulans* like IMD-AMPs. A comparison between species detected a remarkable
627 divergence in the Toll activation process, where *D. simulans* employed both PRR-dependent and
628 independent mechanisms, while the closely related *D. melanogaster* solely upregulated genes related to
629 PRR-dependent activation.

630 **3.10 Other immune response molecules and pathways**

631 The innate immune response of Drosophila is a complex interplay of conserved innate immune signaling
632 pathways and other immune molecules, along with the involvement of immune cells. Several Drosophila
633 immune-induced molecules (DIMs) have been identified in the fly hemolymph (Uttenweiler-Joseph et al.
634 1998). Recent research revealed that twelve related immune-induced molecules, known as the
635 Bomanins (Boms), constitute a family that plays a role in defending against certain fungi and bacteria
636 (Clemmons et al. 2015). Three of the twelve Bomanins (Boms) in *D. melanogaster*—*BomT1*, *BomS5*, and
637 *BomBc3*—are expressed in a sex-differential manner (*p*-adj < 0.05). (Figure S5, File S11). *BomT1* is
638 upregulated to a greater degree in females (log2FC = 2.59) relative to males (log2FC = 1.53) (*p*-adj <
639 0.05). Opposing sex differences were observed for *BomS5*, where females upregulated, and males
640 downregulated expression (*p*-adj < 0.05). *BomBc3* was downregulated in both sexes in *D. melanogaster*,
641 but in males, it was downregulated to a greater extent than in females (*p*-adj < 0.05). However, only
642 *BomS2* and *BomS4* showed significant sex differences in *D. simulans* (*p*-adj < 0.05). In the case of *BomT1*,
643 upregulation was observed in both females (log2FC = 1.18) and males (log2FC = 1.03) in *D. simulans*.

644 *BomBc3* expression is different from that of *D. melanogaster,* where *BomBc3* is downregulated in
645 females (log2FC = -1.36) and males (log2FC = -1.10) (Figure S5, File S11). The expression of the Boms and
646 their regulators showed marked species differences among the sexes.

647 Melanization is one of the extraordinary features of insect innate immunity and is regulated by Toll
648 pathways in terms of the gene expression of serine proteases (SPs) and serpins (Spn) (Dudzic et al.
649 2019). PGRP-SA and GNBP1/ β-Glucan Recognition Proteins (βGRP) can directly modulate the expression
650 of the melanization cascade genes (Matskevich et al. 2010). In the case of core serine proteases, *Hayan*
651 and *SP7*/*MP3* both show significant sex differential expression in *D. simulans* (*p*-adj < 0.05) (Figure S5,
652 File S11). Although *D. melanogaster* females and males upregulated Hayan and SP7 more than wound
653 control, no significant sex differences were observed. In both species, the level of *SP7* upregulation is
654 lower than that of *Hayan*. Although key SPs are upregulated, there is downregulation of the key
655 *prophenoloxidases* (*PPO*), *PPO1*, and *PPO2* (Figure S5, File S11). In addition, there is significant
656 upregulation of the negative regulator of melanization, *Spn28Dc*, in both species. With higher
657 upregulation in females (log2FC = 2.99) relative to males (log2FC = 1.15) in *D. melanogaster* (*p*-adj <
658 0.05) and a similar pattern found in the females (log2FC = 3.78) and males (log2FC = 2.07) of *D. simulans*
659 (*p*-adj < 0.05) (Figure S5, File S11). These data strongly suggest that the melanization cascade was not
660 activated; thus, Hayan and SP7 might be utilized by other immunological processes, such as Toll
661 activation, as has been observed in other studies (Yamamoto-Hino and Goto 2016; Dudzic et al. 2019).

662 In *Drosophila*, the Jak-STAT pathway is crucial for regulating antimicrobial peptide (AMP) production in
663 response to bacterial infection and antiviral response (Myllymäki & Rämet, 2014). The cytokine-like
664 ligands *upds*, signal receptor *domeless* (*Dome*), *Janus kinase* (*JAK*), *hopscotch (hop)*, and the Drosophila
665 STAT transcription factor, *stat92E*, were not sex-differentially expressed in either species (Figure S7, File
666 S10-11). Despite the lack of significant sex differences in transcript level in upstream parts of this
667 pathway, some of the effector molecules, such as *Turandot C* (*TotC*), *TotA*, *TotX*, and *TotM*, were
668 significantly upregulated compared to the wound control in males of *D. melanogaster (p*-adj < 0.001*)*;
669 this pattern is absent in females of *D. melanogaster* and both sexes of *D. simulans* (Figure 10-11). The
670 study found no sex differences in components of negative regulators in either species (Figure S7, File
671 S11). JNK is also known as the TNFalpha-Eiger pathway in Drosophila, which, like Jak-STAT, has a diverse
672 set of functions with a key role in immune responses, development, and morphogenesis (Gan et al.
673 2021). The gene expression analysis found that JNK receptors, protein kinase, and regulators showed no
674 significant sex differences (Tafesh-Edwards and Eleftherianos 2020)(Figure S7, File S11).

675 Finally, apart from PGRPs of the IMD and Toll pathways, our data found *PGRP-SB1*, an antibacterial
676 PGRP, was more upregulated in females (log2FC = 2.90) relative to males (log2FC = 2.11) in *D.*
677 *melanogaster* (*p*-adj < 0.05) (File S11). The upregulation is also conserved in *D. simulans*, where females
678 (log2FC = 1.71) upregulate *PGRP-SB1* more than males (log2FC = 0.84) (*p*-adj < 0.05) (File S11). Prior
679 works on *PGRP-SB1* report that it can be significantly upregulated upon IMD pathway activation by the
680 gram-negative bacterial infection, which is consistent with our findings (Zaidman-Rémy et al. 2011). The
681 analysis of *thioester-containing proteins* (*Teps*) identified that only *Tep2* showed differential expression
682 compared to wound control in females (log2FC = 2.38) and males (log2FC = 1.98) in *D. melanogaster*
683 (Figure S6, File S11). The females (log2FC = 0.44) and males (log2FC = 0.32) also upregulated *Tep2* in *D.*
684 *simulans*, but the degree of upregulation is significantly lower than in *D. melanogaster* counterparts
685 (Figure S6, File S11).

686 **4. Discussion**

687 The emergence of sex differences in immune responses is thought to originate from complex
688 evolutionary dynamics including trade-offs and sexual conflict (Zuk and Stoehr 2002; Scharf et al. 2013;
689 Schwenke et al. 2016). The interplay between the immune system and various physiological processes
690 can result in trade-offs between immunity and other energetically costly life-history traits, such as
691 reproduction (Zuk and Stoehr 2002; Schwenke et al. 2016). Immune responses can also be sexually
692 dimorphic, with males and females showing different responses to the same pathogens in identical
693 environments (Lazzaro and Little 2009; Morrow 2015). Recently, there has been a growing interest in
694 adult immunity and the role of sex-biased gene expression in immune response pathways in generating
695 sex dimorphism in the overall immune response due to its impact on the medical sector (Fischer et al.
696 2015; Wilkinson et al. 2022). However, many studies in immunology have focused predominantly on one
697 sex, often males (Belmonte et al. 2020). The importance of including both sexes in immunological
698 studies is increasingly acknowledged and enables researchers to achieve a comprehensive
699 understanding of sex dimorphism, evolution, and molecular mechanisms of the immune response.

700 Our investigation of sex differences in immune response against *P. rettgeri* in *D. melanogaster* and *D.*
701 *simulans* found that survival differences may not be conserved, and sexes differ in survival in *D.*
702 *melanogaster*. Interestingly, we also find that although both sexes express similar immune response
703 genes for conserved immune pathways and some other immune response components, there is a strong
704 pattern of a robust response in *D. melanogaster* females relative to males. In *D. simulans*, females also
705 mounted higher immune gene expression, but the expression difference between the two sexes is not

706 as high as in *D. melanogaster* and did not confer a significant survival advantage. Moreover, both species
707 upregulate similar genes to inhibit infection, but broadly, the degree of up or downregulation is lower in
708 *D. simulans* than in *D. melanogaster*. Overall, these findings highlight species-specific variations in the
709 effectiveness of the immune response and the physiological impact of the infection. In addition, the
710 downregulation of metabolism-related gene sets in males of both species, and the downregulation of
711 reproductive genes in *D. simulans* suggests that each sex uses different strategies to modulate other
712 processes in response to the infection. Lastly, the evolutionarily conserved bacterial pathways IMD and
713 Toll exhibited sex-specific variation, encompassing not only effector molecules but also regulators, such
714 as those involved in negative feedback, in the context of infection progression. This suggests that the
715 IMD pathway responds more in a controlled manner in *D. melanogaster* females than in the rest of the
716 three groups. Moreover, in *D. simulans,* infected animals used two distinct strategies at sixteen hours
717 post-infection, canonical PRR-SPE-Spz, and microbial proteases-based Persephone cascade, to activate
718 the Toll pathways.

719 **4.1 Survival of unmated males and females differs between the sexes and species**

720 The survival rates of unmated male and female flies were monitored over a 36-hour period following
721 infection. In *D. melanogaster*, females showed significantly higher survival rates than males from 18hpi
722 onward. In *D. simulans*, while females had slightly higher mean survival rates than males, the difference
723 was not statistically significant. In previous studies, females have been observed to represent higher
724 survival rates than males, for example, in gram-positive *S. aureus* infection in *D. melanogaster* (Duneau
725 et al. 2017b). However, this same study also found that *D. melanogaster* females were more susceptible
726 to *P. rettgeri* (gram-negative), *P. alcalifaciens* (gram-negative), and *E. faecalis* (gram-positive) infections
727 (Duneau et al. 2017b). In addition, two individual studies of gram-negative *Pseudomonas aeruginosa*
728 infections showed opposite-sex survival bias direction (Chowdhury et al., 2019; Vincent & Sharp, 2014).

729 Sex dimorphism in survival rates appears to depend on sex, host strains, type of infection, and external
730 conditions in *Drosophila* (Howick and Lazzaro 2014; Rai et al. 2023). It has also been shown that even
731 when the pathogen and host are identical, mating status, diet, age, and injection site, among other
732 differences, can alter the outcome of the infection (Lazzaro and Little 2009; Short and Lazzaro 2010;
733 Howick and Lazzaro 2014; Rai et al. 2023). Therefore, results from different studies may vary due to
734 differences between laboratories or due to specific differences in the study design.

735 In our study, both males and females were unmated, whereas in the Duneau et al. 2017 study, males
736 were mated, and then compared to mated or unmated females (Duneau et al. 2017b). Studies have

737 indicated that mated females exhibit increased susceptibility to infection, elevated pathogen loads, and
738 reduced capacity to initiate immune responses, which could explain why unmated females in our study
739 had better outcomes than in studies that focused on mated animals (Short and Lazzaro 2010; Schwenke
740 and Lazzaro 2017; Rai et al. 2023).

741 To the best of our knowledge, the post-infection survival of *D. simulans* has not been previously
742 reported; thus, a direct comparison to existing studies is not possible. However, when comparing
743 between species, *D. melanogaster* females had significantly higher survival rates relative to *D. simulans*
744 females after the infection was established. Similarly, *D. melanogaster* males showed significant
745 differences in survival rates compared to *D. simulans* males from 18hpi. Overall, there are both sex- and
746 species-based differences in survival rates, with *D. melanogaster* females showing the highest survival
747 rates. Previous studies have shown variation within the same species based on different conditions, as
748 our results also show compared to prior studies. Here, we have different species in the same
749 environment and with concurrent experiments where both sexes and species can be directly compared.
750 This means that the difference we observe between *D. melanogaster* and *D. simulans* is likely to be
751 genetic, although we note that evaluation of additional strains is needed to establish fixed species-level
752 differences. This study showed that the impact of sex on survival may have diverged between these
753 species and both sex and species effects may be contributing to differences observed.

754 **4.2 Distinct patterns of up and down gene regulation are concordant with differences in survival**

755 The up and downregulation of gene expression in the context of stress is a common phenomenon
756 (Lecheta et al. 2020; Girgenti et al. 2021). There are fewer upregulated genes than downregulated for
757 both females and males in both species. *D. melanogaster* females downregulated 977 genes compared
758 to 2269 in males, which is consistent with lower survival rates of males and may indicate higher stress
759 conditions in males relative to females. Compared to the 1871 genes downregulated in *D. simulans*
760 males and 1683 genes in females, *D. melanogaster* females downregulated fewer genes. A similar
761 pattern of up and down-regulation was observed upon Sigma Virus infection in *D. melanogaster*, where
762 males downregulated 336 genes compared to 88 genes in females (Carpenter et al. 2009).

763 The enrichment analysis indicated differences between sexes in which types of genes were
764 downregulated in *D. melanogaster*. Only one GO-BP category was significantly enriched among
765 downregulated genes in females, whereas 17 metabolism-related downregulated categories were
766 identified in males. This is consistent with the idea of higher stress levels in infected males relative to

767 females in *D. melanogaster*. Likewise, *D. simulans* males downregulated 15 GO-BP categories, among
768 which metabolism-related genes were predominant.

769 Previous studies also found a trade-off between immune and metabolic processes in males upon
770 bacterial infection in *D. melanogaster* (Schlamp et al. 2021). Schlamp et al. showed that the activation
771 of the IMD pathways affects the metabolic homeostasis of *Drosophila* males (Schlamp et al. 2021). *D.*
772 *simulans* females may also have been under increased levels of stress because of infection, given that
773 there was extensive downregulation of reproductive GO: BP terms, such as eggshell formation and
774 reproductive developmental processes. Multiple studies have reported trade-offs between immune
775 response and reproduction in both males and females in *Drosophila* as well as other insects (McKean
776 and Nunney 2001; Short et al. 2012; Schwenke et al. 2016). The previous research on *Drosophila*
777 infected with *P. rettgeri* revealed that females halt egg-laying during the acute phase of infection, with a
778 return to normalcy observed during the chronic phase (Howick and Lazzaro 2014) and upon DNA virus
779 Kalithea (KV) infection, females experience degeneration of ovarian tissue and reduced fecundity
780 (Palmer et al. 2018). Additionally, viral-induced female infertility was found upon flock house virus (FHV)
781 infection due to the destruction of oocytes (Thomson et al. 2012). Overall, *D. melanogaster* females
782 downregulated fewer genes, and had fewer functional categories that were consistently downregulated,
783 while at the same time upregulating immune-related genes to a greater degree, which may be related to
784 their higher survival rates.

785 **4.3 Sex and species differences in the IMD pathway**

786 IMD receptors and effector molecules were upregulated in females and males in both species. In
787 general, females showed higher levels of expression than males, especially the effector molecules such
788 as AMPs. There is a noticeable degree of higher upregulation in *D. melanogaster* flies compared to *D.*
789 *simulans*. As the IMD pathway is specialized in response to gram-negative bacterial infection, *P. rettgeri*
790 infection is expected to elicit a higher level of IMD-regulated AMP expression than other core immune
791 response pathways. There was a consistent pattern of female-biased sex differential expression in both
792 species for IMD-AMPs; however, it was also evident that the sexes of *D. simulans* consistently showed
793 lower levels of upregulation than *D. melanogaster* sexes.

794 The species-level expression difference is also visible in the positive and negative regulators of the IMD
795 pathway; for instance, PGRP-SD, primarily associated with the Toll pathway, significantly contributes to
796 the positive regulation of the IMD pathway by amplifying the recognition of peptidoglycans (Tanji and Ip
797 2005; Myllymäki et al. 2014). Our results showed that *D. melanogaster* males significantly upregulate

798 the gene *Charon,* which can directly activate the NF-kB/Relish at AMP promoters in males via a PARP-
799 1/Relish/Charon-dependent innate immune response (Ji et al. 2016). Alternatively, the knockdown of
800 *Charon* expression plays a vital role in the translocation of Relish to the nucleoplasm, and blocks that
801 function making flies vulnerable to bacterial attack (Ji et al. 2016). The influence of the PARP-
802 1/Relish/Charon-dependent response might act in conjunction with greater upregulation of NF-
803 kB/Relish in *D. melanogaster* males, relative to females; males may amplify the response to compensate
804 for lower upregulation of signal receptors (PRRs) (File S11). This indicates that sex differences are likely
805 to be complex, and interactions between different genes and pathways will need to be untangled to
806 delineate key mechanisms (Ji et al., 2016).

807 The precise modulation of energetically demanding immune pathways is crucial to halt the progression
808 of infection. Over-activation of the IMD pathways can severely affect survival (Bischoff et al. 2006). A
809 comprehensive analysis of the negative regulators of IMD pathways was conducted, revealing that *D.*
810 *melanogaster* and *D. simulans* exhibited sex differences in expression of the genes encoding certain
811 proteins, namely PGRP-LB, PGRP-SC2, PGRP-LF, and Pirk, which play roles in the negative regulation of
812 IMD pathways. *D. melanogaster* females and males both upregulated PGRP-LB, PGRP-SC2, PGRP-LF, and
813 Pirk, but differed in the degree of upregulation. PGRP-LB works as an activator of the IMD pathway, but
814 excessive upregulation of PGRP-LB can trigger a negative feedback loop for the IMD pathway (Bischoff et
815 al. 2006; Vincent and Dionne 2021). *D. melanogaster* females significantly upregulated PGRP-LB more
816 than males, and both sexes of *D. simulans. D. melanogaster* females also upregulated IMD-regulated
817 AMPs at a significantly higher level than the rest of the groups. The significant overexpression of PGRP-
818 LB could either activate the IMD pathway to more efficiently eliminate pathogens, or lead to the
819 pathway's downregulation via negative feedback in *D. melanogaster* females (Figure S2, File S11).

820 In addition to PGRP-LB, PGRP-SC2, another negative regulator of IMD pathways, also showed
821 significantly higher upregulation in *D. melanogaster* females relative to others. Another member of the
822 PGRP, PGRP-LF, is a membrane-associated protein, but it cannot bind to peptidoglycans directly (Persson
823 et al. 2007). PGRP-LF interacts with PGRP-LC and functions as a negative regulator of IMD signaling
824 (Maillet et al. 2008; Basbous et al. 2011). Both females (log2FC = 2.61) and males (log2FC =2.27)
825 upregulated PGRP-LF higher than control in *D. melanogaster*, with no significant difference between the
826 sexes. Interestingly, PGRP-LF is highly upregulated in *D. simulans* females compared to the other three
827 groups (Maillet et al. 2008). Another PGRP-LC interacting protein, Pirk, which is involved in the negative

828 regulation of IMD, was upregulated at significantly higher levels in females and males of both species
829 (Vincent and Dionne 2021).

830 Overall, the IMD pathway was employed in a sex-differential manner in *D. melanogaster* and *D.*
831 *simulans*. However, upon comparing these four "brakes" (PGRP-LB, PGRP-SC2, PGRP-LF, and Pirk) of IMD
832 across experimental groups, a noticeable trend emerges where *D. melanogaster* females exhibit higher
833 upregulation compared to other groups; this might indicate that *D. melanogaster* females activated the
834 IMD pathways at a level where negative regulation was initiated, whereas other experimental groups
835 were unable to reach that elevated level. The reason behind the significant upregulation of PGRP-LF and
836 Pirk in *D. simulans* remains unclear. This is particularly ambiguous considering the survival data, where it
837 is evident that *D. simulans* was not effectively handling the infection at 16hpi (Figure 2-3, Figure 10-11,
838 File S11).

839 Despite the upregulation of negative regulators, *D. melanogaster* females upregulate effector genes of
840 the IMD pathway to a higher degree than males or *D. simulans;* this may reflect greater control of
841 expression of effectors, as well as specific dynamics in female *D. melanogaster* in which upregulation of
842 negative regulators is a response to the high levels of effector expression at 16hpi. This would then be
843 expected to reduce effector expression at later time points, which was not possible to test from this
844 experimental data. The key transcription factor Relish/NF-κB is upregulated less than males at 16hpi for
845 *D. melanogaster* females; this might suggest there would be downregulation of the AMPs at later time
846 points than those measured (Zaidman-Rémy et al. 2006). Eventually, the increased expression of genes
847 involved in negative feedback loops regulating IMD in *D. melanogaster* females would be expected to
848 result in reduced signaling and potentially reduced expression of other genes in the IMD pathway (Tanji
849 and Ip 2005; Zaidman-Rémy et al. 2006; Vincent and Dionne 2021). These findings provide valuable
850 insights into the intricate mechanisms underlying sex-specific immune responses and suggest a balance
851 between gene activation and suppression as part of an adaptive strategy that contributes to increased
852 survival in females.

853 **4.4 Sex and species differences in the Toll pathway**

854 Toll is another conserved signaling pathway and is involved in many functionally different processes,
855 including innate immunity. Broadly, Toll-regulated AMPs were also upregulated in a female-biased
856 manner in both species. The comparison between species revealed higher upregulation in *D.*
857 *melanogaster* relative to *D. simulans,* which is also observed for IMD-regulated AMPs. However, the
858 level of Toll-AMP upregulation is lower than it is for IMD-regulated AMPs in both sexes for *D.*

859 *melanogaster* and *D. simulans.* This is expected because Toll is a more specialized response against
860 gram-positive bacterial and fungal infections (Valanne et al. 2011). In the IMD pathway, the core
861 transcription factors Relish showed sex differential expression for both species, but neither of the key
862 transcription factors (Dif and Dorsal) in the Toll pathway were expressed differentially in either species.
863 This suggests that the expression of Dif and Dorsal are not directly responsible for sex differences in the
864 expression of AMPs, although sex differences may result from post-transcriptional regulation or
865 differences in transcript levels prior to 16hpi (Schlamp et al., 2021). In addition, the Toll-associated
866 AMPs might be regulated by other noncanonical transcriptional mechanisms to elicit sex dimorphism
867 (Tanji and Ip 2005).

868 Sex differential regulation was detected for signal receptors and cellular response genes for both
869 species. Among these genes involved in *spz* activation, PGRP-SA, PGRP-SD, and Spirit were upregulated
870 in females and males of both species. This is expected to be associated with PRR-dependent Toll
871 activation. In addition, Clip-SP Hayan, one of the serine proteases of the melanization cascade, was
872 significantly overexpressed in females of both species relative to males (p-adj < 0.001) (Figure S5, File
873 S11). The analysis also detected a notable trend where females (7-Fold) and males (4-Fold) of *D.*
874 *simulans* drive significant upregulation of Clip-SP Psh (File S10). Both Clip-SP Hayan and Clip-SP Psh play
875 a vital role in regulating the Toll pathway downstream of Clip-SP Grass, ModSP, and PRRs (Yamamoto-
876 Hino and Goto 2016; Issa et al. 2018; Dudzic et al. 2019).

877 Persephone, a circulating serine protease, functions as an immune sensor for microbial proteolytic
878 activities upstream of the Toll pathway by detecting exogenous proteases (Chamy et al. 2008; Issa et al.
879 2018). Upon detection, Persephone can elicit Toll activation in two different ways, through Psh-SPE-Spz
880 or Psh-Protease-Spz (SPE independent) activation (Ligoxygakis et al. 2002; Gottar et al. 2006; Chamy et
881 al. 2008; Yamamoto-Hino and Goto 2016). Although it is not established that Hayan can detect microbial
882 proteases, it plays a vital role in both PRR-independent activation mechanisms (Dudzic et al. 2019).
883 Previous studies showed that animals with SPE null mutations can perform the pro-Spz to Spz cleavage,
884 suggesting that another SP can cleave Spz beyond SPE (Yamamoto-Hino and Goto 2016; Dudzic et al.
885 2019).

886 It is also possible that Hayan is upregulated to promote the melanization cascade, but the other key
887 members of the Hayan-dependent blackening reaction, PPO1, and PPO2, were downregulated in *D.*
888 *simulans* (Binggeli et al. 2014; Dudzic et al. 2015, 2019). In addition, the regulation of Hayan is strictly
889 controlled by the serpins (Spn) for melanization (Binggeli et al. 2014). Analysis of the negative regulators

890 of the melanization cascade reveals that Spn28Dc, a member of the Serpin family, which can negatively
891 regulate the melanization process, is significantly upregulated in females and males of *D. simulans* and
892 *D. melanogaster* (Ligoxygakis 2002; Tang 2009; Binggeli et al. 2014). This indicates that Hayan is more
893 likely to be involved in the PRR-independent Toll activation rather than melanization.

894 Based on the observation, we hypothesize that significant downregulation of SPE in females (log2FC = -
895 1.31) and males (log2FC = -0.44) in *D. simulans* coincides with the use of the alternate Toll activation
896 pathways, whereas females (log2FC = 0.91) and males (log2FC = 0.65) of *D. melanogaster* might
897 sufficiently upregulate SPE; thus *D. melanogaster* is not depending on the microbial proteases-based
898 Psh-SPE-Spz or Psh-Protease-Spz (SPE independent) activation at 16hpi (Issa et al. 2018; Dudzic et al.
899 2019). In contrast, Toll responses in *D. simulans* might be enhanced by the additional microbial protease
900 based Psh-SPE-Spz or Psh-Protease-Spz (SPE independent) activation allowing maintenance of
901 appropriate Spz levels (Yamamoto-Hino and Goto 2016; Issa et al. 2018; Dudzic et al. 2019). We note
902 that it is not possible to make conclusions from our single timepoint data, although our results support
903 the investigation of the hypothesis that SPE at 16hpi is downregulated, with *D. simulans* flies using Psh-
904 Protease-Spz (SPE independent) activation.

905 Immune responses incur significant energetic costs for organisms. Therefore, the success of an organism
906 depends not only on the activation of immune pathways but also on their effective suppression. Among
907 the suppressors of Spz activation and the Toll pathway, two of the Serpins family members named
908 Spn88Ea and Spn42Dd were overexpressed in both sexes of *D. melanogaster* (p-adj < 0.001). The role of
909 the Spn88Ea and Spn42Dd is to negatively regulate the production of antifungal peptides via the Toll
910 pathway (Reichhart et al. 2011). As mentioned earlier, there is less expression of the fungal-related
911 AMPs except for *Drosomycin (Drs),* which has multiple roles as antipathogenic peptides, including
912 function against bacterial infection (Hanson and Lemaitre 2020; Lin et al. 2020). These findings also
913 explain the downregulation of the fungal cell wall pattern recognition receptor GNBP3 in the infected
914 females (log2FC = -0.60) and males (-0.61) relative to wound control in *D. melanogaster* (Figure S2, File
915 S10).

916 **5. Conclusion**

917 Our data showed that similar genes and pathways are upregulated in males and females, but the degree
918 of upregulation differs between sexes and species. The comparative analysis revealed that these sex
919 differences in the upregulation of immune-related biological processes are conserved between the two
920 closely related species examined; interestingly, the sexes appear to suppress different sets of genes,

921 indicating potential differences in trade-offs and resulting strategies. However, there are differences
922 between species, both *D. melanogaster* sexes show higher upregulation of immune genes relative to *D.*
923 *simulans*. Moreover, among the four experimental groups, the *D. melanogaster* female upregulated
924 immune genes at the highest level relative to males and *D. simulans* of both sexes – which is concordant
925 with the differences observed in survival. The downregulation of biological processes in females and
926 males showed significant differences in both species. Although the pattern of downregulated GO terms
927 is similar in males between species, the categories corresponding to downregulated genes in females
928 are significantly different.

929 It has been reported in prior studies that Toll and IMD are responsible for the sex differences in *D.*
930 *melanogaster*, and our results showed there were sex differences in the regulation of both conserved
931 bacterial infection response pathways, IMD and Toll, which supports these previous studies (Duneau et
932 al. 2017b; Vincent and Dionne 2021). Intriguingly, our data strongly suggest that *D. melanogaster* and *D.*
933 *simulans* employ two mechanisms to activate Toll pathways differently during infection by *P. rettgeri*,
934 with *D. melanogaster* using the canonical PRR-based Toll activation, and *D. simulans,* the microbial
935 protease recognition-based Persephone pathway to stimulate Toll effectors along with the canonical
936 path. The AMP expression level of *D. melanogaster* is consistent with the hypothesis that PRR-SPE-Spz
937 activation alone can mount a heightened immune response in *D. melanogaster* relative to both
938 processes in *D. simulans*. The IMD pathway genes showed sex-differential expression and varied in the
939 degree of expression across species. The analysis of the negative regulators showed *D. melanogaster*
940 females were upregulating four key "brakes" (PGRP-LB, PGRP-SC2, PGRP-LF, and Pirk) to prevent the
941 overstimulation of IMD pathways at 16hpi. Overall, our finding suggests that sex differences in the
942 expression of immune genes are typically conserved in the two species, for most of the upregulated
943 genes with the key exception of negative regulators but noticeably different for downregulation; also,
944 there were significant sex differences in the fold-change of up- or downregulation between *D.*
945 *melanogaster* and *D. simulans*.

946 This study will significantly contribute to our understanding of the evolution of sex differences and point
947 to specific testable hypotheses that might explain observed species and sex differences in survival.
948 Further research involving both within and between-species comparisons is essential to ascertain
949 whether these differences are consistent across various contexts. The observed variation in infection
950 outcomes among different reports, even when using the same species and pathogens, emphasizes the
951 significance of considering multiple sources of variation within individual studies. Additionally,

952 conducting comparisons across a diverse range of time points, rather than relying on a single snapshot,
953 would provide a more comprehensive view of sex dimorphisms occurring at different stages of disease
954 progression.

955 **Data availability**

956 All raw and processed sequencing data generated in this study can be found on NCBI Gene Expression
957 Omnibus (GEO; https://www. ncbi.nlm.nih.gov/geo/) (in processing). The authors confirm that all data
958 supporting this article's conclusions are included in the article, figures, supplement figures, and
959 supplement tables.

960 **Acknowledgments**

961 We extend our gratitude to the Bloomington Stock Center for providing the flies. We also thank Brian
962 Lazzaro from the Department of Ecology and Evolutionary Biology at Cornell University for supplying the
963 bacterial strain. Our appreciation goes to Blake Rochester, Bayley Atkins, Isabella Blair, and Audrey
964 Powell for their assistance with fly work, and to the other members of the Graze lab for their valuable
965 discussions. RNA sequencing was conducted at the Genomic Sequencing and Analysis Facility, University
966 of Texas at Austin.

967 **Funding**

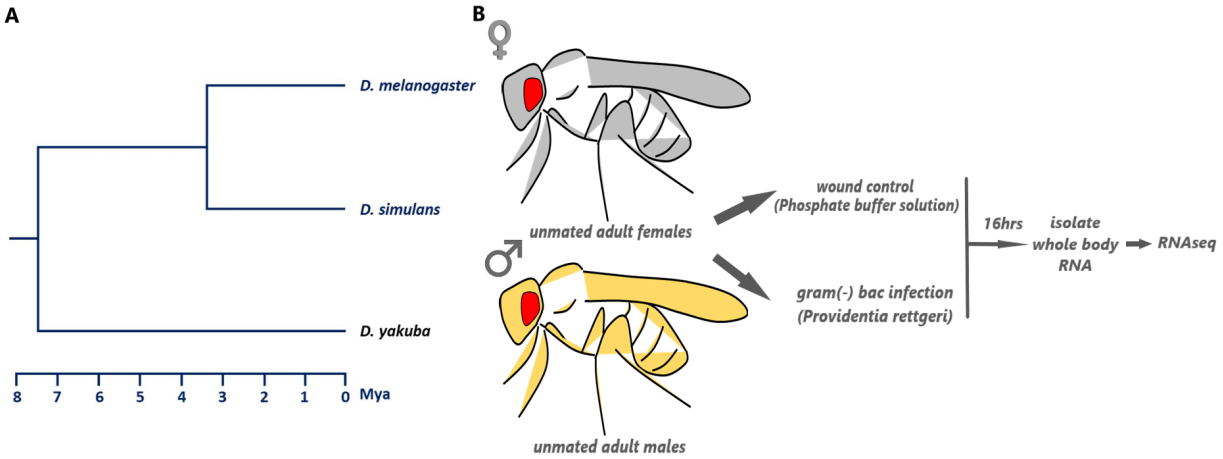
968 This work was supported by grants to RMG from the National Science Foundation (NSF), grant number
969 DEB-1751296.

970 **Conflict of Interest**

971 The author(s) declare no conflict of interest.

972 **Figures and Tables**

973



974

975 **Figure 1:** Phylogenetic relationship and experimental design. **A.** Phylogeny (represented as a schematic
976 diagram) of *D. melanogaster* and *D. simulans*, based on (Bhutkar et al. 2007; Paris et al. 2013; Li et al.
977 2022). *D. melanogaster* and *D. simulans* were used for the experiments, and *D. yakuba* is shown as an
978 outgroup. Mya: millions of years ago **B.** Experimental Design. The unmated adult flies were infected with
979 *P. rettgeri* (PRET) for the treatments and phosphate buffer solutions (PBS) for wound control. RNAseq
980 was performed from RNA isolated from pooled flies (whole body) at 16hpi in four replicates.

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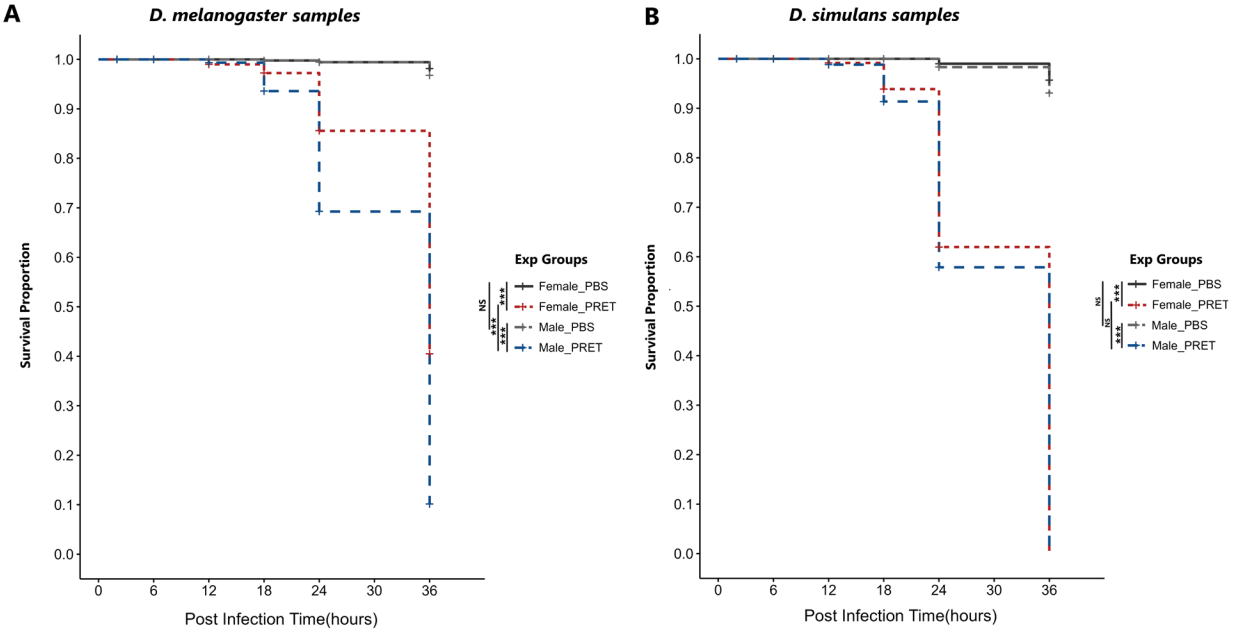


982

983 **Figure 2:** Determination of survival. **A.** Survival curve of *D. melanogaster* (Mel) upon infection with *P.*
984 *rettgeri* (PRET) and wound control (PBS). **B.** Survival curve of *D. simulans* (Sim) upon infection with *P.*
985 *rettgeri* (PRET) and wound control (PBS). The survival curve was generated with the Kaplan–Meier
986 estimator, and the statistical analysis was performed using Cox regression. The asterisks (***)
987 demonstrated the *p*-values < 0.001, and NS represented no significance. All the experimental flies of the
988 *D. simulans* were identified dead at 36hpi for females and males.

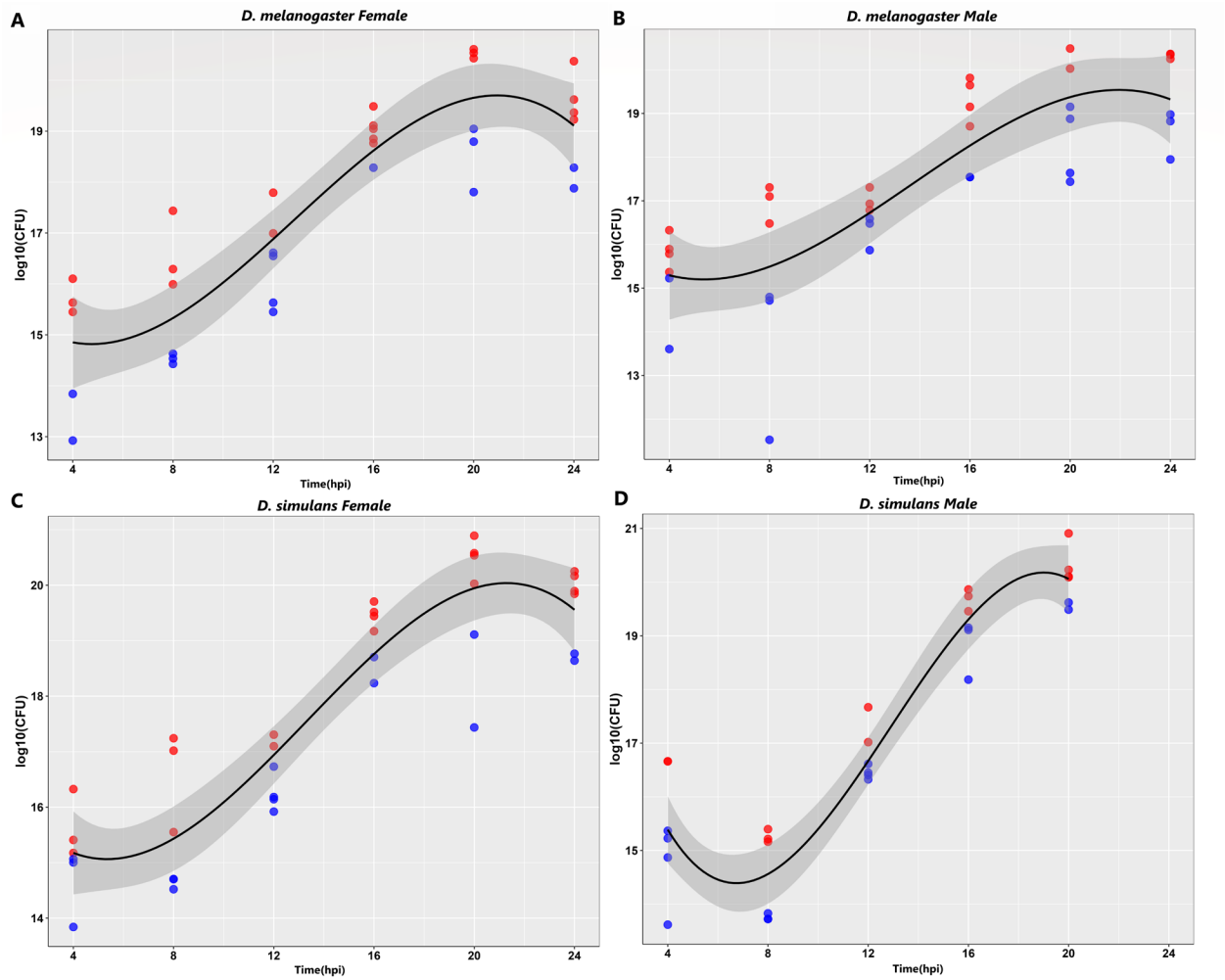


Figure 3: Bacterial load for *D. melanogaster* and *D. simulans* sexes. Determination of bacterial load for females and males at 4-hour intervals upon *P. rettgeri* (PRET) infection in *D. melanogaster* and *D. simulans*. The data were represented for the initial (4, 8, 12hpi) and later (16, 20, 24hpi) stages of the infection in log10(CFU/ML) between the initial and later stages. None of the sample groups showed significant differences at any individual time points (4, 8, 12, 16, 20, and 24hpi) for the bacterial load at p-value < 0.05. Polynomial regression was employed to fit a polynomial curve, where bacterial loads above the curve were marked in red and those below in blue. All *D. simulans* males died at 24hpi. There are 6 replicates for each group, except the dmel female at 4 hrs, which has 5 Replicates (one replicate was dropped as an outlier). The wound control data is not shown; it is available in File S14.

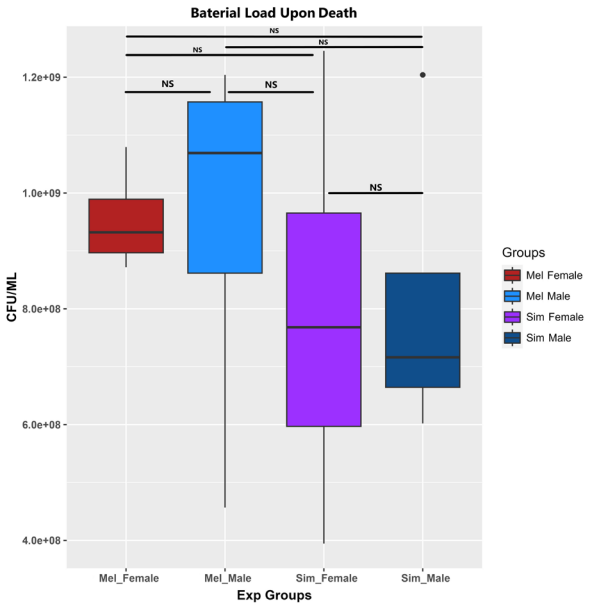


Figure 4: BLUD for *D. melanogaster* and *D. simulans* sexes. Determination of Bacterial Load Upon Death (BLUD) for females and males upon *P. rettgeri* (PRET) infection. The wound control (PBS) showed no death for the experimental groups. The asterisks (*) demonstrated the *p*-values < 0.05, and NS represented no significance.

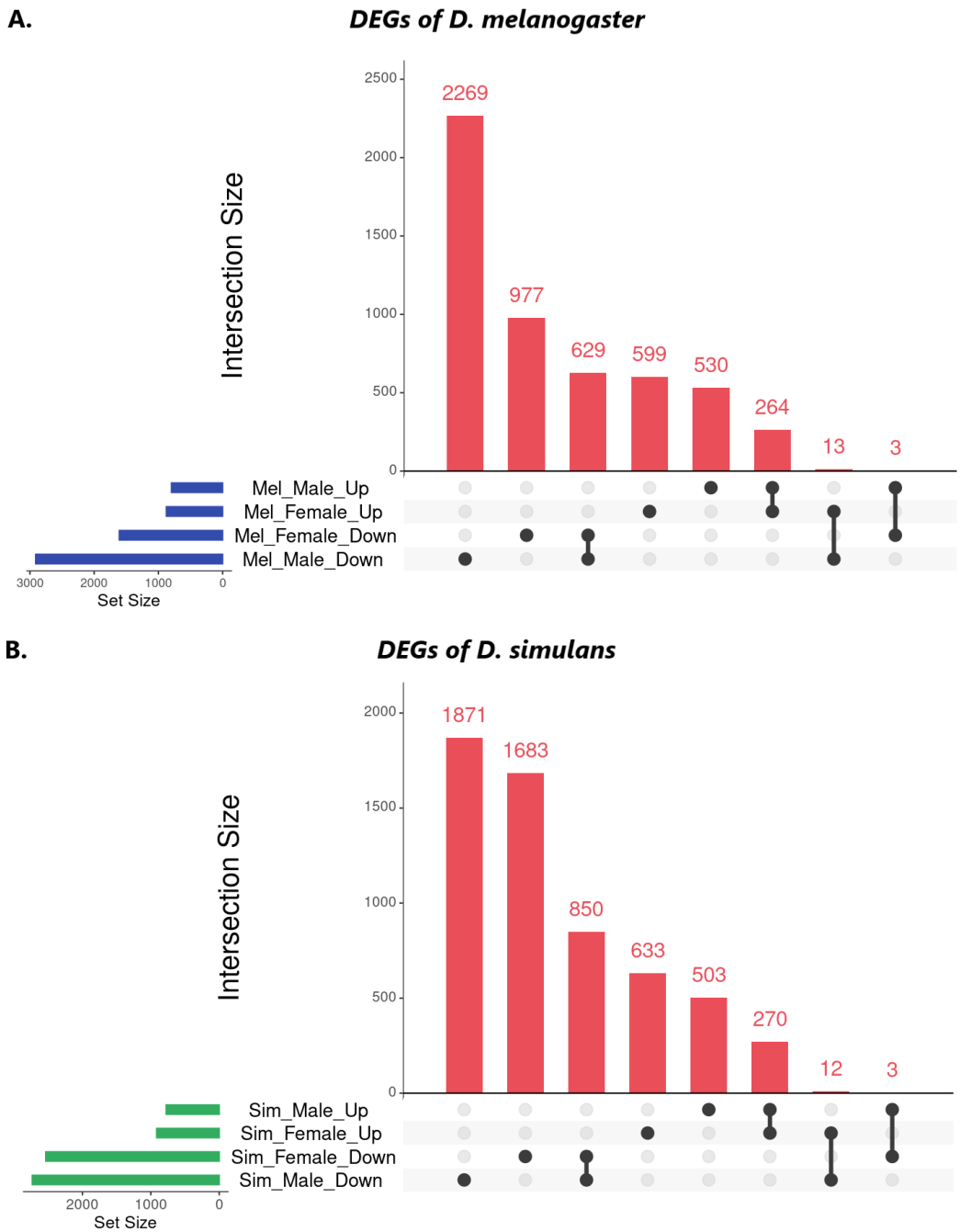


Figure 5: The upset plots represent up- and down-regulated gene counts. **A.** Comparison within species for females and males in *D. melanogaster*, **B.** Comparison within species for females and males in *D. simulans*. The plots also show the synergistically and antagonistically expressed gene numbers for each sex in *D. melanogaster* and *D. simulans*. The figure uses an intersection-based representation (for details, File S4-S5, Table S1).

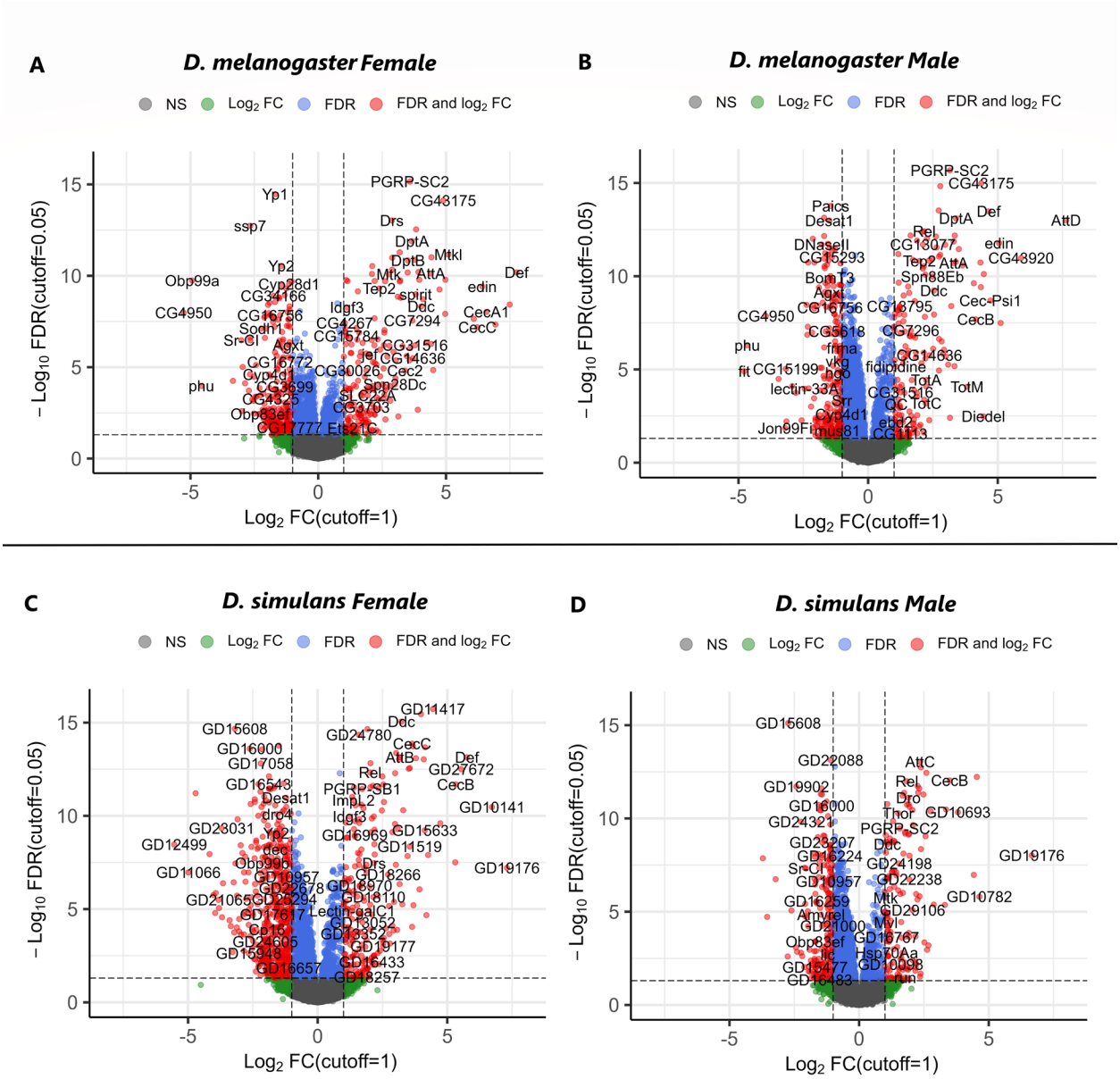


Figure 6: The volcano plots represent DEGs. **A.** *D. melanogaster* females, **B.** *D. melanogaster* males, **C.** *D. simulans* females, and **D.** *D. simulans* males represented upon *P. rettgeri* (PRET) infection compared to wound control (PBS). For *D. melanogaster*, 13588 Normalized Genes, and for *D. simulans*, 13588 Normalized Genes were used to represent DEGs with a cutoff: log2Fold Change ≥ 1; FDR < 0.05. The *D. melanogaster* female also expressed *AttD* (log2FC = 10.52), which is out of the figure range (for details, File S4-S5).

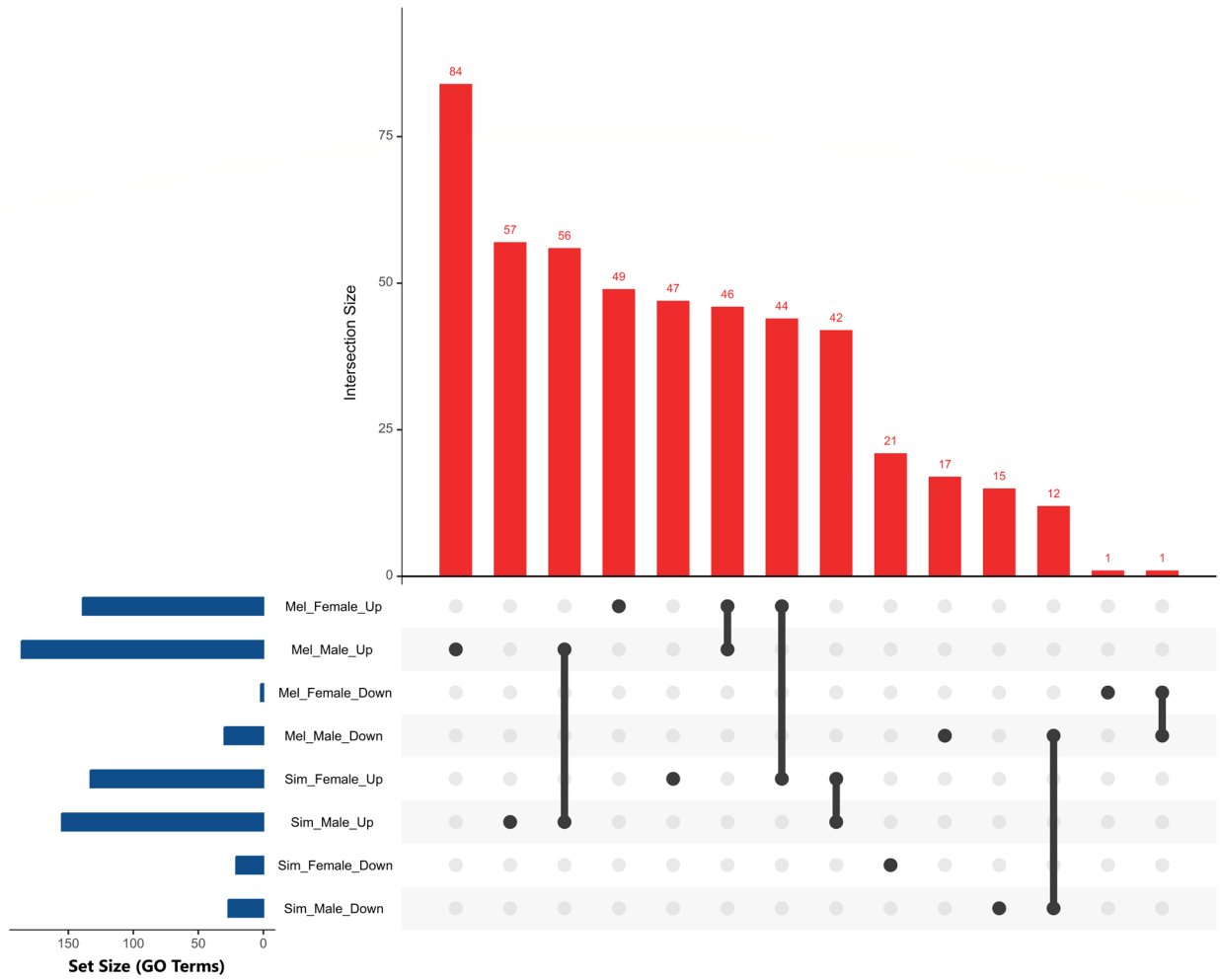


Figure 7: Up- and down-regulated enriched gene ontology terms biological process (GO: BP) in males and females. Mel=*D. melanogaster*, Sim=*D. simulans* (for details, Table S2, File S9). The figure uses an intersection-based representation.

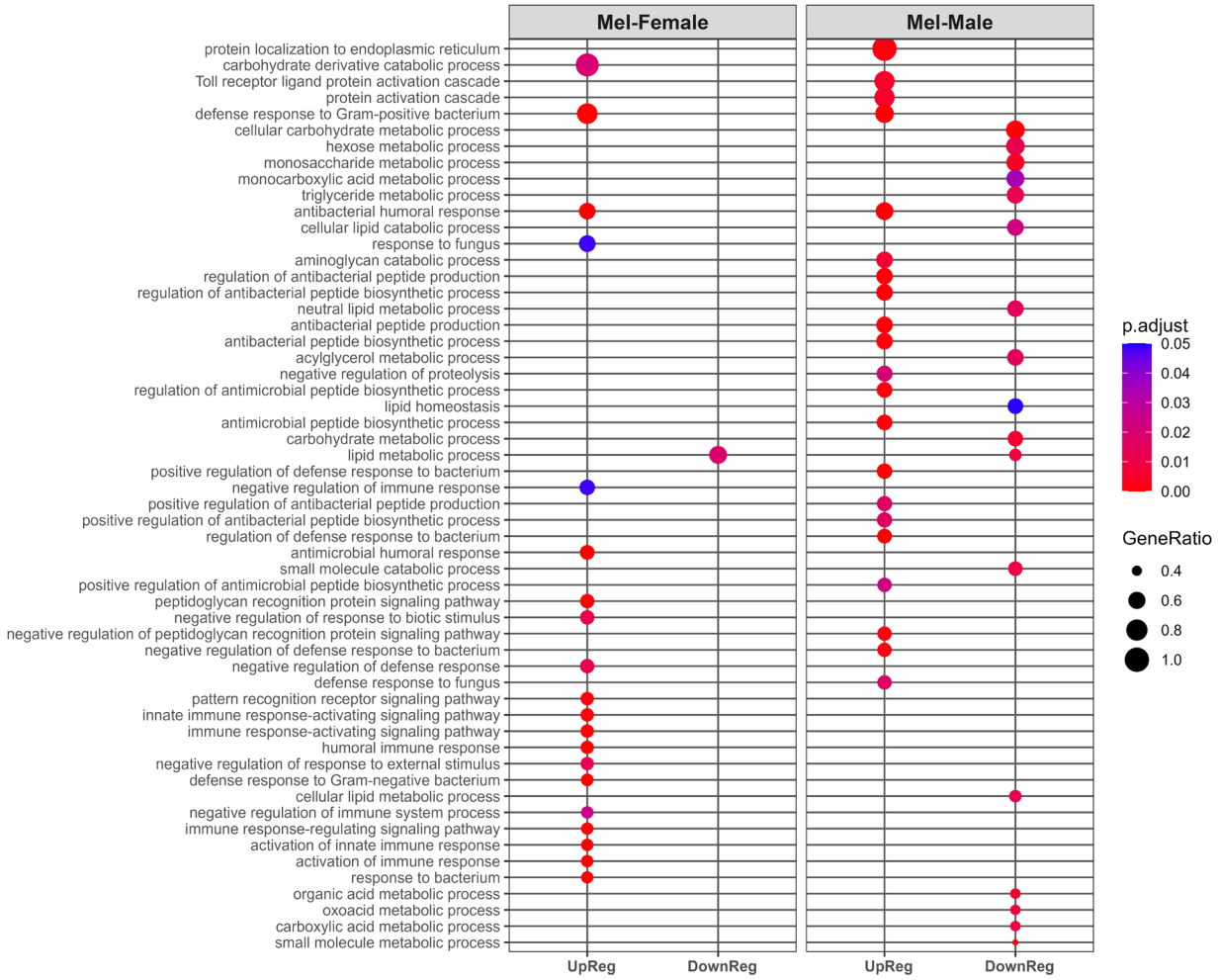


Figure 8: The top 20 up- and down-regulated enriched gene ontology terms (gseGO: BP) in females and males of *D. melanogaster* upon P. rettgeri (PRET) infection (for details, File S9). Mel = *D. melanogaster*, UpReg = Up-Regulation, DownReg = Down-Regulation.

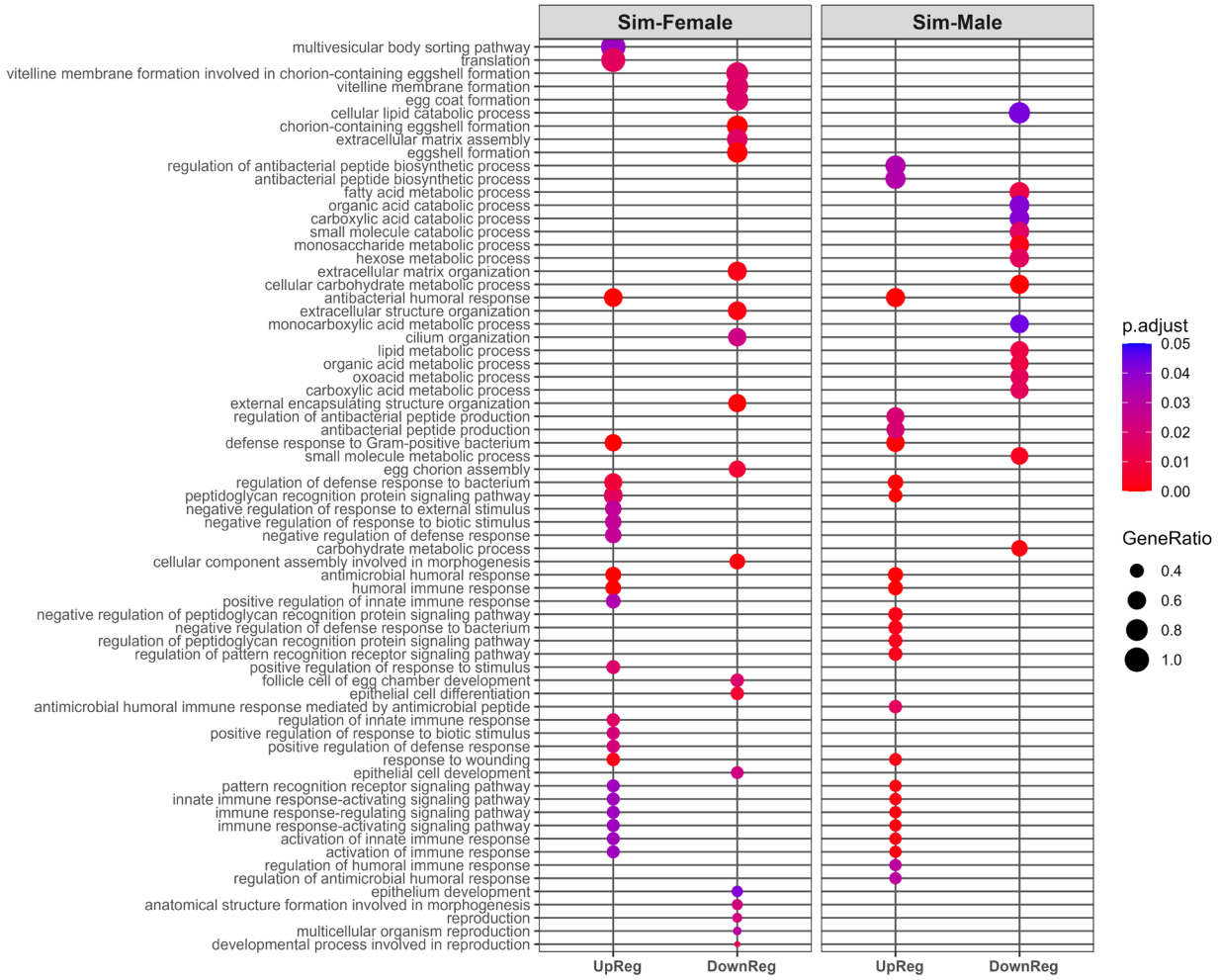
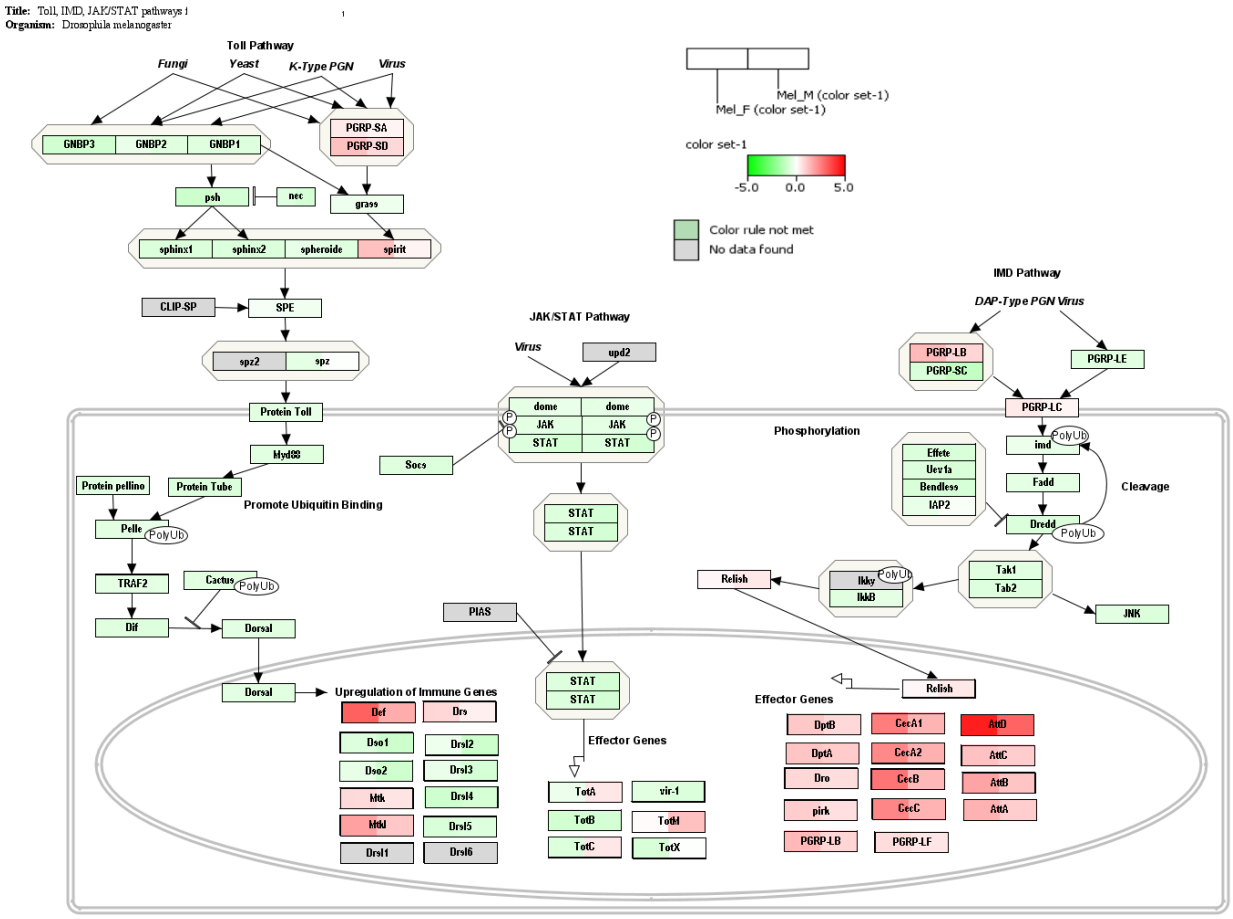


Figure 9: The top 20 up- and down-regulated enriched gene ontology terms (gseGO: BP) in females and males of *D. simulans* upon P. rettgeri (PRET) infection (for details, File S9). Sim = *D. simulans*, UpReg = Up-Regulation, DownReg = Down-Regulation.



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1031 **Figure 10:** DEGs in females and males of *D. melanogaster* in response to *P. rettgeri* infection for genes in
1032 the Toll-IMD-Jak/Stat signaling pathway (wiki pathway ID: WP3830). The expression (z-score) of the
1033 pathway is represented without statistical cut-off. Red represents upregulation, green represents
1034 downregulation, and white represents no change compared to wound control (for details, File S10,
1035 Figure S2).

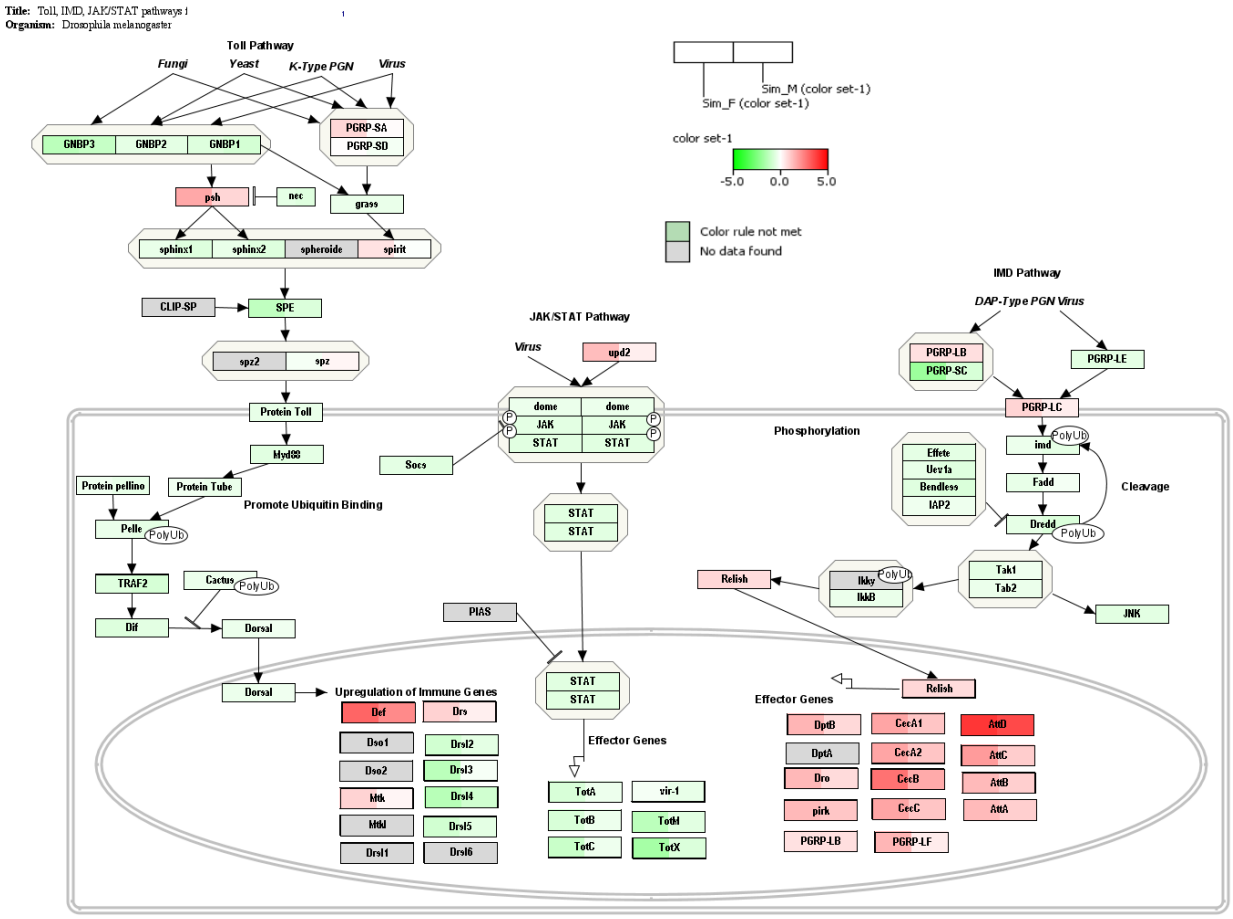

Figure 11: DEGs in females and males of *D. simulans* in response to *P. rettgeri* infection for genes in the Toll-IMD-Jak/Stat signaling pathway (wiki pathway ID: WP3830). The expression (z-score) of the pathway was represented without statistical cut-off. Red represents upregulation, green represents downregulation, and white represents no change compared to wound control (for details, File S10, Figure S2).

1042 **Supplement Information, Figures, and Tables**

1043 **Table S1:** The number of genes upregulated, downregulated, and not significant at FDR < 0.05 for *D.*
1044 *melanogaster* and *D. simulans*. f=Female, m=make, pbs=phosphate buffer solution, pret= *P. rettgeri*,
1045 Mel= *D. melanogaster*, Sim= *D. simulans*, DownReg= Down-Regulation, UpReg= Up-Regulation, NotSig=
1046 Not significant, FDR= False Discovery Rate (for details, File S4-S5)

Experimental Group (Contrast)	Number of Genes	Species	Sex	Regulation /Types
f_pret_VS_f_pbs_FDR0.05_down	977	Mel	Female	DownReg
f_pret_VS_f_pbs_FDR0.05_up	599	Mel	Female	UpReg
f_pret_VS_f_pbs_FDR0.05_not_sig	12012	Mel	Female	NotSig
m_pret_VS_m_pbs_FDR0.05_down	2269	Mel	Male	DownReg
m_pret_VS_m_pbs_FDR0.05_up	530	Mel	Male	UpReg
m_pret_VS_m_pbs_FDR0.05_not_sig	10789	Mel	Male	NotSig
f_pret_VS_f_pbs_FDR0.05_down	1683	Sim	Female	DownReg
f_pret_VS_f_pbs_FDR0.05_up	633	Sim	Female	UpReg
f_pret_VS_f_pbs_FDR0.05_not_sig	10694	Sim	Female	NotSig
m_pret_VS_m_pbs_FDR0.05_down	1871	Sim	Male	DownReg
m_pret_VS_m_pbs_FDR0.05_up	503	Sim	Male	UpReg
m_pret_VS_m_pbs_FDR0.05_not_sig	10636	Sim	Male	NotSig

1047

1048 **Table S2:** Up- and down-regulated enriched gene ontology terms biological process (GO: BP) in males
1049 and females. Mel=*D. melanogaster*, Sim=*D. simulans*, Com. En. w= Commonly Enriched with, UpReg=Up
1050 Regulation, DwReg=Down Regulation (for details, File S9)

Exp Groups	Total Enriched	Up Reg	Com. En. w Opposite Sex-UpReg	Com. En. w compared Species-UpReg	Dn Reg	Com. En. w Opposite Sex-DnReg	Com. En. w compared Species-DnReg
Mel Female	50	49	46	44	1	1	0
Mel Male	101	84	46	56	17	1	12
Sim Female	68	47	42	44	21	0	0
Sim Male	72	57	42	56	15	0	12

1051

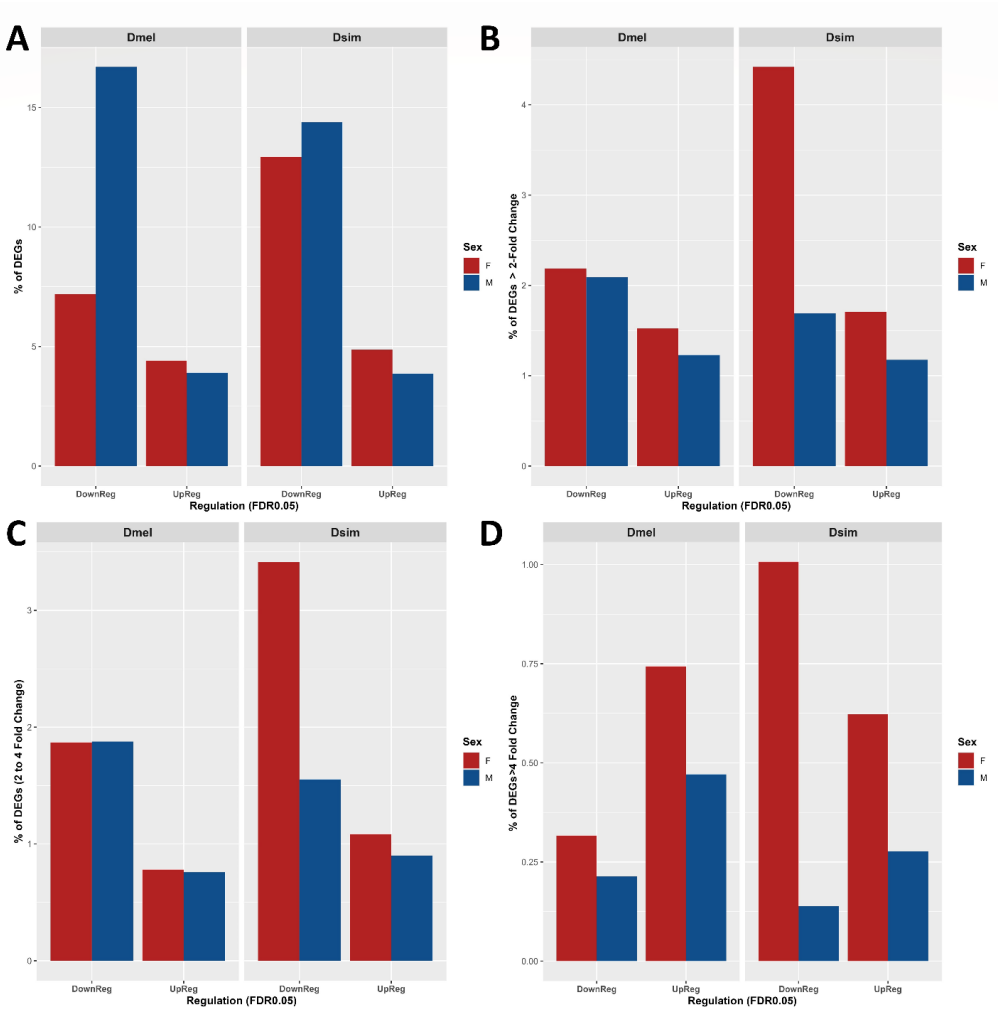

Figure S1: Percent of differentially expressed gene (DEGs) in the females (F) and males (M) in *D. melanogaster* (Dmel) and *D. simulans* (Dsim) upon *P. rettgeri* (PRET) infection for different ranges of fold change (FC). **A.** Percentage of up- and downregulated DEGs (without FC cutoff), **B.** Percentage of up- and downregulated DEGs at > 2FC**, C.** Percentage of up- and downregulated DEGs at 2 to 4FC, **D.** Percentage of up and downregulated DEGs at > 4FC. The percentage of expressed genes showed differential expression with cut-off FDR < 0.05 when compared to the wound control (PBS). The expressed genes were defined after filtering for low expression, the number of expressed genes was 13588 and 13010 for *D. melanogaster* and *D. simulans*, respectively (for details, File S4-S5). The percent count of the more than 2-FC downregulated genes showed the difference between females and males in *D. melanogaster* is much more comparable, in contrast to the pattern for downregulation overall (without FC) (Figure S1). In *D. simulans,* downregulation at more than 2-FC showed the opposite direction than overall downregulation (without FC). For the greater than 2-FC cutoff, upregulation is comparable in both species for females and males. For genes with the largest differences in expression, > 4 FC, in *D. simulans*, many more genes are downregulated in females than in males, and a similar pattern is revealed in upregulation. These suggest that while overall downregulation was more prevalent in males for *D. simulans*, when the degree of suppression is dependent on sex, females showed more highly downregulated genes (at > 2-FC, 2-FC < FC < 4-FC, > 4-FC). This is also true for the *D. melanogaster* (at > 2-FC and > 4-FC), although the magnitude of the difference is not as high as in *D. simulans* (Figure S1).

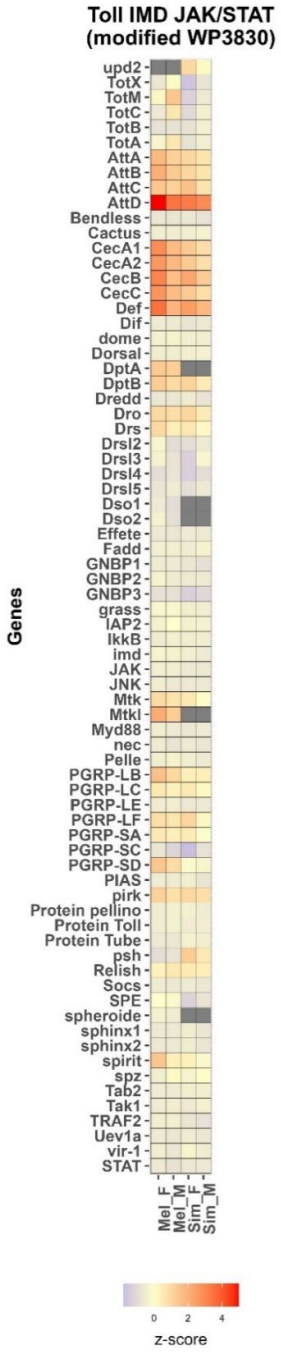

Figure S2: Sex differences in the Toll-IMD-Jak/Stat signaling pathway (wiki pathway ID: WP3830) in *D. melanogaster* and *D. simulans* upon *P. rettgeri* infection. Mel=*D. melanogaster*, Sim=*D. simulans*, F=Female, M= Male. The expression (z-score) of the pathway was represented without statistical cut-off (for details, File S10)

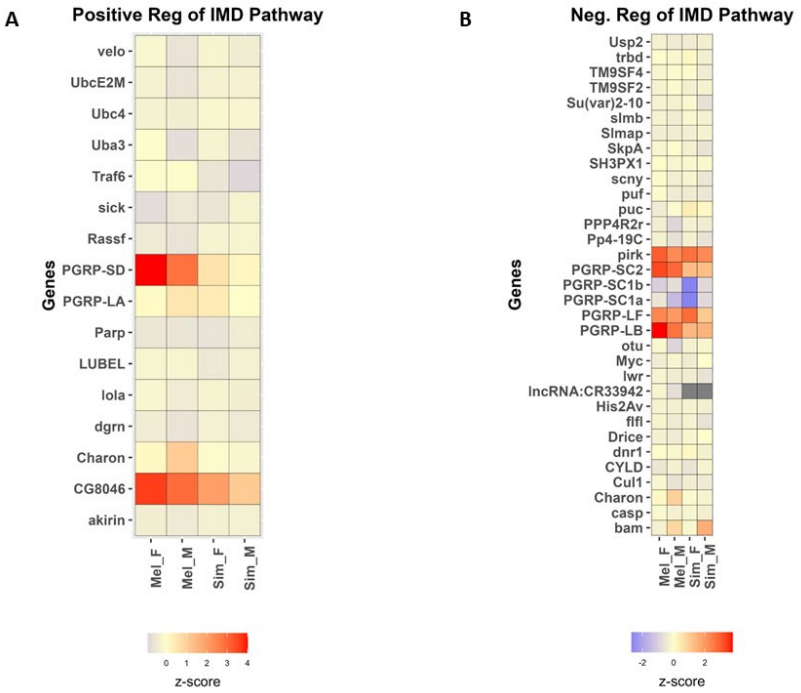

Figure S3: Sex differences in **A.** positive and **B.** negative regulators of the IMD pathways in *D. melanogaster* and *D. simulans* upon *P. rettgeri* infection. Mel=*D. melanogaster*, Sim=*D. simulans*, F=Female, M= Male. The expression (z-score) of the pathway was represented without statistical cut-off (for details, File S11).

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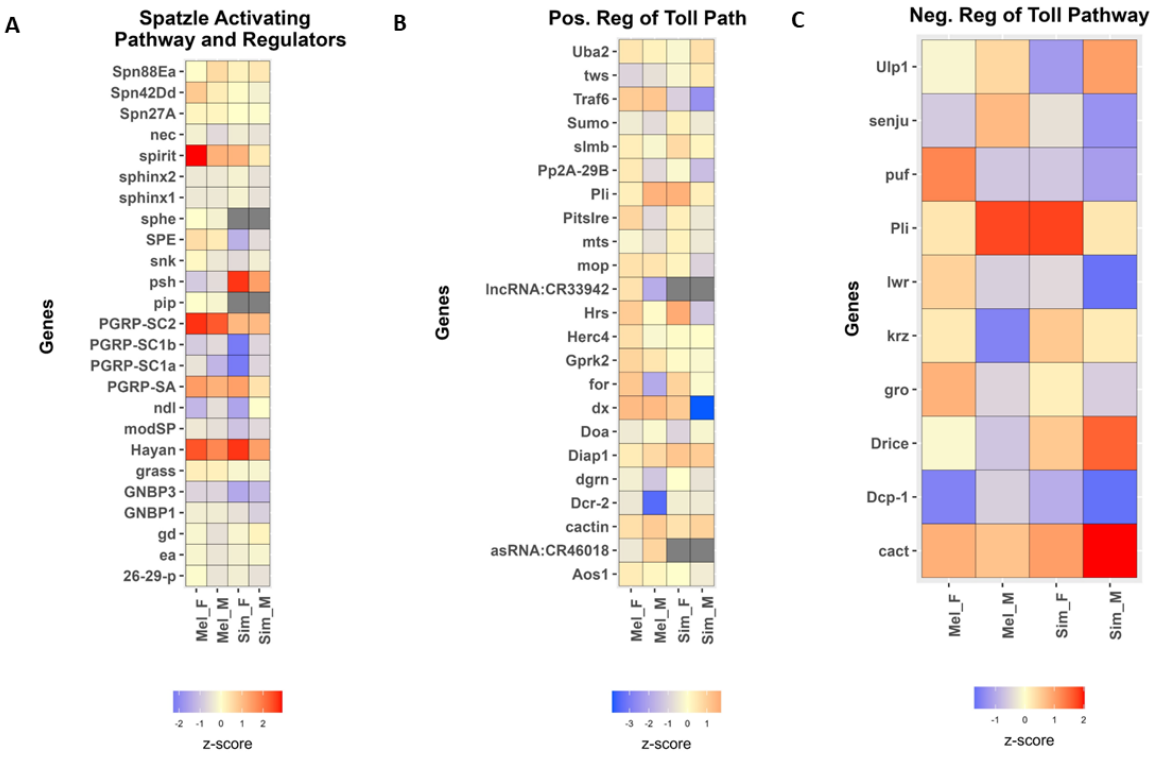
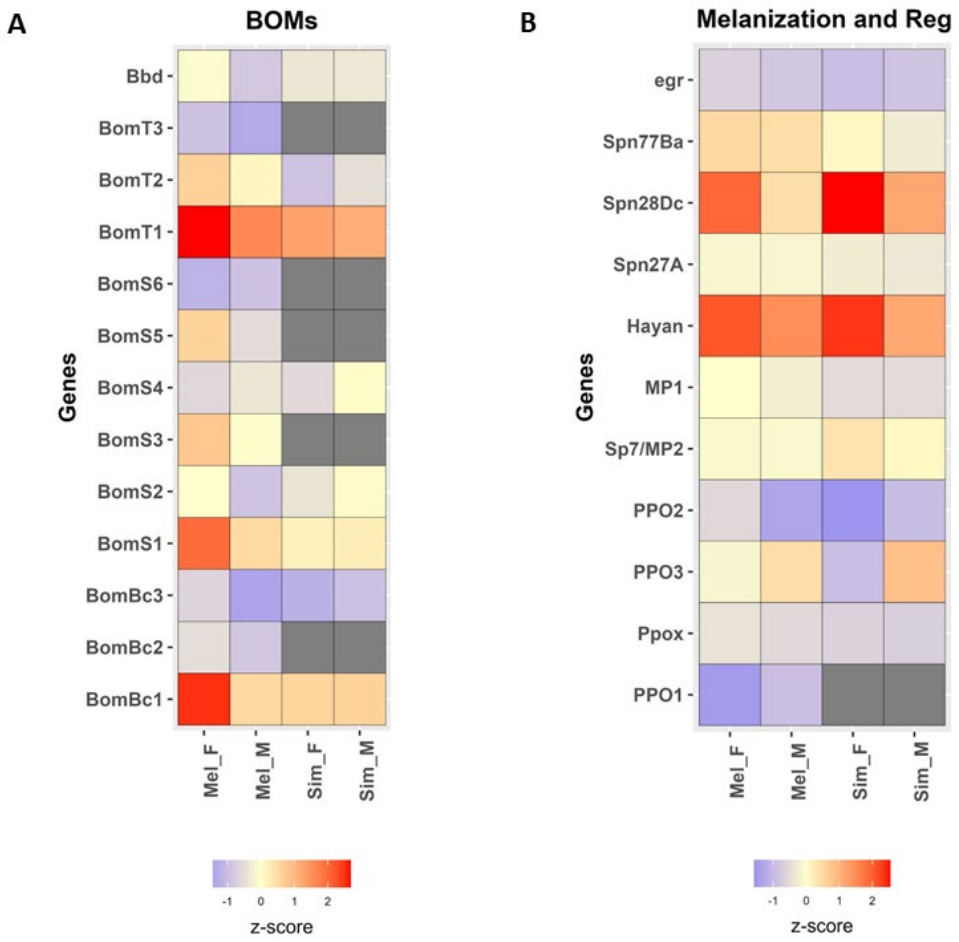


1082

1083 **Figure S4:** Sex differences in **A.** *spz* activating pathway, **B.** positive and **C.** negative regulators of the Toll
1084 pathways in *D. melanogaster* and *D. simulans* upon *P. rettgeri* infection. Mel=*D. melanogaster*, Sim=*D.*
1085 *simulans*, F=Female, M= Male. The expression (z-score) of the pathway was represented without
1086 statistical cut-off (for details, File S11).



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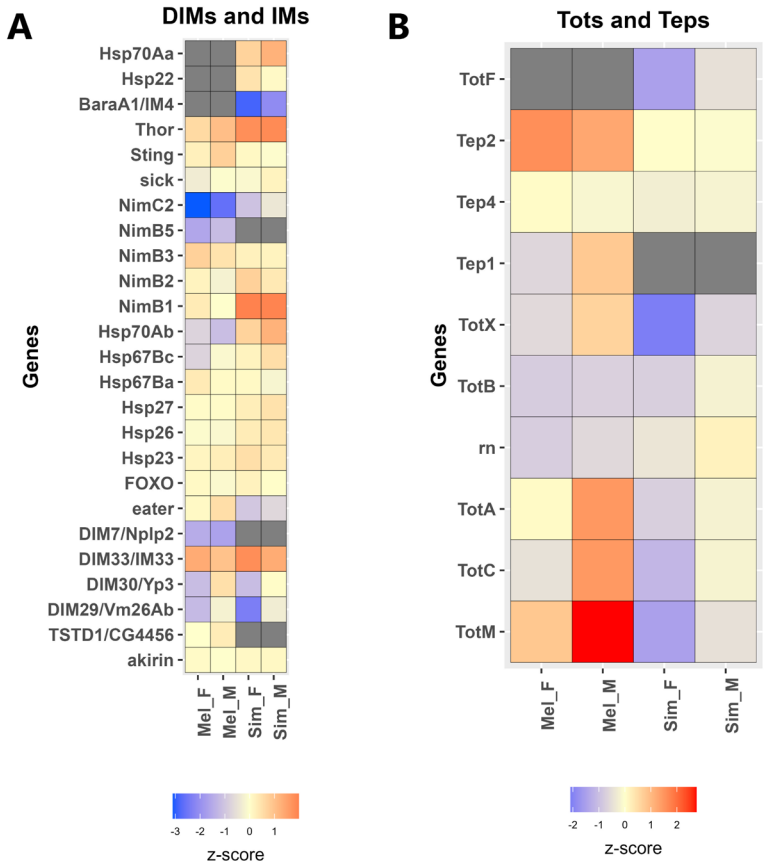
1088 **Figure S5:** Sex differences in **A.** Bomanins (Boms) and **B.** melanization in *D. melanogaster* and *D.*
1089 *simulans* upon *P. rettgeri* infection. Mel=*D. melanogaster*, Sim=*D. simulans*, F=Female, M= Male. The
1090 expression (z-score) of the pathway is represented without statistical cut-off (for details, File S11).

1091



1092

1093 **Figure S6:** Sex differences in **A.** DIMS and IMs, and **B.** Tots, and Teps in *D. melanogaster* and *D. simulans*
1094 upon *P. rettgeri* infection. Mel= *D. melanogaster*, Sim= *D. simulans*, F= Female, M= Male, DIMs=
1095 Drosophila immune-induced molecules, IMs= Immune Molecules, Tots= Turandot, Teps= Thioester-
1096 containing protein, AMPs= Antimicrobial peptides. The expression (z-score) of the pathway was
1097 represented without statistical cut-off (for details, File S11).

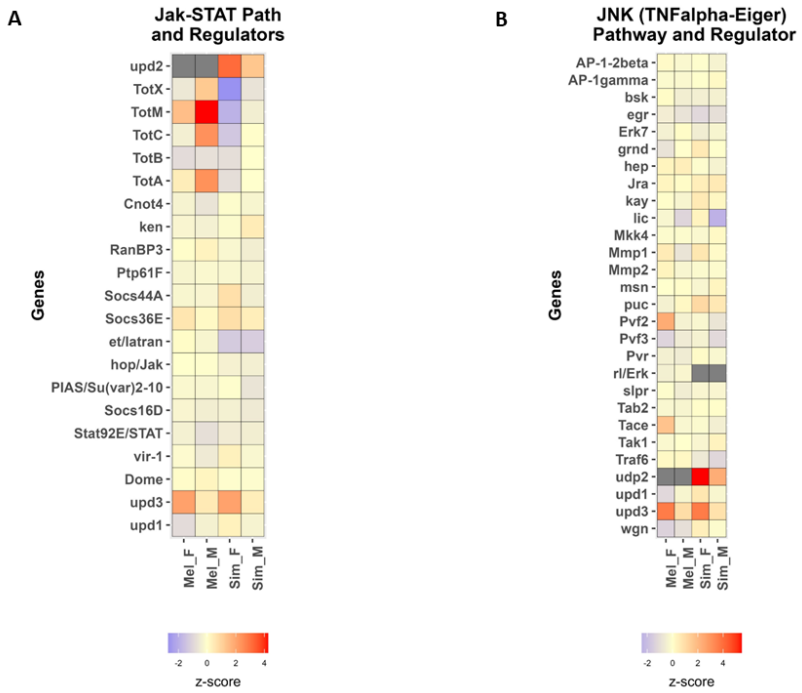


Figure S7: Sex differences in **A.** JAK-STAT (hop-STAT92E) and **B.** Jnk (TNFa-Eiger) pathway with regulators in *D. melanogaster* and *D. simulans* upon *P. rettgeri* infection. Mel=*D. melanogaster*, Sim=*D. simulans*, F=Female, M= Male. The expression (z-score) of the pathway was represented without statistical cut-off (for detail, File S10-S11).

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