Two avian *Plasmodium* species trigger different transcriptional responses on their vector *Culex pipiens*

Running title: *Cx. pipiens* response to avian *Plasmodium* infection

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Abstract

Malaria is a mosquito-borne disease caused by protozoans of the genus *Plasmodium* that affects both humans and wildlife. The fitness consequences of infections by avian malaria are well known in birds, however, little information exists on its impact on mosquitoes. Here we study how *Culex pipiens* mosquitoes transcriptionally respond to infection by two different *Plasmodium* species, *P. relictum* and *P. cathemerium*, differing in their virulence (mortality rate) and transmissibility (parasite presence in exposed mosquitoes' saliva). We study the mosquito response to the infection at three critical stages of parasite development: formation of ookinetes at 24 hours post-infection (hpi), the release of sporozoites into the hemocoel at 10 days post-infection (dpi), and storage of sporozoites in the salivary glands at 21dpi. For each time point, we characterized the gene expression of mosquitoes infected with each *P. relictum* and *P. cathemerium* and mosquitoes fed on an uninfected bird and, subsequently, compared their transcriptomic responses. Differential gene expression analysis showed most of the transcriptomic changes occurred during the early infection stage (24 hpi), especially when comparing *P. relictum* and *P. cathemerium* infected mosquitoes. Differentially expressed genes in mosquitoes infected with each species were related mainly to the immune response, trypsin, and other serine-proteases metabolism. We conclude that these differences in response likely underlay the differential virulence and transmissibility previously observed in *P. relictum* and *P. cathemerium* in *Cx. pipiens*.

Keywords: avian malaria, *Plasmodium relictum*, *Plasmodium cathemerium*, vector-borne parasites, mosquito transcriptome, RNAseq.

1. Introduction

Vector-borne diseases are a major challenge for both human and animal health, representing around 25% of emerging infectious diseases. Mosquitoes are vectors of relevant pathogens including viruses that cause yellow fever, dengue, or West Nile fever and parasites such as nematode worms which cause lymphatic filariasis, and haemosporidians, like *Plasmodium*, which cause malaria (Lehane 2010). Malaria is one of the most important vector-borne diseases for humans, and only in 2020, it caused around 602,000 deaths (WHO 2021). Malaria parasite species affect humans and related primates as well as other mammals, reptiles, or birds, driving some populations to extinction (van Riper et al. 1986).

Avian malaria is a worldwide distributed mosquito-borne disease caused by *Plasmodium* parasites which use birds as obligate hosts (Valkiūnas 2005). Avian *Plasmodium* is transmitted by mosquitoes, mainly of the genus *Culex,* which are the definitive hosts and where *Plasmodium* reproduces sexually (Valkiūnas 2005). In birds, as a part of their life cycle, *Plasmodium* merozoites invade the host erythrocytes and can differentiate into mature gametocytes (Sinden 1983). When a female mosquito feeds on a *Plasmodium*-infected bird, gametocytes are released from the erythrocytes, and male and female gametes unite resulting first in the zygote, and then in the motile ookinete (Sinden 2002). About 24 hours post-infection (hpi) the ookinete crosses the midgut epithelium and remains located between the epithelial surface and the basal lamina, where it transforms into a sessile oocyst. After about 10 days post-infection (dpi), mature oocysts liberate thousands of sporozoites into the hemocoel. Sporozoites that survive the immune system of the mosquito eventually invade

the salivary glands (Sinden 1983; Sinden 2002; Abraham and Jacobs-Lorena 2004) from where they can be then transmitted to a new avian host upon a mosquito bite.

For an appropriate development and transmission of the parasites, mosquitoes susceptible to the infection need to survive it, allowing parasites to complete their life cycle. Thus, the nature of the interaction between pathogens and mosquitoes determines the ability of a vector to acquire, maintain and transmit parasites to a new host, i.e. the vector competence (Beerntsen et al. 2000; Bonizzoni et al. 2013). Therefore, the vector competence is conditioned on the mosquito response against the pathogen (Higgs and Beaty 2005), which includes a number of defense mechanisms. After a blood meal, mosquitoes synthesize and release serine proteases that constitute a chemical barrier against pathogens (Vizioli et al. 2001; Molina-Cruz et al. 2005; Muller et al. 1995) and form a chitin-containing peritrophic matrix around the blood. This matrix constitutes a physical barrier for harmful food particles, digestive enzymes, and pathogens (Lehane 1997). Despite the existence of these and other barriers, some pathogens manage to reach the mosquito midgut, hemocoel, or internal organs and activate the immune response, which may be categorized into cellular and humoral immune responses (Hillyer 2016). The cellular response includes mechanisms such as phagocytosis, cellular encapsulation, autophagy, melanization, and induction of apoptosis, while the humoral response consists of the activation of signaling pathways that eventually result in the synthesis of factors with antimicrobial activity (Michel and Kafatos 2005; Hillyer 2016). In insects, one of the main immune signaling pathways is the Toll pathway, which is activated by the Spätzle cytokine and

92 results in the activation of the transcription of antimicrobial peptides (AMPs) and
93 other immune effectors that may combat pathogens (Kumar et al. 2018). In
94 addition, two other pathways have been shown to be involved in the response
95 to *Plasmodium*, including avian malaria parasites, the immune deficiency (Imd)
96 and the Janus Kinase signal transducer of activation (JAK-STAT) (Clayton et al.
97 2014, García-Longoria et al. 2022).

98 The infection by *Plasmodium* and the mosquito response against
99 infection ultimately result in a cost to vectors (Ahmed et al. 2002), which might
100 depend, among other factors, on which *Plasmodium* species infects the
101 mosquito. For example, Gutiérrez-López et al. (2020), found a higher survival
102 rate and transmissibility (measured as the presence of parasite DNA in the
103 saliva of mosquitoes) in *Culex pipiens* infected with *Plasmodium cathemerium*
104 compared to those infected with *Plasmodium relictum*. However, the genetic
105 mechanisms that underlay these phenotypic differences are unknown. Part of
106 the heterogeneity in parasite virulence and therefore in the fitness
107 consequences in their hosts (Gutiérrez-López et al. 2020) is due to the
108 remarkable cellular plasticity and transcriptional variation of *Plasmodium*
109 (García-Longoria et al. 2020). Avian *Plasmodium* is an extremely diverse clade
110 with at least 55 species (Valkiūnas and Iezhova 2018) divided into more than
111 1,446 unique genetic lineages (Egerhill 2022). This extensive inter and intra-
112 specific genomic variation will translate into different phenotypic characters,
113 including differences of virulence, which will also determine how the mosquitoes
114 respond to an infection.

115 Although studies on avian malaria that use transcriptomic approaches
116 are increasing in the last few years, they have mainly focused on the avian host,

addressing either the bird response to infection (e.g. Videvall et al. 2015) or the gene expression of *Plasmodium* infecting birds (e.g. Videvall et al. 2017; García-Longoria et al. 2020; Videvall et al. 2021). In contrast, to the best of our knowledge, only three studies have analysed the gene expression of mosquitoes infected by avian *Plasmodium* (Zou et al. 2011; Ferreira et al. 2022; García-Longoria et al. 2022). However, none of them focus on *Cx. pipiens*, which is the main vector of avian *Plasmodium* in Europe. Another significant limitation is that all of these studies focus on *P. relictum* and consequently do not take into consideration the effect of the differences in virulence and transmissibility between *Plasmodium* lineages.

Here, we analyse the gene expression of *Cx. pipiens* infected with two widely distributed species of avian *Plasmodium* with different characteristics namely, *P. relictum* (lineage SGS1) and *P. cathemerium* (lineage PADOM02). To that end, we obtained RNA-seq data of mosquitoes infected with the two *Plasmodium* species at three time points corresponding with key stages of parasite development in the vector. These stages are (1) during the formation of ookinetes, (2) during the release of sporozoites into the hemocoel, and (3) after sporozoites invade and are stored in the salivary glands. This study uses a natural avian malaria system that will help to address the current knowledge gaps on molecular mechanisms occurring during *Plasmodium* infections in mosquitoes. In addition, these results will allow us to further understand the differences in virulence and transmissibility between these two *Plasmodium* species (Gutiérrez-López et al. 2020) and how this might affect vector competence.

2. Results

Transcriptomics data description

We obtained 36 mRNA-seq libraries: four replicates for three infection status at three time points. The mean number of reads per library was 33,177,082, ranging from 15,406,828 to 45,332,626 reads for raw samples. Raw data has been made publicly available through to the European Nucleotide Archive ENA database (https://www.ebi.ac.uk/ena/browser/home) under project accession number PRJEB1609, Study ERP125411. The percentage of reads kept after trimming ranged from 80.56% to 99.52%, (12,411,723 to 44,887,231 reads see Table S1). The MultiQC report showed a mean quality score above q30 in all base calls across the read. On average, 80.87% of reads mapped to the genome of *Cx. quinquefasciatus***.** For downstream analyses, we removed two samples taken at 24 hpi that had barcoding errors due to lab processing.

Sample clustering reveals early transcriptomic response to infection

The principal component analysis (PCA) results show that most of the transcriptome variation is contained in the PC1 (85% var.) driven by the time post-infections, under both *Plasmodium* species infections. At 24 hpi, there were clear differences (PC2, 3% var.) between control samples, *P. cathemerium*, and *P. relictum* infected mosquitoes. By contrast, there were no clear transcriptomic differences at later stages, such as between 10 dpi and at 21 dpi, nor between infection statuses (Figure 1).

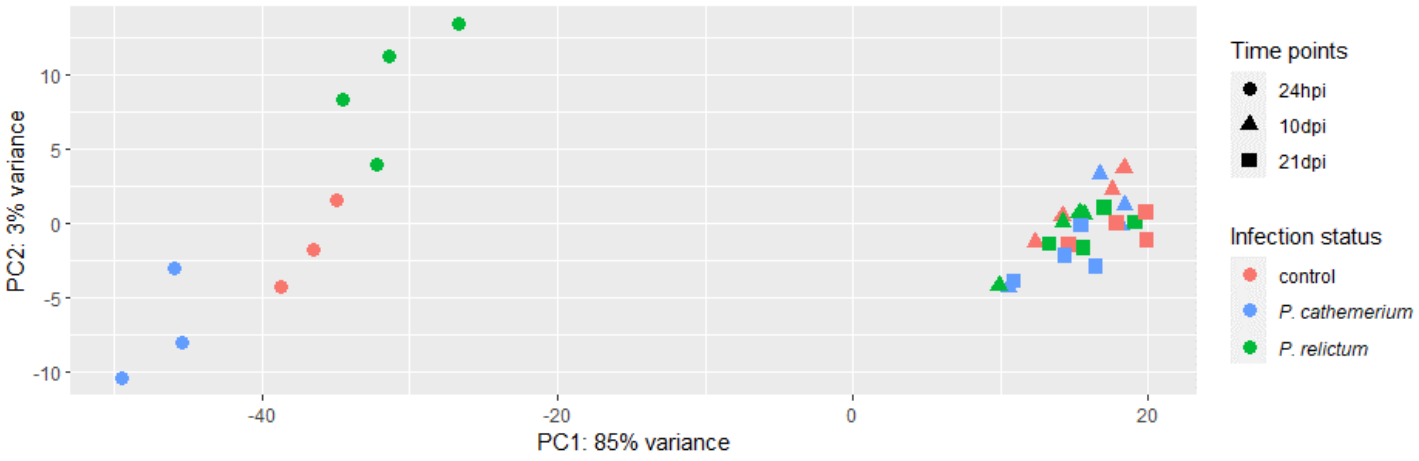

Figure 1. Principal component analysis (PCA) of transcriptome variation *in Cx. pipiens* at three time points after feeding on either an uninfected bird (control), a *P. cathemerium*-infected bird and a *P. relictum*-infected bird. Time points analysed were 24 hours post-infection (hpi), 10 days post-infection (dpi) and 21 dpi. The x-axis shows the first principal component score, which captures 84% of variation and the y-axis shows the second principal component score, which captures 3% of variation.

Differential gene expression analysis and enrichment analysis unveil the clues of the mosquitoe transcriptomic response to Plasmodium infections

Overall, 2,038 genes were differentially expressed in *Cx. pipiens*. Most of the transcriptomic differences were found at an early infection stage (24 hpi), especially when comparing *P. relictum*-infected mosquitoes vs *P. cathemerium*-infected mosquitoes, and *P. relictum*-infected mosquitoes vs controls. No differences were found between mosquitoes infected by *P. cathemerium* and those infected by *P. relictum* at 10 dpi and between mosquitoes infected with *P. relictum* and controls at 21 dpi. At 24 hpi, the comparisons *P. relictum*-infected mosquitoes vs controls and *P. relictum* vs *P. cathemerium*-infected mosquitoes shared 460 differentially expressed genes (Figure 2).

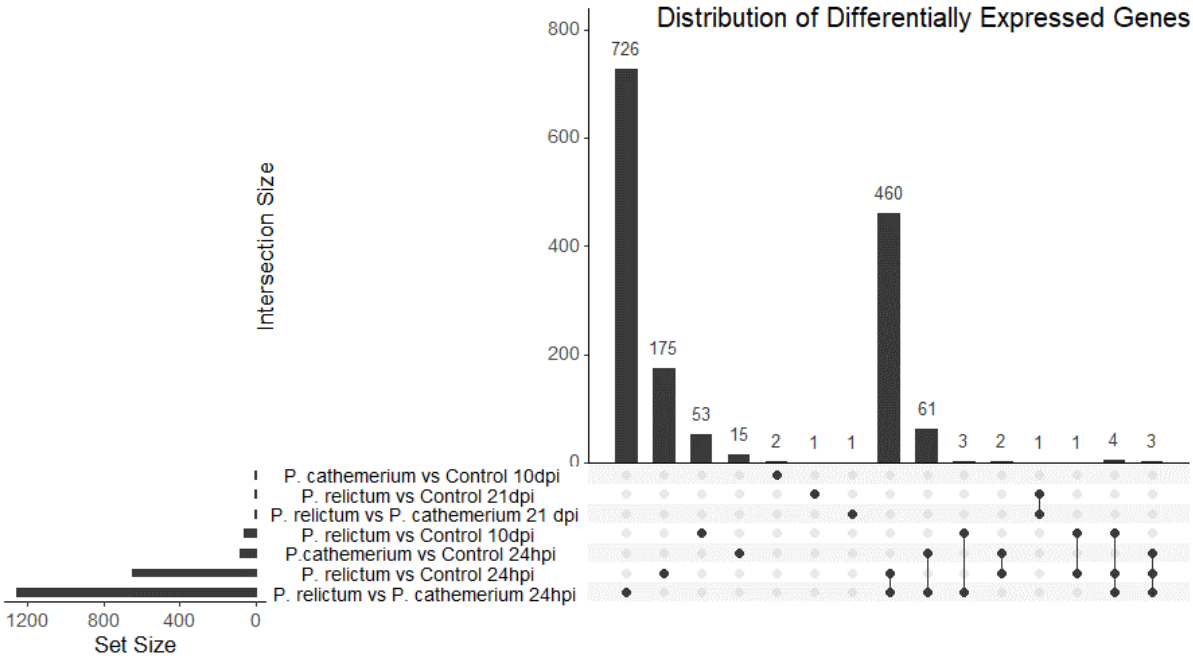

Figure 2. UpSet plot showing overlap size of sets of differentially expressed genes for i) *P. cathemerium* infected mosquitoes vs controls, ii) *P. relictum infected* mosquitoes vs controls, and iii) *P. relictum* infected mosquitoes vs *P. cathemerium* infected mosquitoes at three time points (24 hpi, 10 dpi and 21 dpi). The top vertical bar plot shows the number of genes (y-axis) contained in each intersection (x-axis). The horizontal bar plot at the bottom shows the number of differentially expressed genes for each comparison.

a) *Cx. pipiens* gene expression response to *P. relictum* infection

At 24 hpi, *P. relictum*-infected *Cx. pipiens* had 638 genes differentially expressed when compared to the controls (Figure S1A). Exposition to *P. relictum* triggered the expression of 106 genes, including a cecropin gene (CPIJ005108), the *spätzle* gene (CPIJ006792) and a mitochondrial NADH-ubiquinone oxidoreductase gene (CPIJ009076). The 532 downregulated genes included an apoptosis inhibitor (CPIJ004812), one trypsin (CPIJ007075), chymotrypsins (e.g. CPIJ003915, CPIJ018205, CPIJ007838 and CPIJ006568) and serine proteases genes (CPIJ004984 and CPIJ002112).

At 10 dpi, 54 genes had higher expression levels and 15 genes had lower expression levels in infected mosquitoes compared to controls (Figure S1B). At 21 dpi, a single gene corresponding to an uncharacterized protein was up-regulated, and a testicular acid phosphatase precursor gene was down-regulated.

At 24 hpi, the GO categories within biological processes related to cellular mechanisms and mitochondrial chain complex assemblies presented the greatest number of up-regulated genes. Within down-regulated genes, the enriched biological processes included molecules binding, metabolism, transport and location (Figure 3). The molecular functions enriched for up-regulated genes were cation binding, metal ion binding and ion binding. Protein, ATP, and nucleic acids binding molecular functions (including purine ribonucleoside triphosphate, purine nucleotide, purine ribonucleotide, adenyl nucleotide and adenyl ribonucleotide, among others) were enriched for down-regulated genes (Figure 4).

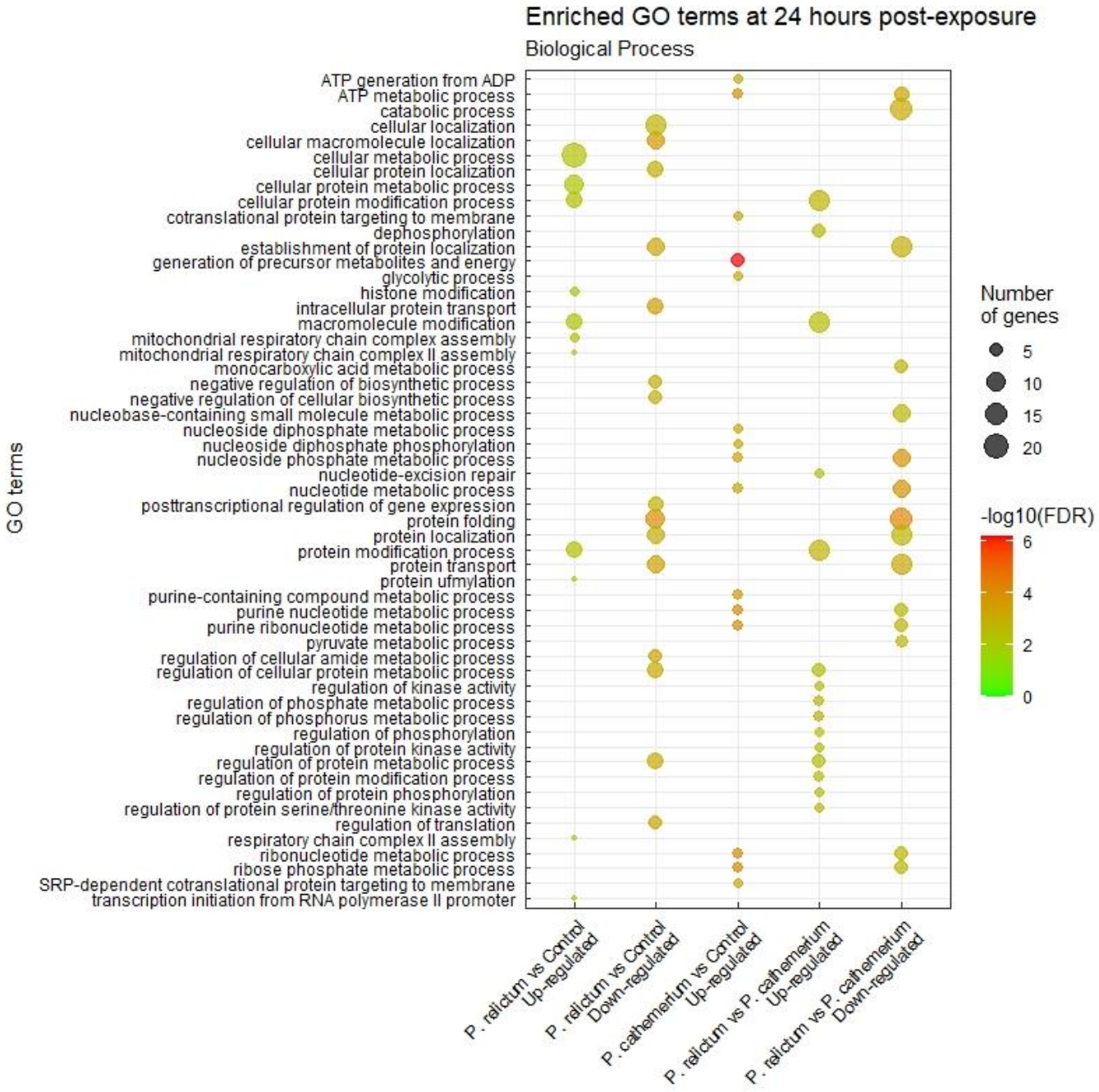

Figure 3. Dot plot of significantly (FDR adjusted p-value <0.01) enriched GO biological processes for differentially expressed genes (up and down-regulated) at 24 hpi for i) *P. cathemerium* infected mosquitoes vs controls, ii) *P. relictum* infected mosquitoes vs controls, and iii) *P. relictum* infected mosquitoes vs *P. cathemerium* infected mosquitoes. We did not find enriched GO terms for down-regulated genes for *P. cathemerium* infected mosquitoes vs controls. Larger dots correspond to a higher number of significant genes, and the color gradient goes from green for the least significant terms to red for the most significant terms.

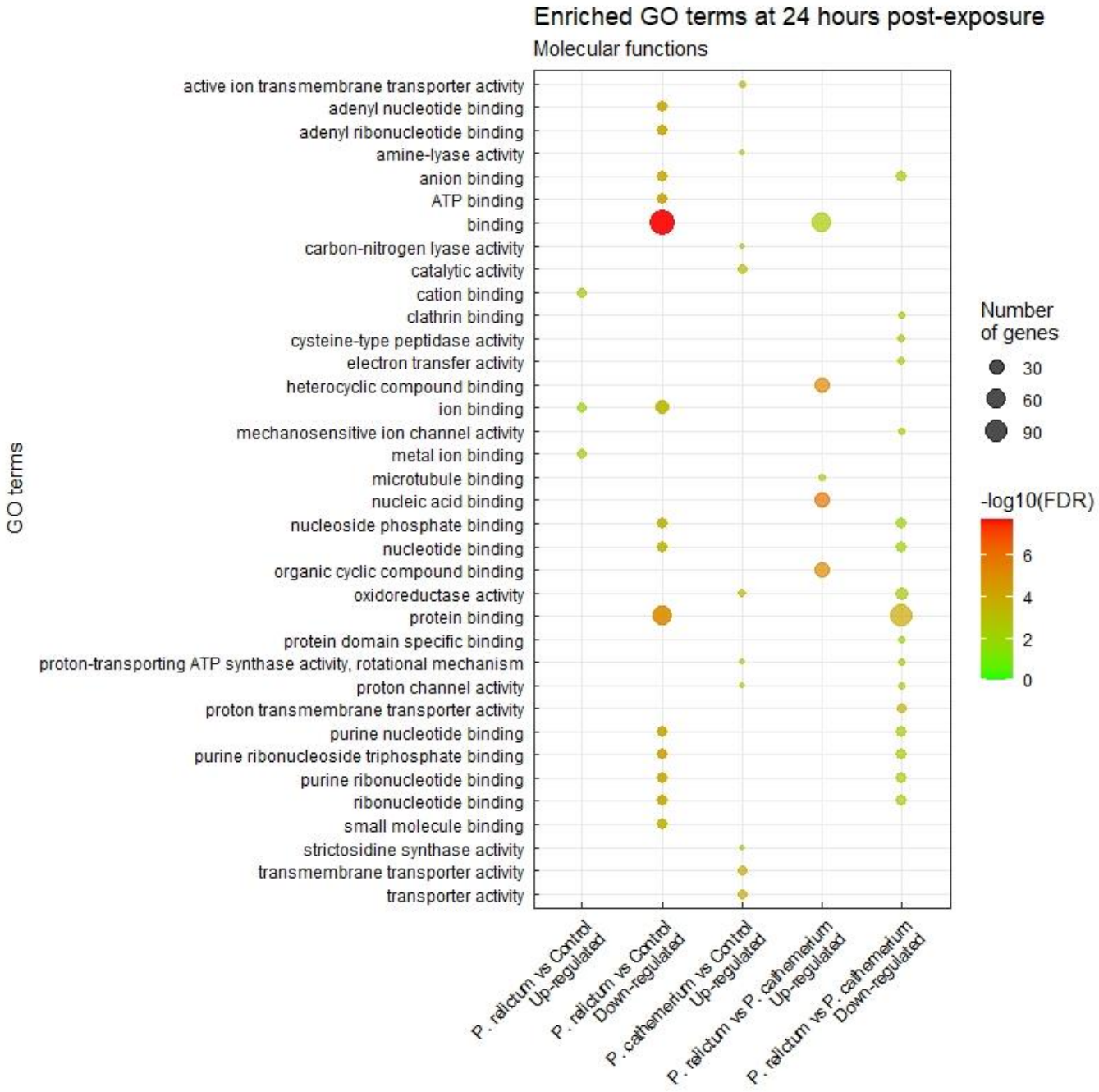

Figure 4. Dot plot of significantly (FDR adjusted p-value <0.01) enriched GO molecular functions for differentially expressed genes (up and down-regulated) at 24 hpi for i) *P. cathemerium* infected mosquitoes vs controls, ii) *P. relictum* infected mosquitoes vs controls, and iii) *P. relictum* infected mosquitoes vs *P. cathemerium* infected mosquitoes. We did not find enriched GO terms for down-regulated genes for *P. cathemerium* infected mosquitoes vs controls.

At 10 dpi enriched biological processes were related to amino acid biosynthesis for up-regulated genes and to protein geranylation for the

down-regulated genes (Figure S2). Molecular functions included actin monomer binding and GTPase activity for up-regulated genes and DNA binding for down-regulated genes (Figure S3).

b) *Cx. pipiens* gene expression during response to *P. cathemerium* infection

In *P. cathemerium* infected mosquitoes, 79 genes had a significant differential expression, 76 genes were up-regulated and 3 down-regulated, when compared with the control group at 24 hpi (Figure S4). Among the up-regulated genes with a greater log fold change there were genes related to digestive enzymes like *trypsin 2 precursor* (CPIJ005273), two protein G12 precursors (CPIJ012846 and CPIJ012848), and a GRIM19 gene (CPIJ009571), which product is a cell death-regulatory protein. Several genes related to the mitochondrial electron chain were also up-regulated, including a ADP-ATP carrier protein (CPIJ005941), several ATP synthase subunits (e.g. CPIJ018457, CPIJ005682, CPIJ005682 and CPIJ018208), and a NADH-ubiquinone oxidoreductase (CPIJ009280). The gen *pom1* gen (CPIJ019938) was down-regulated. At 10 dpi only one gene had differential gene expression and was up-regulated but was uncharacterized. There was no differential gene expression at 21 dpi.

Enriched terms were found only for up-regulated genes at 24 hpi. GO terms associated with biological processes were mostly related to the generation of precursor metabolites and energy, ATP generation, and nucleic acids metabolic processes, including nucleoside phosphate, ribose phosphate, nucleotide, ribonucleotide, purine nucleotide and purine ribonucleotide metabolic processes (Figure 3). GO terms associated with molecular functions

included ion and transmembrane transporter, oxidoreductase and proton-transporting ATP synthase activities (Figure 4).

c) *Cx. pipiens* respond differently to *P. relictum* and *P. cathemerium* infections

At 24 hpi 1,247 genes were found to be differentially expressed in *P. relictum* and *P. cathemerium* infected mosquitoes (Figure S5). Mosquitoes infected with *P. relictum* shown 327 up-regulated and 920 down-regulated genes compared to mosquitoes infected with *P. cathemerium*. Mosquitoes infected by *P. relictum* compared to those infected by *P. cathemerium* showed down-regulated genes related to four protein G12 precursors (CPIJ012848, CPIJ012844, CPIJ012845, and CPIJ012846), two chitin synthases (CPIJ014268 and CPIJ014269), a peritrophic membrane chitin binding protein (CPIJ007042) and a number of serine proteases including four chymotrypsin precursors (CPIJ003915, CPIJ018205, CPIJ007838 and CPIJ006568), three trypsin precursors (CPIJ005273, CPIJ006019, and CPIJ004660) and one maltase precursor (CPIJ013170), among others. No differentially expressed genes were found at 10 dpi and only 2 genes at 21 dpi.

At 24 hpi, biological processes related to metabolic regulation were enriched for up-regulated genes, while down-regulated genes included nucleic acid nucleotide metabolic processes (Figure 3). Molecular function transporter activities and microtubule binding were enriched in up-regulated genes in mosquitoes infected by *P. relictum* compared to those infected by *P.*

cathemerium. Most down-regulated enriched molecular functions were related to protein and nucleotides binding (Figure 4).

3. Discussion

How mosquitoes respond to infection and the impact of such infection will ultimately influence the transmission of different *Plasmodium* species. For example, *P. relictum* and *P. cathemerium* infections in *Cx. pipiens* have a different impact on the mosquitoes' fitness. In particular, *P. relictum* causes higher mortality than *P. cathemerium* infections in *Cx. pipiens*, and the transmissibility of *P. relictum* is lower than that of *P. cathemerium* (Gutiérrez-López et al. 2020). But little is known about the genetic underpinnings of mosquito response to Plasmodium infections. Here, we compare the transcriptional response of *Cx. pipiens* infected by these two avian *Plasmodium* species. Our results show that although responses of infected *Cx. pipiens* by these *Plasmodium* species share some common pathways, there are key differences in gene expression associated with the immune system, serine-protease synthesis, and nucleic acid metabolism that may explain the differences found in virulence and transmission.

Early and different response to the infection

When a mosquito feeds on a vertebrate host, a number of processes involving changes in gene expression are triggered (Dana et al. 2005). We found that the vast majority of the differentially expressed genes between infected and uninfected mosquitoes were found at 24 hpi. Between 18-24 h after blood feeding ookinetes form, invade the peritrophic matrix leaving behind the blood bolus and start the midgut epithelium cell invasion (Cirimotich et al. 2010; Valkiūnas et al. 2015; Baia-da-Silva et al. 2018). Ookinete formation and

308 invasion of the midgut epithelium are considered critical steps that will
309 determine the success of the infection. Parasite abundance drops drastically
310 during this step due to lumenal and epithelial immune responses mounted by
311 the mosquito (Cirimotich et al. 2010), which may explain the significant
312 differences in gene expression between uninfected and infected mosquitoes at
313 24 hpi (Ferreira et al. 2022). In addition, Vlachou et al. (2005) using the *P.*
314 *berghei* - *Anopheles gambiae* model found that 7% of the mosquito
315 transcriptome was differentially regulated during ookinete invasion of the
316 midgut.

317 At 24 hpi, for both mosquitoes infected with *P. cathemerium* and *P.*
318 *relictum*, we found an increase in differentially expressed genes and enriched
319 GO terms related with the mitochondrial respiratory chain activity that ultimately
320 produces reactive oxygen species (ROS) (Kowaltowski et al. 2009). Small
321 regulatory changes in the mitochondrial respiratory chain can drastically affect
322 ROS generation (Korshunov et al. 1997; Kowaltowski et al. 2009). For example,
323 *Anopheles stephensi* and *An. gambiae* increased the levels of ROS in response
324 to *Plasmodium* infection (Han 2000; Kumar et al. 2003), and higher levels of
325 ROS improve mosquito survival after a bacterial infection (Molina-Cruz et al.
326 2008). At 24 hpi we also found most of the differences in gene expression
327 between mosquitoes infected by each of these parasites.

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Differential immune response between mosquitoes exposed to *P. relictum* or *P. cathemerium*

We found important differences in the expression of genes associated with the immune response between *P. relictum*- and *P. cathemerium*-infected mosquitoes. At 24 hpi in *Cx. pipiens* infected by *P. cathemerium,* we found that two of the most up-regulated genes were protein G12 precursors. G12 transcripts accumulate in the midgut of mosquitoes after blood feeding (Shao et al. 2005; Bonizzoni et al. 2012), and may aid in erythrocyte digestion given their hemolytic activity (Foo et al. 2020). G12 transcripts have been suggested to play a role in immune function because: i) G12 protein in Ae*. Aegypti* has a high level of identity with cockroach allergens (Morlais et al. 2003), ii) it is up-regulated after flavivirus infection via the JAK-STAT pathway (Etebari et al. 2017), and iii) it has a cytolytic effect on flaviviruses and several types of eukaryotic cells (Foo et al. 2020). In fact, a G12 protein gene was found up-regulated in *Ae. aegypti* 12 hours after feeding on blood infected with *Plasmodium gallinaceum* (Morlais et al. 2003), suggesting a potential role in the immune response against these parasites as well.

When compared to the controls, mosquitoes exposed to *P. cathemerium* also had up-regulation of a GRIM19 gene. The cell death-regulatory protein GRIM19 is a well-known subunit protein of the mitochondrial complex I that acts as a tumor suppressor in humans and is highly conserved in eukaryotes, including insects (Nallar and Kalvakolanu 2017). The GRIM19 protein is involved in the innate immune response producing proinflammatory cytokines (Chen et al. 2012), which act controlling the growth of parasites and their elimination. An enrichment of GRIM19 sequence was found in *Ae. aegypti* and

Armigeres subalbatus mosquitoes exposed to pathogenic bacteria (Bartholomay et al. 2004).

In *P. relictum*-infected mosquitoes several of the up-regulated genes at 24 hpi were also related to the innate immune response when compared to the controls. Some of these genes included the Spätzle and Cecropin N proteins. In insects, when a pathogen is recognized, the extracellular Spätzle cytokine activates the Toll receptors, which regulate the antimicrobial peptides (AMPs), an essential innate immune response (De Gregorio 2002; Shia et al. 2009). The AMPs eventually kill pathogens by a number of strategies including disrupting the microbial membrane (Shen et al. 2018). Spätzle protein activates a Toll receptor in *Ae. aegypti* mosquitoes when infected with the fungus *Beauveria bassiana* (Shin et al. 2006) and in other insects after bacterial and fungal exposure (Bae et al. 2021). Cecropins are one of the largest groups of insect AMPs found in the orders Diptera, Lepidoptera, and Coleoptera among others (Vizioli et al. 2000; An et al. 2009; Memarpoor-Yazdi et al. 2013). The activation of the Toll pathway (Frolet et al. 2006) and specifically cecropin-analogs may kill *Plasmodium* parasites (Jaynes et al. 1988) and disrupt sporogonic development by aborting the normal development of oocysts (Gwadz et al. 1989; Kim et al. 2004). Our results showing the role of the Toll pathway are consistent with a recent study addressing the effect of *P. relictum* (lineage SGS1) on the immune system of *Cx. quinquefasciatus* (García-Longoria et al. 2022). They found that over 50% of immune genes identified as being part of the Toll pathway in *Cx. quinquefasciatus* were up-regulated after exposure to *P. relictum*.

Differential expression of genes associated with trypsin and serine metabolism

Serine proteases have several functions in insects including blood digestion (Borovsky and Schlein, 1988) and mediation in the immune response (melanization, cytokine activation, and antimicrobial peptides; Jiang et al. 2010). Trypsins and chymotrypsins, two types of serine proteases, are two essential digestive enzymes in mosquitoes (Molina-Cruz et al. 2005; Borges-Veloso et al. 2012). The synthesis of trypsin and chymotrypsin is triggered after mosquito blood feeding to digest the chitin-containing peritrophic matrix (Vizioli et al. 2001; Muller et al. 1995) which is fully formed at 24 h post feeding (Hegedus et al. 2009). However, the production of trypsin and chymotrypsin may be down or up-regulated by the infection with different parasites (Borovsky and Schlein 1987; Shahabuddin et al. 1996; Serrano-Pinto et al. 2010). Mosquito trypsin may be a signal for *Plasmodium* ookinetes to cross the peritrophic matrix at the right time for proper *Plasmodium* development (Shahabuddin et al. 1996). In particular, mosquito trypsin proteases play a fundamental role in allowing *P. gallinaceum* to cross the peritrophic matrix by activating a *Plasmodium* prochitinase enzyme (Shahabuddin et al. 1996). In fact, trypsin inhibitors block the development of *Plasmodium* oocysts (Shahabuddin et al. 1996), suggesting that mosquito trypsin is a key molecule for pathogen infection.

Mosquitoes exposed to *P. cathemerium* up-regulated genes associated with trypsin and serine metabolism, while mosquitoes exposed to *P. relictum* down-regulated genes from the same family. Furthermore, when comparing gene expression between mosquitoes exposed to each species, we found several trypsin and chymotrypsin precursors down-regulated in mosquitoes exposed to *P. relictum*. This differential expression was found at 24 hpi,

406 therefore coinciding with the moment when the peritrophic matrix is fully formed
407 and the ookinetes are crossing it. Agreeing with the findings of Shahabuddin et
408 al. (1996) for the infection by *P. gallinaceum*, we also found that trypsin (*trypsin*
409 *2 precursor*; CPIJ005273) was up-regulated in mosquitoes infected with *P.*
410 *cathemerium* when compared with uninfected mosquitoes. This suggests the
411 potential role of the trypsin proteases to allow *P. cathemerium* to cross the
412 peritrophic matrix. In contrast, mosquitoes infected with *P. relictum* had a
413 number of down-regulated genes characterized as serine proteases, including
414 several chymotrypsin and one trypsin. Ferreira et al. (2022) also found serine-
415 type endopeptidase activity enriched for down-regulated genes when studying
416 the expression of *Cx. quinquefasciatus* infected by *P. relictum.* We hypothesize
417 that the lower levels of trypsin in response to *P. relictum* infection might lead to
418 a lower number of oocysts and would explain why the transmissibility of *P.*
419 *relictum* is lower than that of *P. cathemerium* (Gutiérrez-López et al. 2020).

420 In addition, an under-expression of serine-proteases related to blood
421 digestion such as chymotrypsins should partly block the digestion of the blood,
422 which is very rich in proteins (Kumar et al. 2017). This would make it difficult to
423 obtain nutrients needed for various processes like egg formation and could lead
424 to digestive dysregulation in the mosquito which might increase mortality. Here
425 mosquitoes seem to be down-regulating important processes due to the
426 infection with *P. relictum* which might have a cost for the mosquito, induce
427 damage and decrease the tolerance to infection.

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Differential expression of genes with nucleic acids metabolism activity and binding function

At 24 hpi, GO biological processes related to nucleic acids metabolism were enriched for up-regulated genes in mosquitoes infected with *P. cathemerium* compared to controls. At the same time point, molecular functions related to nucleic acids binding were enriched for down-regulated genes in both mosquitoes infected with *P. relictum* vs controls and mosquitoes infected with *P. relictum* vs *P. cathemerium*. When a mosquito is infected by a pathogen after a blood meal, the gut epithelium suffers cellular damage (Nászai et al. 2015) and a group of specialized cells regenerates these epithelium cells (Taracena et al. 2018; Janeh et al. 2019). After a *Plasmodium* infection, the regeneration of the midgut cells would require the up-regulation of the nucleic acid metabolism to synthetize the genetic material, as we observed in *Cx. pipiens* after *P. cathemerium* infection. On the other hand, mosquitoes infected with *P. relictum* under-expressed molecular functions related to nucleic acids binding, which may hinder the regeneration of the mosquito's damaged cells, reducing its tolerance to infection. This is consistent with reduced survival of *Cx. pipiens* infected with *P. relictum* compared to those infected with *P. cathemerium* observed by Gutiérrez-López et al. (2020).

Reduced differential gene expression at 10 and 21 days post-infection

An unexpected result was the strong decrease of differential gene expression between infected and uninfected mosquitoes at 10 dpi. We chose this time point because at about 10 dpi *Plasmodium* mature oocysts release the

454 sporozoites into the mosquito hemocoel (Cirimotich et al. 2010). However, at
455 this time point, only *P. relictum*-infected mosquitoes compared to controls
456 showed differences in gene expression and the number of differentially
457 expressed genes decreased significantly compared to 24 hpi. Similar results
458 were obtained by Ferreira et al. (2022) when studying gene expression of *Cx.*
459 *quinqueafasciatus* exposed to *P. relictum*. In addition, the absence of differential
460 expression in mosquitoes exposed to *P. relictum* compared to those exposed to
461 *P. cathemerium* may be due to differences in the developmental time of
462 different species of *Plasmodium* (Sinden 1983). *P. cathemerium* produces
463 sporozoites faster than *P. relictum* when infecting *Cx. pipiens* mosquitoes
464 (Kazlauskienė et al. 2013; Aly et al. 2020), and therefore they may not be
465 exactly at the same point of development within the mosquito at 10 dpi.

466 By 21 dpi, the differences in gene expression were drastically reduced. With
467 only two genes differentially expressed in mosquitoes infected with *P. relictum*,
468 and none in those infected with *P. cathemerium*. This decrease in differential
469 expression towards the end of the infection has been found before in *Cx.*
470 *quinqueafasciatus* infected with *P. relictum* (Ferreira et al. 2022, García-
471 Longoria et al. 2022). Although the causes behind this decrease are not clear,
472 we hypothesize that the response towards the end of the infection might be
473 localized to the salivary glands, and by analysing whole mosquitoes we are
474 diluting any potential effect.

475 *Comparisons across avian malaria studies*

476 Although studies analysing the response of mosquitoes to avian malaria
477 are still scarce we can already see some common patterns and differences.
478 Two other recent studies have analysed the response to *P. relictum* infection in

Cx. quinquefasciatus, a species closely related to *Cx. pipiens*. A common pattern found in all studies is the progressive reduction in the differential gene expression at 10 and 21 dpi with respect to 24 hpi. In addition, like García-Longoria et al. (2022), we found the activation of the Toll-like receptor pathway in response to *P. relictum SGS1* infection. Interestingly, Ferreira et al. (2022) did not find this pattern. Although different factors may be influencing this result, like experimental procedures or the genetic differences between the populations of *Cx*. *quinquefasciatus* used, one of the main factors might have been the lineage used for the infection. Ferreira et al. (2022) infected the mosquitoes with another lineage, *P. relictum* GRW4, which dominates in America. Like Ferreira et al. (2022), the response to infection we found for mosquitoes infected with *P. cathemerium*, was different. But, a similar pattern has been found before. Shahabuddin et al. (1996) reported the important role of a mosquito trypsin in the passage of *P. gallinaceum* through the peritrophic matrix, as our results indicate for *P. cathemerium*. Altogether these result suggests that different *Plasmodium* species or lineages may trigger a differential immune response in mosquitoes.

Concluding remarks

Because we used naturally infected wild birds as *P. cathemerium* and *P. relictum* donors and wild collected mosquitoes, the results obtained here represent a good example of a natural system. In this respect, we found a different transcriptomic response to infections, especially at 24 hpi. This time point coincides with one of the key stages of *Plasmodium* development in mosquitos, when the ookinetes form, cross the peritrophic matrix and start to invade the midgut epithelium. Although both elicit an innate immune response,

the response seems to be stronger in mosquitoes exposed to *P. relictum* with the activation of the Spätzle and Cecropin N proteins which result in a reduction in the number of oocysts in the mosquito midgut. In addition, the lower levels of trypsin in mosquitoes exposed to *P. relictum* may also affect the parasite development within the mosquito, which may affect parasite transmission (Gutiérrez-López et al. 2020). If the cost of this response is high, this can also potentially lead to higher mosquito mortality. In particular, the proteases and trypsin are necessary to digest the blood meal, and if levels are too low this might increase mosquito mortality. Future studies are necessary to understand how these differences may be related to the different ecology and incidence of *Plasmodium* lineages/species in the wild.

4. Material and Methods

Sampling and experimental conditions

We captured juvenile house sparrows (*Passer domesticus*) with mist nets in September 2020 at Granja Escuela de Trigueros (Huelva province, Spain). We ringed, weighted and measured the individuals before bringing them into captivity at the animal facilities of the Doñana Biological Station, following the ethical guidelines (article 34 RD 53/2013).

In the field, we took blood samples from each bird jugular vein using a sterile syringe. Blood samples were used to molecularly identify the blood parasite infections and the parasite lineage identity. To do that, we extracted genomic DNA from blood samples using a Lithium Chloride protocol (Gemmell and Akiyama 1996) and detected parasite infections following Hellgren et al. (2004). We sequenced the amplified products for positive samples on both

strands using Capillary Electrophoresis Sequencing by Macrogen (Madrid, Spain). We analysed the sequences using Geneious v. 2020.0.3 (Kearse et al. 2012) and identified lineages in MalAvi (http://130.235.244.92/Malavi/blast.html). After molecular analyses, we chose three birds for further analyses, namely: A bird that was not infected by *Plasmodium*, *Haemoproteus* nor *Leucocytozoon* (control), a bird infected with *P. relictum* lineage SGS1, and a bird infected with *P. cathemerium* lineage PADOM02.

We collected mosquito larvae on October 2020 in Aljaraque (Huelva province) and reared them following Gutiérrez-López et al. (2020). We maintained larvae in dechlorinated water and fed *ad libitum* with Hobby-Mikrozell 20 ml/22 g (Dohse Aquaristik GmbH & Co.101 KG, D-53501, Gelsdorf, Germany) and Hobby-Liquizell 50 ml (Dohse Aquaristik GmbH & Co.101 KG, D-53501, Gelsdorf, Germany). After emergence, we identified adults to the species level and sexed them following Gunay et al. (2018). We kept adult *Cx. pipiens* females in separate cages of 50 individuals maximum and fed them with a 10% sugar solution. We maintained both larvae and adult mosquitoes under controlled conditions (26ºC ± 1, 55-60% relative humidity (RH) and 12:12 light:dark photoperiod cycle).

We divided the 11-day-old adults (± 1 day) into 3 groups mixing individuals originating from the different cages and allowed them to feed overnight on a *P. relictum*-infected bird, a *P. cathemerium*-infected bird and an uninfected control bird. Only one individual of each category was used in this experiment and they were exposed to mosquitoes only one time. In the morning

after exposure, we separated the fed females of each group into three different cages and maintained them under the conditions described above.

For transcriptome analyses, we processed mosquitoes at three time points after exposure (24 hpi, 10 dpi and 21 dpi). At each time point, we created pools of 5 mosquitoes of each infection status capturing the mosquitoes alive and immediately transferring them to dry ice. We preserved the mosquitoes at -80C until RNA extractions were carried out. We collected a total of 36 samples including controls (4 pools x 3 time-points x 3 conditions).

RNA extraction, library preparation, and sequencing

We extracted RNA and DNA from pools of 5 mosquitoes using TRIzol® (Invitrogen, Carlsbad, CA, USA) followed by column purification using RNeasy mini kit® (QIAGEN, Hilden, Germany) following Ferreira et al. (2022). We tested the remaining DNA for the presence of *Plasmodium* following Hellgreen et al. (2004) confirming that parasite DNA was present in all positive samples and not present in negative samples. We prepared RNAseq libraries at the Polo d'Innovazione di Genomica, Genetica e Biologia, Siena (Italy) using and Illumina library. Then, we quantified samples using Qubit® 4.0 Fluorometer and checked RNA integrity using the Fragment Analyzer to measure RNA Quality Number (RQN) specific for mosquitoes. Finally, we prepared the libraries following the QIAseqTM Stranded mRNA Selected Kit Handbook for Illumina Paired-End Indexed Sequencing. Indexed DNA libraries were sequenced in an Illumina NextSeq550 Flowcell, using the Illumina chemistry V2.5, 2x75bp run.

578 *Data analysis*
579

580 To check the quality of the reads we used FastQC (ver. 0.11.9; Andrews
581 et al. 2010) and MultiQC (Ewels et al. 2016). Then, we filtered low quality and
582 under 36 bp reads using Trimmomatic (Bolger et al. 2014). Since the reference
583 genome and annotations of *Cx. pipiens* are not published yet, we used the
584 reference genome and annotations of phylogenetically closest species that
585 were available in Ensembl, *Cx. quinquefasciatus*
586 (https://metazoa.ensembl.org/Culex_quinquefasciatus/Info/Index). We used
587 STAR (Dobin et al. 2012) to map the short reads to the reference genome of
588 *Cx. quinquefasciatus* and RSEM (Li and Dewey 2011) to quantify gene
589 abundances.

590 Following steps were carried out in R (R Core Team 2021) using
591 Bioconductor packages (Huber et al. 2015). We carried out a Variance
592 Stabilizing Transformation (VST) of the counts to represent the samples on a
593 PCA plot. Then, we used the DESeq2 package (Love et al. 2014) to perform the
594 differential gene expression analysis comparing: i) *P. cathemerium* infected
595 mosquitoes vs controls, ii) *P. relictum* infected mosquitoes vs controls, and iii)
596 *P. relictum* infected mosquitoes vs *P. cathemerium* infected mosquitoes. We
597 kept those genes with an adjusted p-value < 0.01 and sorted them by the log2
598 fold change estimations to consider the strength of up- and down-regulation.
599 We finished the differential gene expression analysis visualizing differentially
600 expressed genes by MA plots and an UpSet plot.

601 Finally, we performed a Gene Ontology (GO) enrichment analysis for the
602 up- and down-regulated genes using the topGO package (Alexa and

603 Rahnenfuhrer 2010) including the “biological processes” and “molecular
604 functions” categories from VectorBase (Giraldo-Calderón et al. 2015). For the
605 Enrichment analysis we used classical algorithm and Fisher’s exact test and
606 considered enriched the GO terms with p-value < 0,01. We used the ggplot2
607 (Wickham 2016) package to visualize the enriched GO terms as described by
608 Bonnot et al. (2019).

609 **5. Data Access**

610 Raw sequences generated in this study have been submitted to the European
611 Nucleotide Archive ENA database (https://www.ebi.ac.uk/ena/browser/home)
612 under project accession number PRJEB1609, Study ERP125411.

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