Mutations of γCOP Gene Disturb Drosophila melanogaster Innate Immune Response to Pseudomonas aeruginosa

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Abstract: Drosophila melanogaster (the fruit fly) is a valuable experimental platform for modeling host–pathogen interactions. It is also commonly used to define innate immunity pathways and to understand the mechanisms of both host tolerance to commensal microbiota and response to pathogenic agents. Herein, we investigate how the host response to bacterial infection is mirrored in the expression of genes of Imd and Toll pathways when D. melanogaster strains with different γCOP genetic backgrounds are infected with Pseudomonas aeruginosa ATCC 27853. Using microarray technology, we have interrogated the whole-body transcriptome of infected versus uninfected fruit fly males with three specific genotypes, namely wild-type Oregon, γCOP 5057302/TM6B and γCOP 14a/γCOP 14a. While the expression of genes pertaining to Imd and Toll is not significantly modulated by P. aeruginosa infection in Oregon males, many of the components of these cascades are up- or downregulated in both infected and uninfected γCOP 5057302/TM6B and γCOP 14a/γCOP 14a males. Thus, our results suggest that a γCOP genetic background modulates the gene expression profiles of Imd and Toll cascades involved in the innate immune response of D. melanogaster, inducing the occurrence of immunological dysfunctions in γCOP mutants.

Keywords: γCOP; D. melanogaster; P. aeruginosa; innate immunity; Toll; Imd; microarray

1. Introduction

Pseudomonas aeruginosa is a metabolically versatile opportunistic pathogen, with a very wide host spectrum that includes plants, nematodes, insects, and vertebrates. P. aeruginosa can cause a wide range of severe human infections, with high mortality rates, particularly in immuno-depressed patients. A diverse arsenal of P. aeruginosa virulence factors, which can have different expression profiles depending on the infection localization and the host immune response, contribute to its high competitiveness [1]. Moreover, P. aeruginosa is included in the lists of the most threatening antibiotic resistant pathogens, such as ESCAPE (Enterococcus faecium, Staphylococcus aureus, Clostridium difficile, Acinetobacter baumannii, Pseudomonas aeruginosa, and Enterobacteriaceae) and the WHO list of priority one, critical pathogens [2,3]. Besides exhibiting both intrinsic and acquired resistance mechanisms to all currently available antibiotics, P. aeruginosa has a high capacity to form biofilms, which are known for their high phenotypic resistance or tolerance to different antimicrobial agents including antibiotics, antiseptics, or host effectors [4].

P. aeruginosa infection triggers an intense innate host response in mammals, mainly mediated by neutrophils, resulting in significant host tissue damages. A higher incidence of severe P. aeruginosa infections in patients with acquired and primary immunodeficiencies and the better prognoses of the infections with P. aeruginosa for patients at the mature age reiterate the importance of the innate immune responses [5–7].
Therefore, a better understanding of the interactions among the bacterial and host factors is expected to contribute to the development of improved therapeutic strategies for *P. aeruginosa* infections.

The bacterial infection process and the development of an innate immune system in *Drosophila melanogaster* and mammals have many similarities [8]. An apparent absence of the adaptive immune system and the impressive arsenal of genetic and molecular tools qualify *D. melanogaster* as a powerful experimental platform for investigating the host’s innate immune response [9–12]. Previous microarray experiments revealed that 283 of the approximately 400 known genes involved in the immune response in *D. melanogaster* are regulated by either one or several signaling pathways [11]. These genes are encoding antimicrobial peptides (AMPs), proteins involved in iron metabolism and sequestration, opsonization, melanization and coagulation processes, production of reactive oxygen species (ROS), signaling via the Jun kinase (JNK) pathway, stress response, and proteases [11]. Toll and Imd cascades are two of the major regulators of the humoral innate immune response in *D. melanogaster* [13]. These pathways are involved in the synthesis and temporary release of AMPs by the cells of the fat body, which is functionally equivalent to the mammalian liver.

In our study, we have harnessed microarray technology in order to investigate the non-lethal effects of experimental infection with *P. aeruginosa* on fruit fly strains with different genetic backgrounds. A goal of this study was to evaluate the impact of normal and disturbed expression of the *γCOP* gene on Toll and Imd pathways consecutive to the infection of males. *γCOP* is an essential, highly conserved gene in eukaryotes and is involved in retrograde vesicular transport between endoplasmic reticulum (ER) and Golgi [14]. Perturbations of the ER functions result in an unbalanced immune response, as ER was linked to numerous processes involved in regulating innate and adaptive immune responses. The main such processes are: the production of inflammatory cytokines, trimming of peptide ligands and assembling of the major histocompatibility complex (MHC) class I molecules, and the transport of both MHC class I and class II complexes to the cell surface [15–18]. Moreover, *γCOP* is important for the ER homeostasis and hence for the innate immunity, but also for the adaptive immunity as revealed in humans [19].

Our inquiry was relying on a set of particular facts that support a link between the genetic background and the severity of infections. For example, the gut microbiome displays distinct features in complex human diseases and some microbial groups have significant heritability estimates [20]. Noticeably, the survival of mice to *P. aeruginosa* infection is affected by their genetic background, which critically influences the extent of the early cell-mediated innate immunity activation [21–23]. Last but not the least, we previously demonstrated that the genetic background of *D. melanogaster* has an impact on commensal microbiota diversity and argued that the natural resistance to the external microbiota colonization could be compromised [24].

2. Results

The microarray experiment generated expression data for a total number of 14,437 targets employed by FlyChip procedure based on FL003 arrays. The microarray probes that do not match the target transcripts or match in multiple locations can introduce inconsistency in quantifying gene expression [25]. Therefore, we used for data analysis only single hit/matched genes assigned to the oligonucleotide sequence. Consecutively, we focused on Imd, Toll, and Imd-JNK pathways and considered for further analyses 99 specific genes and *γCOP*.

An overview of the 51 genes significantly (*p* < 0.05) modulated in at least one category of uninfected and infected *γCOP*<sup>5057302/TM6B</sup> or *γCOP*<sup>14a</sup>/ *γCOP*<sup>14a</sup> mutant males (Table 1 and Figure 1) suggests that a *γCOP* genetic background influences the innate immune response, even in the absence of infection. We also considered for further analyses the gene expression of *DrsII* in uninfected *γCOP*<sup>5057302/TM6B</sup>, for which we found a *P* value of 0.059, bringing the total number of represented genes to 52. Regarding *AttB* and *CecA2*,

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we deemed as significant their gene expression in uninfected $\gamma$COP$^{5057302}$/TM6B and, respectively, uninfected $\gamma$COP$^{14a}$/$\gamma$COP$^{14a}$ mutant males ($p = 0.059$ and $p = 0.056$).

Table 1. The genes associated with Toll, Imd, and Imd-JNK pathways with significant modulation of expression in at least one experimental condition.

| Gene Name                      | Gene Symbol | Biological Process                                      |
|--------------------------------|-------------|---------------------------------------------------------|
| **Toll Pathway**               |             |                                                         |
| Baramicin A2                   | BaraA2      | defense response                                        |
| Bomanin Short 1                | BomS1       | response to bacteria                                    |
| Bomanin Short 2                | BomS2       | defense response                                        |
| Bomanin Short 3                | BomS3       | response to bacteria                                    |
| Cactus                         | cact        | negative regulation of Toll signaling pathway          |
| Defensin                       | Def         | humoral immune response                                 |
| Dorsal                         | dl          | dorsal/ventral axis specification                       |
| Drosomycin                     | Drs         | defense response to protozoa                            |
| Drosomycin-like 1              | Drsl1       | defense response to fungi                               |
| **Gram-negative bacteria binding protein 1** | Gnb1 | carbohydrate metabolic process                          |
| **G protein-coupled receptor kinase 2** | Gprk2 | protein phosphorylation                                 |
| **Gram-positive Specific Serine protease** | grass | proteolysis                                             |
| Kurtz                          | krz         | locomotory exploration behavior                         |
| Modular serine protease        | modSP       | proteolysis                                             |
| Metchnikowin                   | Mtk         | defense response to Gram-positive bacteria              |
| Peptidoglycan recognition protein SA | Pgrp-SA | innate immune response                                 |
| Peptidoglycan recognition protein SD | Pgrp-SD | innate immune response                                 |
| Peptidoglycan recognition protein SA | Pgrp-SA | innate immune response                                 |
| Peptidoglycan recognition protein SD | Pgrp-SD | innate immune response                                 |
| Pelle                          | pll         | Toll signaling pathway                                  |
| Spatzle-Processing Enzyme      | SPE         | defense response to Gram-positive bacteria              |
| Spheroid                       | sphe        | proteolysis                                             |
| Spatzle                        | spz         | defense response to Gram-positive bacteria              |
| Thioester-containing protein 4 | Tep4        | innate immune response                                 |
| Toll-9                         | Toll-9      | signal transduction                                     |
| Ulp1                           | Ulp1        | negative regulation of Toll signaling pathway          |
| Wnt inhibitor of Dorsal        | wntD        | defense response to Gram-positive bacteria              |
| **Imd Pathway**                |             |                                                         |
| Attacin-A                      | AttA        | humoral immune response                                 |
| Attacin-B                      | AttB        | humoral immune response                                 |
| Attacin-C                      | AtlC        | antibacterial humoral response                          |
| Attacin-D                      | AttD        | response to wounding                                    |
| Bendless                       | ben         | positive regulation of tumor necrosis factor-mediated signaling pathway |
| Cecropin pseudogene 1         | Cec-Ψ1      | pseudogene                                              |
| Cecropin 2                     | Cec2        | pseudogene                                              |
Table 1. Cont.

| Imd Pathway                        | Gene Name                  | Gene Symbol | Biological Process                                      |
|------------------------------------|----------------------------|-------------|--------------------------------------------------------|
|                                    | Cecropin A2                | CecA2       | antibacterial humoral response                          |
|                                    | Cecropin B                 | CecB        | defense response to Gram-positive bacteria              |
|                                    | Diptericin A               | DptA        | response to bacteria                                    |
|                                    | Diptericin B               | DptB        | response to wounding                                    |
|                                    | effete                     | eff         | germ-line stem cell population maintenance             |
|                                    | Fas-associated death domain| Fadd        | peptidoglycan recognition protein signaling pathway     |
|                                    | Immune deficiency          | imd         | response to bacteria                                    |
|                                    | Peptidoglycan recognition protein LB | PGRP-LB | innate immune response                                 |
|                                    | Peptidoglycan recognition protein LC | PGRP-LC | regulation of synaptic plasticity                       |
| poor Imd response upon knock-in   | pirk                       |             | negative regulation of peptidoglycan recognition protein signaling pathway |
| Plenty of SH3s                    | POSH                       |             | response to peptidoglycan                               |
| Relish                            | Rel                        |             | peripheral nervous system neuron development           |

| Imd-JNK Pathway                    | Gene Name                  | Gene Symbol | Biological Process                                      |
|------------------------------------|----------------------------|-------------|--------------------------------------------------------|
|                                    | Activating transcription factor-2 | Atf-2      | positive regulation of transcription by RNA polymerase II |
|                                    | Octopamine receptor in mushroom bodies | Oamb       | cellular calcium ion homeostasis                        |
|                                    | basket                     | bsk         | cellular response to oxidative stress                   |
|                                    | hemipterous                | hep         | positive regulation of cell death                       |
|                                    | Jun-related antigen        | Jra         | wound healing                                           |
|                                    | licorne                    | lic         | JNK cascade                                             |
|                                    | Phospholipase C at 21C     | Pcle21C     | lipid metabolic process                                 |
|                                    | Signal-transducer and activator of transcription protein at 92E | Stat92E | receptor signaling pathway via JAK-STAT                |

As revealed in Figure 1, γCOP expression is not affected in either infected or uninfected heterozygous mutant males. Nevertheless, it deserves mentioning that γCOP have a tendency to downregulation in both categories of γCOP<sub>S057302</sub>/TM6B males as revealed by the fact that all eight log<sub>2</sub>FC values standing for the 4 × 2 biological replicates are negative. The biological processes indicated in Table 1 are retrieved from FlyBase [26] and are in accordance to the FlyBase release FB2022_02.

Hence, we obtained four sets of differentially expressed genes for the four categories of mutant males, namely 27 genes for infected γCOP<sub>S057302</sub>/TM6B, 24 genes for uninfected γCOP<sub>S057302</sub>/TM6B, 35 genes for the infected γCOP<sup>14a</sup> / γCOP<sup>14a</sup>, and 25 genes for uninfected γCOP<sup>14a</sup> / γCOP<sup>14a</sup>. A summation of the gene expression profiles in the considered experimental conditions is presented in Figure 1. Strikingly, none of the selected genes is significantly up- or downregulated in the infected Oregon males.

No less than 10 genes from the Toll pathway have an affected expression pattern in both infected and uninfected γCOP<sup>14a</sup> / γCOP<sup>14a</sup> males. In fact, out of these genes, six are upregulated (dl, cact, Tep4, modSP, spz and PGRP-SD) and four are downregulated (SPE, BaraA2, Toll-9, and Gprk2). Both categories of γCOP<sub>S057302</sub>/TM6B males share only seven modulated genes, as dl, cact, Tep4, and wntD are upregulated, while SPE, BaraA2, and Ulp1 are downregulated. On the other hand, three upregulated genes (dl, cact, and Tep4) and two downregulated genes (SPE and BaraA2) are common for both homozygous and heterozygous mutant males.
14 genes modulated in the uninfected γ (16 genes versus only 10 genes), suggesting that the JNK pathways. For each infected (I) or uninfected (UI) experimental condition, downregulated genes are colored in red, while the upregulated genes are indicated with blue. The white squares indicate that the expression variation was not statistically significant. The gene expression is expressed as log2FC.

A similar analysis of Imd/Imd-JNK pathways revealed that, out of 27 considered genes, only 3 of them are mutual (imd, Fadd, and Plc21C) and are downregulated in all four categories of mutant males. When considering the γCOP14a/γCOP14a-infected and uninfected males, in a group of seven shared genes we found four of them to be upregulated (Cec2, bsk, PGRP-LB, and PGRP-LC) and three downregulated (imd, Fadd, and Plc21C). On the other hand, there are 11 genes displaying a similar expression pattern in both categories of imd/Imd males, where 1 gene is upregulated (hep) and 10 genes are downregulated (imd, Fadd, Plc21C, AttB, Atf-2, DptA, DptB, AttA, AttD, and Stat92E).

In the Toll pathway, the number of genes with impacted expression is bigger for the default (uninfected) condition in γCOP14a/γCOP14a compared to γCOP5057302/TM6B males (16 genes versus only 10 genes), suggesting that the γCOP14a/γCOP14a males set up an enhanced response to a commensal microbiota challenge. When scrutinizing inside of the same genetic background, there are only very small numeric differences (Δ) of the affected genes in infected versus uninfected flies (for γCOP14a/γCOP14a males Δ = 0 and for γCOP5057302/TM6B males Δ = -2). Remarkably, six of the genes modulated in the infected γCOP5057302/TM6B males are also affected in the infected γCOP14a/γCOP14a flies. Only wntD and Ulp1 genes are specifically responsive to infection with P. aeruginosa in heterozygous flies.

Concerning the Imd/Imd-JNK pathways, uninfected γCOP14a/γCOP14a males seem to be less reactive, as long as they exhibit by default only 9 affected genes comparative to 14 genes modulated in the uninfected γCOP5057302/TM6B flies (Δ = -5). Conversely, there is a double increment in the number of the affected genes in the infected γCOP14a/γCOP14a mutant males (Δ = 10; from 9 to 19 genes) comparative to the infection response of

Figure 1. Heatmaps of genes having a significant expression variation in at least one mutant background. The gene names are indicated on the left side of each heatmap (Toll pathway and Imd/Imd-JNK pathways). For each infected (I) or uninfected (UI) experimental condition, downregulated genes are colored in red, while the upregulated genes are indicated with blue. The white squares indicate that the expression variation was not statistically significant. The gene expression is expressed as log2FC.
In the Toll pathway, the number of genes with impacted expression is bigger for the γCOP5057302/TM6B mutant males (Δ = 5; from 14 to 19 genes). Coincidentally, 19 genes (Δ = 0) from Imd/Imd-JNK pathways are modulated in both infected γCOP14a/γCOP14a and infected γCOP5057302/TM6B males. Out of these 19 affected genes, the two categories of infected males share six upregulated genes (hep, bsk, PGRP-LB, PGRP-LC, pirk and lic) and six downregulated genes (imd, Fadd, Plc21C, Aft1B, Aft1 and Aft1C). It is somehow difficult to avoid noticing the symmetry of this numbers.

In the absence of infection, the Toll pathway appears to be more active in γCOP14a/γCOP14a mutants comparative to the heterozygous males, but for both considered genetic pathways, the experimental infection impacts more on gene modulation in the γCOP14a/γCOP14a mutant males. However, the upregulation of some genes encoding for pathway inhibitors invite careful interpretation of these aggregate data (Figure 2).

**Figure 2.** An overview of the differential expression of immune genes in γCOP mutant males. For every infected (I) or uninfected (UI) experimental condition, we counted the number of genes pertaining to Imd, Toll, and Imd-JNK pathways that were significantly down- or upregulated. Red (for downregulated genes) and blue (for upregulated genes) lines are highlighting the differences between the I/UI heterozygous and homozygous males.

### 3. Discussion

#### 3.1. An Overview of the Mutant γCOP Background in D. melanogaster

Herein, we investigate if the essential gene γCOP is involved in modulation of the innate immune response in D. melanogaster. In order to test our hypothesis, we performed experimental infection of males from Oregon, γCOP5057302/TM6B, and γCOP14a/γCOP14a lines with the P. aeruginosa ATCC 27853 strain.

In our experiments, we used the fruit fly strain γCOP5057302/TM6B, which contains the null lethal allele γCOP5057302 balanced over the TM6B chromosome. Therefore, the γCOP5057302 allele is unable to migrate in the TM6B balancer and is maintained in its location on the original l(3)S057302 chromosome [27]. The l(3)S057302 chromosome is a specific third chromosome of D. melanogaster obtained by insertional mutagenesis [27] and harbors an insertion of transposon P{lacW}5057302 in the 5'UTR region of the γCOP gene [28]. The insertion determines a recessive lethal allele as revealed by obtaining precise viable excisions of P{lacW}5057302—that proof that there is no other hidden mutation...
in the l(3)S057302 chromosome accountable for the lethal phenotype [28]. Moreover, we found that \( \gamma COP^{S057302} \) fails to complement the lethality of \( \gamma COP^{\Delta 114} \) (located in a chromosome 3 with a different mutational background than l(3)S057302), confirming data of Jayaram et al. [29] and the fact that \( \gamma COP \) is indeed an essential gene, being also involved in fruit flies’ fertility [30]. The crosses among \( \gamma COP^{14a}/\gamma COP^{14a} \) males and females are always sterile, but both homozygous males and females are fertile when crossed with flies having at least one wild-type (WT) copy of \( \gamma COP \) (\( \gamma COP^{WT} \)).

TM6B balancer contains multiple inversions and rearrangements, but also recessive lethal genetic markers causing the homozygous individuals to die as embryos. These chromosomal aberrations prevent recombination by meiotic crossing over with the normal homologous chromosome 3. Herein, the term “normality” refers only to the natural linkage order of the genes, but not to alleles and or to other small-scale mutations. A balanced chromosome is actually a haploblock, which keeps in cis configuration not only the lethal recessive alleles, but also other known or hidden mutations, considering that the balancer is not leaky [31]. TM6B contains the spontaneous inversion Int(3R)C, which is also present in many natural populations but not in the Oregon strain [26]. The breakpoints of Int(3R)C are 92D and 100F; \( \gamma COP \) is located in 100C, close to the distal breakpoint of this inversion. Detailed data concerning the multiple inversions’ effects on the expression of genes located in fruit fly balancer chromosomes were reported [32]. Misexpression of genes is determined either by their proximity to breakpoints of inversion or because they are physically disrupted by the inversions [31].

As revealed by Bing et al. [33], the impact of transvection on gene regulation is a complex and a plastic phenomenon. Transvection (genetic trans-interaction) as a particular way of gene regulation is widespread on the \( D. melanogaster \) genome. The same authors prove that trans-interactions at the Malic enzyme (\( Men \)) locus were eliminated by inversions of two different chromosomal fragments that contain \( Men \) [33]. The authors report that the sole wild-type \( Men \) allele is upregulated by the enhancers of a null \( Men \) allele, and the relative quantity of the specific mRNA and enzyme recovers to those of homozygous wild-type organisms. Bing et al. [33] further proved that \( Men \) upregulation of the wild-type was indeed a transvection, as inversions containing \( Men \) wild-type locus prevents its compensatory upregulation. The temperature was also an impeding factor, pointing to an environmental influence on the plasticity of transvection aspects.

Since the \( \gamma COP^{S057302}/TM6B \) strain contains a null allele and a relocated copy of \( \gamma COP^{WT} \), impairments of the anterograde and retrograde transport between Golgi and ER may be reasonably considered if the \( \gamma COP^{WT} \) allele’s physiology is disturbed by position effects or by defective trans-interactions. On the other hand, \( \gamma COP^{14a}/\gamma COP^{14a} \) males are homozygous for two hypomorphic alleles [30]; hence, neither of the two mutant strains contains a typical WT copy of the \( \gamma COP \) gene comparative to the Oregon control flies.

We suppose that alternative compensatory mechanisms are acting in the mutant background of the two strains. The overexpression of \( \gamma COP \) in \( \gamma COP^{14a}/\gamma COP^{14a} \) males does not necessarily result in an overproduction of \( \gamma COP \) protein since the \( \gamma COP^{14a} \) allele proved to be functionally hypomorphic. The qRT-PCR and microarray data show a compensative overexpression of an abnormal mRNA encoded by the \( \gamma COP^{14a} \) allele, actually conducting a net deficit of the protein comparative to Oregon males [30]. On the other hand, \( \gamma COP^{S057302} \) is an embryo lethal null allele, so an upregulation is not expected for it, but it is for the wild-copy allele, by a transvection phenomenon similar to the one reported for the \( Men \) gene [34]. Hence, it is plausible to consider that in the heterozygous males, In(3R)2C impedes on the ability of the specific cis-regulating sequences to enhance the expression of \( \gamma COP \) by trans-regulation of the normal allele. Alternatively, this inversion may also expose \( \gamma COP \) to the action of local non-specific suppressors, resulting in a slight downregulation of gene expression [35].

In a previous work focusing on a commensal microbiota load of our mutant strains [24], we found that \( \gamma COP^{14a}/\gamma COP^{14a} \) males harbored the lowest bacterial load as compared to both \( \gamma COP^{14a}/TM6B \) and Oregon flies, the latter having the highest microbial charge [24].
Lowering the rearing temperature to 18 °C amplifies the bacterial load differences between γCOP14a/γCOP14a and Oregon individuals. These differences may be a consequence of impaired intracellular vesicle cargo protein transport that could impact protein secretion and the assembly of receptors involved in microbial recognition.

The data fit the same hierarchy of the allelic severity scale that we previously reported for lethality and sterility of these γCop alleles [30]. Therefore, the phenotypic severity modulation of lethality, sterility, and commensal microbiota load are reasonably account-

able for the differences between the two alleles of γCOP. If commensal load data was inconsistent with the severity of γCOP alleles, we would have suspected a hidden mutation in l(3)S057302 chromosome.

A recent paper [19] reports that homozygous missense mutation p.K652E in human γ1-

COP impedes on the ability of COPII complex to bind to the KDEL receptor (KDEL), a key step in the retrieval of chaperones containing the KDEL domain from Golgi to endoplasmic reticulum (ER). The KDEL chaperone proteins are required in ER to assist the proper folding of the secretory proteins as antibodies and cytokines, and their absence contributes to ER stress. Both human patients and model homozygous Copg1K652E mice are affected by combined immunodeficiency (CID), defined by disturbed humoral and cell-mediated immunity. Therefore, the authors demonstrate that p.K652E in the homozygous condition is responsible for CID and reveal that the COPG1 gene is required for a normal adaptive immune response.

In this study, we have shown that, upon P. aeruginosa ingestion, the γCOP heterozygous or homozygous mutants exhibit a different expression profile of many genes encoding for highly evolutionarily conserved innate immune receptors, signaling pathways, and effector molecules, which might affect the response to both pathogenic agents and normal microbiota. When focusing on microarray results for genes from Toll and Imd pathways involved in the innate immunity, we noticed intriguing results. Significant gene expression modulations in either infected or uninfected flies are more frequent in γCOP14a/γCOP14a males as compared to γCOP5057302/TM6B ones, and are more frequent in Toll rather than in Imd pathways (Figures 1 and 2).

Our microarray data reveal that the transcription patterns of Toll and Imd/Imd-JNK pathways in γCOP14a/γCOP14a males are associated with a significant upregulation of γCOP (3.8079X for the infected males and 2.4478X for the uninfected ones). We previously detected a comparative level of overexpression in testes and the oocytes of γCOP14a/γCOP14a individuals, but also in γCOP14a/γCOP14a embryos [30]. These constitutive, correlated expression profiles support once more that γCOP mutant background is, at least partially, responsible for the genes’ expression snapshot noticed in Toll and Imd/Imd-JNK pathways.

The genetic background of the organisms used in biology experiments are always a sensible aspect of data interpretation. Although we rely on complementation tests for assessing the γCOP alleles, hidden or known mutations are to be considered when making conclusions about the results. Sometimes, more or less gratuitous genetic markers are employed in crosses of fruit flies in order to ease the trace back of mutant alleles of the genes of interest. In our case, both γCOP14a/γCOP14a and γCOP5057302/TM6B males have constitutively hemizygous mutations for white, a gene located in the X chromosome and recently reported to be involved not only in red eye pigmentation, but also in the response to some metabolic and cellular stresses [36,37]. To date, there is no information indexed in FlyBase to support or suggest that white is involved either in the immune response of flies or in interactions with γCOP or COPI. Nevertheless, in order to exclude a potential contribution of white to the activation of Toll and Imd—JNK pathways in our experiments, we took advantage to the fact that γCOP5057302/TM6B males contain a single functional copy of mini-white (wr+mc) allele in the P{lacW1057302} transposon [26]. The wr+mc is a key genetic marker used to confirm a P{lacW} insertion in the genome of fruit flies with white defective genetic background. Since all of the γCOP5057302/TM6B males have deep orange eyes instead of white ones, it is evident that wr+mc is functional in these transgenic
organisms. Our microarray data reveal that the expression level of white in the uninfected and infected heterozygous males is indeed the same as in the Oregon ones, which contain a wild-type copy of white. The γCOP^{+}_14a allele was obtained by inducing an imprecise excision of P[w]^{S057302}, therefore γCOP^{+}_14a/γCOP^{+}_14a males no longer contain the w^{+}mc allele. As expected, the microarray data show a significant similar downregulation of white expression in both uninfected and infected γCOP^{+}_14a/γCOP^{+}_14a, but the behavior of Toll and Imd/Imd−JNK are still similar to the w^{+}mc, γCOP^{S057302}/TM6B males. Altogether, these genetic and molecular data reasonably rule out a significant contribution of the white background in the activation of Toll and Imd pathways in the γCOP^{S057302}/TM6B males, or in keeping these pathways in a dormant state in the wild-type white background of Oregon flies. Although over a thousand specific alleles are reported in FlyBase, white is not an essential gene, as no lethal alleles of it are documented so far. Conversely, excepting for eCOP, lethal alleles are reported for 7 out of 8 genes encoding for COPI complex in D. melanogaster.

It is remarkable that none of the genes scrutinized in this paper are either up- or downregulated in infected Oregon males. Plausibly, the γCOP background is the genetic driver that modulates the expression of many Toll and Imd genes in the uninfected mutant homozygous and heterozygous males, but not in the Oregon ones. Our findings are consistent with the essential role of γCOP proteins in the assembly of COPI vesicles; γCOP is the main interactor of the ADP-ribosylation factor at 79F (Arf79F), the fruit fly ortholog of mammalian ARF-1, which, in its turn, is a key protein for both assembly of the COPI complex and for anchoring it to the membrane of Golgi. The other interactor of Arf79F is βCOP (encoded by βCOP in D. melanogaster) and disruption by RNAi of either gene influence the immune system response of D. melanogaster [38,39]. A very conspicuous aspect is that the depletion of Arf79F by RNAi activates the Toll pathway in fruit flies, contributing to the overexpression of Drs and Mik [38] even in the absence of an experimental infection with Gram-positive bacteria. These data partially mirror our data for uninfected γCOP^{S057302}/TM6B males, where Mik and Drs1 are constitutively downregulated, but dl, cact, Tep4, and wntD are upregulated.

Arf79F, βCOP, and γCOP represent the key triplet involved in COPI assembly. Since Arf79F and βCOP are associated with the immune system GO term in FlyBase, it is logical to learn that γCOP is also involved in the immune response of D. melanogaster.

Our experiments revealed that Toll and Imd/Imd−JNK pathways are not activated in the infected Oregon males mainly because they are wild-type for γCOP. Oregon males respond to per os infection with P. aeruginosa but without involving genes of Toll and Imd pathways. The Oregon males exhibit an immune response to these bacteria, as ventral veins lacking, Listericin, Turandot A and Turandot C genes are modulated consecutively with infection. On the other hand, experimentally infected Oregon strain may not activate some genes involved in the innate immune response, most probably if a distinct infection is interpreted as a relatively mild one. As an example, a per os infection with the flagellate parasite Crithidia spp. activates CecA1 and Dipterisin genes from the Imd pathway and Def and Drs from the Toll pathway only in the IIL97 wild-type strain but not in the Oregon [40] flies.

The biological significance of the mutant background of γCOP (at least in males) is that disturbances in the normal physiology of γCOP impact Toll and Imd/Imd−JNK pathways and induce modulations of the transcriptional profiles for many of the constituent genes. Albeit the homozygous mutants present a robust overexpression and the heterozygous display a questionable downregulation of γCOP, it appears that both conditions have an impact on the pathways. Since misregulation of γCOP^{14a} is stronger and significant, it is expected to have a greater impact on the transcriptional landscape of both pathways. Overexpression of γCOP^{14a} in γCOP^{14a}/γCOP^{14a} males determines a more consistent perturbation of the Toll pathway and a bigger difference between infected versus uninfected homozygous males.
We inquire further about the genes from the Toll and Imd/Imd-JNK pathways that had a significantly modulated gene expression in our experiment.

3.2. Toll Pathway

In contrast to most vertebrate species, the *D. melanogaster* anti–infectious defense is represented by highly conserved, complex, and interrelated innate immunity mechanisms, which are activated by the recognition of pathogenic viruses, bacteria, fungi, and parasites [41]. Regarding *P. aeruginosa*, previous studies have shown that besides the well-known pro-inflammatory effects of LPS, many other virulence factors, including proteins involved in protein folding, quorum sensing, transcriptional regulators, efflux (multidrug transporters) systems, biosynthesis of redox active compounds (periplasmic thiol-disulfide oxidoreductase), toxins (exotoxin A, phospholipase C, repeats-in-toxin exoproteins), and proteins of unknown function are essential for maximum pathogenicity in *D. melanogaster*, triggering the activation of an innate immune response and secretion of proinflammatory cytokines [42–44].

Toll and Imd are the major innate immunity pathways regulating the production of AMPs and other immune responses in fruit flies [45].

The Toll/Cactus signaling pathway is mainly triggered by fungal infections, while the Imd cascade, including TAK1, Dredd, IKK complex, and Rel, is activated in bacterial infections [46,47]. The Toll pathway (Figure 3) is activated upon the binding of spz cytokine to the Toll-1 receptor ectodomain. Upon receptor signaling, dimerization/oligomerization of TIR (Toll/IL-1R receptor), the intracellular domain of the Toll receptor, allows the recruitment of a complex comprising of Myd88, Tub and pll. This complex is responsible for downstream phosphorylation and degradation of cact, facilitating the nuclear translocation of dl and dorsal-related immunity factors (Dif) [48]. Cact phosphorylation is catalyzed by Gprk2 [49]. Dif/dl nuclear translocation is positively regulated by TNF receptor-associated factor 6 (Traf 6), following interaction with the pll and Pellino (pli) complex [50]. This process is also negatively regulated by a negative feedback loop involving wntD [51] and a krz-Ulp1 sumorylation complex [52]. Dif/dl dimers or heterodimers require a deformed epidermal autoregulatory factor-1 (Deaf1) transcription factor to produce the antimicrobial peptides Drs, Drsl1 and Mtk [48].

Eight other Toll receptors (Toll 2–9) are described in *Drosophila*, although their involvement in the upregulation of AMP genes is not fully understood. However, regarding Toll-9, it was demonstrated that vertebrates express multiple Toll-like receptors (TLRs) resembling *Drosophila* Toll-1 receptor. They are involved in the regulation of different immune responses upon recognition of different pathogen-associated molecular structures, such as Gram-negative bacterial lipopolysaccharides, peptidoglycans, or flagellin [48].

Pathogen recognition is dependent on the interaction between the extracellular modSP and GNBP1, GNBP3 or PGRP-SA, and PGRP-SD [53], leading to an extracellular cascade involving grass and a serine protease complex (spirit, spheroid, sphinx) resulting in the activation of SPE, responsible for spz activation [48]. In our study, the expression of SPE was downregulated in all four mutant lines, irrespective to the infection, demonstrating that the genetic background inhibits the production of spz cytokine, the ligand of Toll-9. Moreover, the constitutive expression of Toll-9 is inhibited in the infected and uninfected homozygous mutants, as well as in the infected heterozygotes, proving the role of the genetic background in the innate immune response. Previously, it was demonstrated that Toll-9 is functionally homologous to Toll-1 inducing a strong dl expression [54].
Figure 3. Biochemical model of Toll pathway activation in *D. melanogaster* cells as a response to microbial infection. The observed gene expression change corresponding to the experimental conditions and mutants is shown in blue (upregulation) or red (downregulation). With orange-filled or orange-bordered squares, we indicate that the corresponding gene significantly varies its expression in infected and, respectively, uninfected γCOP14a/γCOP14a males. With green-filled or green-bordered triangles we indicate that the marked gene significantly varies its expression in infected and, respectively, uninfected γCOP14a/γCOP14a males. Unmarked components of the pathway did not show any statistically significant expression change. Created with BioRender.com (accessed on 24 February 2022).
Regarding the Toll cascade inside the nucleus, we have observed that in both infected and uninfected experimental conditions, \textit{cact} is upregulated. To bind to Toll-1, the circulating zymogen (pro-spz-1) is cleaved by the SPE into spz [55]. Subsequently to spz binding to Toll-1, the I\textsuperscript{-}\kappa\text{B inhibitor cact is phosphorylated and degraded, enabling the translocation of the NF\textsuperscript{-}\kappa\text{B transcription factors Dif and dl into the nucleus where they stimulate the AMP genes expression} [56]. Thus, one would expect that the upregulation of \textit{cact} could block the nuclear translocation of the dl transcription factor. However, in our study the dl expression was increased in all experimental groups.

The \(\gamma\text{COP} \) mutant background is also associated with increased expression of the \textit{wntD}. It was previously shown that \textit{wntD} acts as a feedback inhibitor of dl/NF\textsuperscript{-}\kappa\text{B}, blocking dl nuclear accumulation, independently of the I\textsuperscript{-}\kappa\text{B homologue cact. The \textit{wntD} loss-of-function (LOF) was associated with immune defects and increased levels of Toll/dl signaling [57]. However, in our study both \textit{wntD} and dl expression levels were increased. Thus, our results suggest that in \(\gamma\text{COP} \) mutants, dl expression levels are increased, even though two of the inhibitors of this pathway, i.e., \textit{cact} and \textit{wntD}, are upregulated. This might suggest the involvement of other regulatory pathways.

The downregulation of the SUMO protease, \textit{Ulp1}, correlated with decreased expression of the \(\beta\text{-arrestin} \textit{krz} \), was noticed in the heterozygous \(\gamma\text{COP} \) mutants. The \textit{Ulp1} downregulation could also account for the dysregulation of the Toll signaling pathway and impairment of the immune system homeostasis. Previous studies have shown that \textit{Ulp1} or \textit{krz} LOF were accompanied by an increased inflammatory response in \textit{Drosophila} larvae [52].

The Toll pathway seems to be more severely affected in homozygous \(\gamma\text{COP} \) mutants via the modulation of the events upstream of the Toll receptor, i.e., downregulation of \textit{GNBP1} and upregulation of \textit{PGRP-SA}, \textit{PGRP-SD}, and \textit{ModSP}. \textit{ModSP} is a modular serine protease that was shown to interconnect the circulating recognition molecules with the grass-SPE-spz extracellular pathway upstream of the Toll receptor [53]. Going downstream of the Toll receptor, the innate response of the homozygous \(\gamma\text{COP} \) mutants is additionally impaired by the downregulation of the highly conserved \textit{pll} (coding for a serine/threonine protein kinase), a positive regulator of the nuclear translocation of the NF\textsuperscript{-}\kappa\text{B transcription factors Dif and dl} [58]. Interestingly, \textit{Gprk2} was inhibited only in homozygous mutants. This kinase has an evolutionarily conserved role in normal dl expression through a NF\textsuperscript{-}\kappa\text{B regulation mechanism, which is unrelated to cact degradation. Noticeably, the cytokine expression consequent to \textit{Escherichia coli} infection was impaired in zebrafish embryos for the orthologous \textit{GRK5}, and in \textit{D. melanogaster}, the susceptibility of infections with \textit{Enterococcus faecalis} infection is increased by RNAi silencing of \textit{Gprk2} [49].

Our results demonstrate for the first time that mutations in \(\gamma\text{COP} \) alter the proper function of this conserved NF\textsuperscript{-}\kappa\text{B signaling regulation pathway. Consequently, the fruit fly’s capability to respond adequately to Gram-negative bacterial infections is also perturbed.}

Overall, our data suggest that the \(\gamma\text{COP} \) background interferes with the Toll signaling pathway and with the innate immunity of \textit{Drosophila}. Most probably, this interaction affects the fruit fly’s ability to recognize the pathogen-associated molecular patterns and to further activate the signal transduction cascades and the transcription of effector genes.

### 3.3. Imd Pathway

The second major cascade involved in the host–pathogen crosstalk is the Imd pathway, whose main role is the production of AMPs through the NF\textsuperscript{-}\kappa\text{B transactivator Rel.}

The Imd pathway (Figure 4) shares many features with the mammalian TLR/IL-1 and TNF-R signaling pathways, pointing to a highly evolutionary conserved innate immunity mediated by production of AMPs [59,60].
Figure 4. Biochemical model of Imd pathway activation in *D. melanogaster* cells in response to Gram-negative (G-) infection. The observed gene expression change corresponding to the experiment conditions and mutants is shown in blue (upregulation) or red (downregulation). With orange-filled or orange-bordered squares we indicate that the pinpointed gene significantly varies its expression in infected and, respectively, uninfected \( \gamma \text{COP}^{14a} / \gamma \text{COP}^{14a} \) males. With green-filled or green-bordered triangles we indicate the genes that significantly vary their expression in infected and, respectively, uninfected \( \gamma \text{COP}^{S057302} / \text{TM6B} \) males. Unmarked components of the pathway did not show any statistically significant expression change. Created with BioRender.com (accessed on 24 February 2022).

Imd pathway-mediated pathogen recognition is dependent on the interaction between LPS and other Gram-negative bacterial wall components, such as the meso-diaminopimelic acid (DAP)-type peptidoglycan (PGN) and the PGRP-LC transmembrane receptor [61,62]. Additional PGRP proteins are involved in PGN regulation (PGRP-LB) and receptor binding.
(PGRP-LE and PGRP-LF) [63]. The intracellular membrane bound receptor PGRP-LE plays a crucial part in the immune response against intracellular pathogens by cooperatively regulating expression of intracellular antimicrobial peptide Listericin via a JAK/STAT interaction [64].

Upon receptor activation, an intracellular complex is recruited at the receptor site, consisting of imd, Fadd, and Dredd (caspase-8 homolog Death-related ced-3/Nedd2-like protein). Ubiquitination of Dredd by Diap2, Uev1A, ben, and eff complex activates Dredd for the cleavage of an imd N-terminal site, followed by ubiquitination of cleaved imd [65]. Alternatively, activation of the intracellular membrane bound receptor PGRP-LE by tracheal cytotoxins (TCT) results in similar interactions. At this level, pirk, an imd and RAS/MAPK pathway regulated protein, inhibits imd activation by the transmembrane receptors [66]. Processed imd activates downstream the Tak1, Tab2 complex, which phosphorylates the IKK complex (IKKβ and key), leading to activation of Rel transcription factor [63]. Prior to nuclear translocation, phosphorylated Rel requires Dredd mediated cleavage. Whilst Caspar (casp) inhibits both the Dredd-Rel interaction and Rel nuclear translocation, Traf6 facilitates the latter [63,67]. Transcription factors caudal (cad) [68] and nuclear protein akirin [69] interact with Rel dimers for regulating the production of several types of AMPs, namely diptericins (Dpt, DptB), cecropins (CecA2, CecB, Cec-Ψ1, Cec2) and attacins (AttA, AttB, AttC, AttD).

In our study, both γCOP infected and uninfected homozygous mutants, as well as infected heterozygous mutants exhibited an increased expression of the PGRP-LC. While this upregulation was expected for *P. aeruginosa* infected flies, the expression profile change in the uninfected homozygous males suggests that the γCOP mutant background induces a dysregulation of the humoral immune response in *Drosophila*. The same upregulation profile was recorded for PGRP-LB, an amidase that specifically degrades the Gram-negative PGN. This process downregulates the Imd pathway and provides a fine negative feedback regulation of the immune response to infection, including the modulation of the immune reactivity to ingested bacteria in the gut of the fruit flies [70]. The upregulation of this amidase in uninfected γCOP homozygous flies also supports the hypothesis of an immunodeficiency condition in these mutants. However, an alternative explanation could be that the fruit flies distinguish the low virulence of the infecting strain and thus an exaggerated immune response is prevented. This hypothesis is sustained by a significant upregulation of PGRP-LB in infected versus uninfected homozygous γCOP mutants.

*Fadd* and *imd* expression was inhibited in both infected and uninfected flies with different γCOP mutant genotypes, suggesting a disturbance of the innate immunity response in these mutants. The association of Fadd with imd is required for further activation of the Imd signaling pathway and previous studies have shown that loss of Fadd function was associated with high susceptibility of flies to Gram-negative bacteria infections [71]. Our results show that *Fadd* is strongly downregulated in all of the experimental conditions, and this could severely impair the proper activation of the Imd pathway in γCOP mutants. Moreover, *P. aeruginosa* infection upregulates the expression of *pirk*, a negative regulator of the Imd signaling pathways, in both mutant genotypes, thus enhancing the immunodeficiency condition in these flies. Previous studies have also shown that *pirk* overexpressing fruit flies were indeed more susceptible to Gram-negative bacterial infection than WT ones [66]. Another gene that was downregulated in infected homozygous males is *POSH*, which is essential for the properly timed activation and termination of Imd-mediated immune response [72]. These results are supporting our hypothesis that γCOP mutation induces a dysregulation of the innate immune response against Gram-negative bacterial infection [72].

The main target of the Imd pathway is the transcription factor Rel, which is activated within minutes after infection, triggering the expression of AMPs genes within hours. In order to prevent an exaggerated immune response to harmless commensal microbes, the production of AMPs is tissue specific [73]. In our study, the expression of *Rel* was significantly upregulated in the homozygous infected males and downregulated in
uninfected heterozygous flies. This different expression pattern is visible when all the experimental conditions are considered (FC values of infected $\gamma$COP$^{S057302}$/TM6B, uninfected $\gamma$COP$^{S057302}$/TM6B, infected $\gamma$COP$^{14a}$/\$COP^{14a}$, uninfected $\gamma$COP$^{14a}$/\$COP^{14a}$, and infected Oregon are $-0.2116$, $-0.446$, $0.439$, $0.033$, and, respectively, $0.0322$). These data could suggest: (i) that the lack of functional $\gamma$COP impairs the regulation of $D.\ melanogaster$ response intensity to a non-virulent $P.\ aeruginosa$ strain or/and (ii) particular interaction between the Toll and Imd pathways could synergistically act to overcome some defects affecting one of the two pathways (Figure 5). It was previously shown that Toll activation may increase the level of Rel, and the \textit{Rel} and \textit{spz} double mutant exhibits a higher susceptibility to \textit{E. coli} infection as compared to the \textit{Rel} mutant fruit flies. Alternatively, the Imd pathway could regulate the Toll signaling cascade by controlling the PGRP-SA [74]. These observations are also supported by the upregulation of \textit{Rel} and of PGRP-SA in the infected homozygous $\gamma$COP mutants.

Figure 5. Biochemical model of interactions of Toll and Imd pathways in $D.\ melanogaster$ as a response to microbial infection. The immune response in $D.\ melanogaster$ involves the activation or inhibition of multiple pathways, such as RAS/MAPK, JNK, or JAK/STAT. The observed expression change corresponding to the experiment conditions and mutants is shown in blue (upregulation) or red (downregulation). With orange-filled or orange-bordered squares, we indicate that the respective gene significantly varies its expression in infected and uninfected $\gamma$COP$^{14a}$/\$COP^{14a}$ males. With green-filled or green-bordered triangles we indicate a gene that significantly varies its expression in infected and, respectively, uninfected $\gamma$COP$^{S057302}$/TM6B males. Unmarked components of the pathway did not show any statistically significant expression change. Created with BioRender.com (accessed on 24 February 2022).
The AMPs expression profile was predominantly downregulated in different mutant groups, suggesting that an immunodeficiency condition affecting the innate humoral immune response is mediated by AMPs in γCOP mutants.

However, some exceptions were noticed, i.e., CecA2 upregulation in uninfected homozygous γCOP mutants, CecB upregulation in uninfected homozygous γCOP mutants and infected heterozygous γCOP mutants, and Cec2 upregulation in infected/uninfected homozygous and uninfected heterozygous γCOP mutants. In particular, the upregulation of these cecropins in the uninfected flies suggests a defect in strict regulation of the innate humoral response in the mutant flies.

3.4. Toll and Imd Pathways Interplay

The increased expression levels of the cecropins in the infected heterozygous flies with a normal Rel level, may be explained by the possible involvement of other signaling pathways that could modulate the AMP profile, including the Toll cascade [75]. It was previously shown that Dif and, to a lesser extent, dl gene products trans-activate the CecA1 gene in co-transfection assays [76]. In our study, dl was upregulated in all experimental variants and Dif exhibited expression profiles similar to the uninfected wild-type control (Figure 5). The upregulated or normal levels of these regulators could, at least partially, explain the increased expression levels of the three cecropins. Within the Imd cascade, the Tak1, Tab2 complex creates a bifurcation to the JNK pathway, and the complex activation is inhibited by the POSH scaffold protein [72]. This JNK bifurcation at the Tak1, Tab2 complex positively regulates the activation of the AP-1 transcription factors complex (Ira and kay) via a series of phosphorylations. The AP-1 complex translocates to the nucleus where, together with JAK/STAT controlled Stat92E and dl switch protein 1 (Dsp1), it regulates Rel-dependent expression of AMPs [77]. It was noted that simultaneous activation of Toll and Imd pathways can lead to nuclear heterodimers between Dif, dl and Rel transcription factors [78], which control the expression of AMPs associated with both pathways.

However, since a cumulative or even synergistic effect of different AMPs is needed to eliminate Gram-negative bacteria, the general AMP downregulation landscape revealed by our study indicates a defective innate humoral immune response in the γCOP mutants.

Considering the changes observed for the expression of genes pertaining to Toll and Imd pathways, the γCOP mutant background might alternatively modulate other members of the complex innate immunity network, to allow the fruit flies to resist microbial aggression. In the extracellular space, proteins regulated by the Toll pathway, such as BomS1, BomS2, BomS3 [79], or Baramicin proteins (BaraA1, BaraA2) [80], and by the JAK/STAT pathway, involved in regulation of Tep4 via a positive feedback loop [64,81], act as immune response regulators upstream of transmembrane receptors. In our study, an increased expression of BomS1, BomS2 and BomS3 was observed in uninfected homozygous mutants. This family of secreted peptides is known to mediate the innate immune response activated by Drosophila Toll receptors upon pathogen recognition and it is involved in fruit fly’s resistance to infection [79,82].

All infected and uninfected fruit flies exhibit upregulated levels of Tep4, whose product is involved in the opsonization and elimination of pathogens and in the amplification of the inflammatory response in vertebrates. Tep4 also plays a role in regulating apoptosis, metabolic activities, and pathophysiological effects in fruit flies challenged by Photorhabdus infection [83,84]. The upregulation of Tep4 in both infected and uninfected fruit flies could suggest that the γCOP mutant phenotype triggers augmented and potentially deleterious inflammatory responses to less virulent and commensal microbes. Both infected and uninfected heterozygous γCOP mutants exhibit a downregulation of Stat92E from the JAK–STAT signaling pathway, which was previously shown to be involved in D. melanogaster response against DNA viruses [85]. Stat92E downregulation could partially explain the downregulation of Rel observed in uninfected heterozygous males.

As already shown in Figures 3 and 4, Traf6 is regulated by both Toll and Imd pathways, facilitating nuclear translocation of each pathway’s final transcription factors [50,67]. Both
the krz-Ulp1 sumoylation complex [52], which inhibits nuclear translocation of Dif and dl, and pirk, which inhibits lmd activation by the transmembrane receptors [66], are linked to the RAS/MAPK pathway. The RAS/MAPK pathway is involved in activation of the stress response regulating protein Mekk1, and along with the Tak1 and Tab2 complex, activates the serine/threonine kinase licorne (lic), leading to p38c and Atf-2 phosphorylation and resulting in dual oxidase (Duox) expression [86]. The p38 mitogen-activated protein (MAP) kinase signaling cascade is known to be involved in the physical, chemical, and biological stress response and immunity in different species. Among other substrates, the p38 kinases phosphorylate the ATF family transcription factors. In our study, Atf-2 was downregulated in the infected experimental groups, as well as in the uninfected heterozygous males. The decreased expression levels of Atf-2 could be linked to a lower production of ROS, as a result of a decreased level of the ROS-producing enzyme Duox [86] (Duox exhibited a FC of $-0.145$ in infected $\gamma$COP$^{14a}/\gamma$COP$^{14a}$, almost reaching the significance threshold with a p value of 0.088).

4. Materials and Methods

4.1. D. melanogaster Stocks

To perform the experimental infection and subsequent microarray experiment we employed young adult males from three strains of D. melanogaster that were maintained on the Nutri-Fly Bloomington Formulation medium (Genesee Scientific, San Diego, CA, USA). These strains are: 1. Oregon, standing for the standard WT strain; 2. $\gamma$COP$^{S057302}/$TM6B harboring the insertion of $\gamma$Cop$^{S057302}$ transposon in the 5′UTR region of $\gamma$COP gene (GenBank accession number AJ492220) and causing embryo lethality; 3. The homozygous $\gamma$COP$^{14a}/\gamma$COP$^{14a}$ males were harvested from $\gamma$COP$^{14a}/$TM6B strain and contain the hypomorphic $\gamma$COP$^{14a}$ allele (GenBank accession number DQ279402). Both mutant lines have white defective genetic background.

4.2. Bacterial Strain

The P. aeruginosa ATCC 27853 strain was cultivated on Cetrimide agar at 37 °C and following 24 h of cultivation, bacterial suspensions of $9 \times 10^9$ CFU/mL were obtained.

4.3. Bacterial Ingestion Assay

WT Oregon, $\gamma$COP$^{S057302}$/TM6B, and $\gamma$COP$^{14a}/\gamma$COP$^{14a}$ males were starved for several hours prior to being placed in vials containing 5% sucrose agar covered with sterile paper blotted with either 230 µL of 5% sucrose solution containing $9 \times 10^9$ CFU of P. aeruginosa suspension (infected replicates) or 230 µL of 5% sucrose (uninfected replicates).

A total of 20 Oregon uninfected replicates were considered as reference; for each of the $\gamma$COP$^{S057302}$/TM6B and $\gamma$COP$^{14a}/\gamma$COP$^{14a}$ infected and uninfected experimental variants four biological replicates were used, as well as for the infected Oregon males. Every biological replicate comprised of 25 to 27 individuals.

The males were allowed to feed for about 63 h at 25 °C, then, a total of 25 males/replicate were placed in 1.5 mL Eppendorf tubes, covered with 300 mL TRIZol, and shipped to FlyChip Facility (Cambridge Systems Biology Centre, Cambridge, UK).

4.4. Microarray Project

The D. melanogaster samples (40 biological replicates) were processed at FlyChip according to their standard protocol. Briefly, medium-scale RNA extraction was performed in the first step; then, the 20 control Oregon RNA samples were pooled together and converted into double-stranded DNA used as a reference to compare all experimental conditions against the control. All the samples were submitted to Klenow labelling of 1 µg double-stranded DNA derived from total RNA and subsequent hybridization to amino-modified long oligonucleotide microarrays, using the FL003 (FlyChip_long_oligonucleotide_003—INDAC) array format. The fluorescent spots were analyzed with a GenePix Axon scanner (5 µM pixel resolution).
4.5. Gene Expression Data Set

Microarray data are available online at NCBI’s Gene Expression Omnibus (GEO), accession number GSE80084. Normalization and quality control steps were performed using \textit{vsn} [87] and \textit{limma} [88] software packages (Bioconductor). The resulting values are expressed as log$_2$ ratios of sample/control (log$_2$FC values).

4.6. Microarray Data Analysis

Heatmaps summarizing the up— and downregulated genes associated with Toll and Imd pathways, as well as the overview of the differential expression of immune genes in $\gamma$COP mutants, were generated with GraphPad Prism 5.03 software (GraphPad Software, La Jolla, CA, USA) using the log$_2$FC values.

5. Conclusions

This insight into the whole-body transcriptome of \textit{D. melanogaster} males with specific $\gamma$COP mutant backgrounds revealed that the expression of at least 52 genes of the Imd and Toll pathways and their interactors were significantly modulated upon infection with \textit{P. aeruginosa}. Remarkably, in both infected and uninfected mutant males, the expression pattern of a vast majority of the genes from both pathways is affected when compared to the uninfected Oregon control. A very interesting aspect is that none of these genes are significantly modulated in infected Oregon males. This led us to hypothesize that natural microbiota might act as an environmental trigger in $\gamma$COP mutant background, a condition that signals an alert state. The peculiar microarray pattern may suggest that genes from Toll and Imd pathways respond to this alert by adjusting their transcription rate even in the absence of experimental infection with \textit{P. aeruginosa}. One scenario is that, in the absence of enough functional $\gamma$COP product, the host increases the stringency of the feedback control of commensal microbiota, by triggering an erratic immune response.

Further studies are needed to confirm and refine the contribution of both host’s genetic background and different virulence factors to bacterial pathogenicity and host immune response. In our experimental design, we have selected a laboratory \textit{P. aeruginosa} bacterial strain with low virulence administered by ingestion, in order to evaluate the ability of adult flies to adequately sense and respond to the presence of Gram-negative ubiquitous bacteria relative to their $\gamma$COP genotype. In natural conditions, fruit flies seem to be able to detect Gram-negative components, such as LPS, which is sensed through the transient receptor potential (TRP) channels (e.g., the TRPA1 protein), and thus avoid eating food contaminated with LPS and bacteria [89]. However, whether the activated receptors further activate the fruit fly’s immune response to a potential infection remains to be elucidated. Studies performed on mice have shown that the deletion of TRPA1 decreases pain and inflammation induced by LPS, suggesting that detection of bacterial components in food modulates the innate response to Gram-negative pathogens [90]. Complementarily, research using highly virulent \textit{P. aeruginosa} strains administered by direct injection in the hemolymph [91] could help to elucidate the role of $\gamma$COP mutations in the innate immune response to Gram-negative infections. The $\gamma$COP mutations are affecting the fruit fly’s ability to recognize the pathogen-associated molecular patterns and to further activate the signal transduction cascades and the transcription of effector genes in the Toll cascade. Both inhibitory (downregulation of \textit{GNBP1}, inhibition of \textit{SPE} and \textit{Toll-9}, upregulation of \textit{cact}, \textit{wntD}, and \textit{Gprk2}) and activating (inhibition of \textit{Ulp1}/\textit{krz}, upregulation of \textit{PGRP-SA}, \textit{PGRP-SD}, and \textit{ModSP}) effects were noticed, suggesting a dysregulation of this important innate immunity pathway. The same dual effects were recorded for the Imd cascade, whose expression profiles suggest either inhibition (upregulation of \textit{PGRP-LB}, inhibition of \textit{imd} and \textit{Fadd}, upregulation of \textit{pirk}) or activation (increased expression of the \textit{PGRP-LC} and downregulation of \textit{POSH}). The variable expression profile of transcriptional factors and AMPs in different experimental groups demonstrates the complex interactions between Toll and Imd, as well as with other signaling pathways.
Interestingly, γCOP14a/γCOP14a males are also unable to compensate for the conditional sterility of γCOP14a/γCOP14a females in the respective crosses [30]. We argue that γCOP is the most probable genetic determinant herein, since comparative data we obtained for viability, fertility, and infection tests points to a similar behavior of the inquired γCOP alleles. Basically, γCOP14a behaves as a hypomorph, and therefore is a less severe allele than the γCOPS057302 null, but heterozygous γCOPS057302/TM6B exhibit less severe phenotypes of sterility and immune sensitivity compared to γCOP14a/γCOP14a males.

Overall, our data suggest that the γCOP background interferes with the Imd and Toll signaling pathways at different levels. The γCOP mutations disturb the fine regulation of the innate immune response and hence the fruit fly’s capability to adequately respond to Gram-negative bacterial infections.

Our results reveal in premiere that mutations of the γCOP gene impair not only the adaptative immune response, as in humans and mice, but also the innate immune response of D. melanogaster, reinforcing the power of this organism for modeling infections.

Purportedly, the prognostic of some bacterial infections should be estimated not only by the classical clinical procedures, but also by a quick screening of patients for γCOP mutations, as inferred from recent medical research data [19].

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