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1 # Foreign Ribosome Inactivating Proteins as immune effectors in insects

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7 **Funding information**

8 This work has been funded by grants from CONICET (Consejo Nacional de Investigaciones
9 Científicas y Técnicas) grant numbers PIP 2732, PIBAA 28720210100081CO and UNSL
10 (Universidad Nacional de San Luis) grant number PROICO 02-1720.

11 **Conflict of interest disclosure**

12 The authors declare that they have no conflict of interest.

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15 # Abstract

16 Ribosome inactivating proteins (RIPs) are RNA *N*-glycosidases that depurinate an adenine
17 residue in the conserved alpha-sarcin/ricin loop (SRL) of rRNA. This ribosomal modification inhibits
18 protein synthesis. During the last years, we have reported the existence of these toxins in insects, where
19 their presence is restricted to mosquitoes from the Culicinae subfamily (*e.g. Aedes aegypti*) and whiteflies
20 from Aleyrodidae family (*e.g. Bemisia tabaci*). Combination of phylogeny and synteny analyses showed
21 that both groups of genes are derived from two independent horizontal gene transfer (HGT) events.
22 Interestingly, we found that RIP encoding genes have been evolving under purifying selection, indicating
23 that they have a positive impact on fitness of host organisms. We also demonstrated that *A. aegypti* RIP
24 genes are transcribed and their transcripts are polyadenylated. Although the biological roles of these
25 toxins remain open to speculation, defense activities have been postulated for plant and bacterial RIPs.
26 Based on these pieces of evidence, we hypothesize that RIPs play a similar protective role in insects. In
27 this work, we report the occurrence of a third HGT event in Sciaroidea superfamily, supporting that RIP
28 genes fulfill an important functional niche in insects. Analysis on transcriptomic experiments from the
29 three groups of insects indicate a convergence in expression profiles which are compatible with immune
30 effectors. Finally, we show the induction in RIP expression after infection with pathogens. Moreover, we
31 show transcriptomic evidence of parasite SRL depurination. Altogether, our results strongly support the
32 role of these foreign genes as immune effectors that confer fitness advantage to host insects.

33 **Key words:** Ribosome Inactivating Proteins, Horizontal Gene Transfer, Insects, Immune
34 effectors, RNA *N*-glycosidase.

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36
Introduction

37 Horizontal gene transfer (HGT) consists of the non-genealogical transmission of genetic material
38 (Goldenfeld and Woese 2007). The relevance of this mechanism for evolutionary innovation in bacteria is
39 widely accepted (Goldenfeld and Woese 2007; Lerminiaux and Cameron 2019). In contrast, its impact on
40 the fitness of multicellular organisms (*e.g.,*animals) is still under debate (Dunning Hotopp et al. 2007;
41 Van Etten and Bhattacharya 2020). For the transferred genes to be permanently maintained in animal
42 species, they must be incorporated into germline cells and transmitted to the offspring. Once vertically
43 transmitted, at least two possible fates are expected for HGT-derived genes. They may be eroded by
44 genetic drift or acquire a functional role in the host species (Keeling and Palmer 2008). While multiple
45 examples of putatively functional HGT-derived genes can be found, little is known about their biological
46 role, how they are regulated in the recipient organism, and ultimately what impact they have on host
47 fitness/survival. In insects like aphids, psyllids, whiteflies, and mealybugs, it has been postulated that
48 HGT has played a central role in the adaptation to new diets, contributing to the efficient assimilation and
49 detoxification of their food (Husnik and McCutcheon 2018; Prasad et al. 2021; Xia et al. 2021). Another
50 interesting example of the importance of horizontally acquired genes in insects is the case of the *gasmin*
51 gene, which is required for the lepidopteran *Spodoptera littoralis* to combat its natural enemies and
52 infection (Di Lelio et al. 2019). Recently, we have reported the horizontal acquisition of Ribosome
53 Inactivating Protein (RIPs, EC 3.2.2.22) encoding genes by some species of insects (Lapadula et al. 2017;
54 Lapadula et al. 2020b).

55 RIPs are RNA *N*-glycosidases that irreversibly modify ribosomes through the depurination of an
56 adenine residue in the conserved alpha-sarcin/ricin loop (SRL) of rRNA (Endo and Tsurugi 1988). This
57 modification in a key component of the ribosomal elongation-cycle machinery prevents the binding of the
58 elongation factor 2 to the ribosome, arresting protein synthesis (Nilsson and Nygard 1986). It is known
59 that RIP encoding genes are found in plant, bacterial and fungal lineages (Lapadula and Ayub 2017). The
60 recent exponential increase in database information has boosted the power of homology searches allowing
61 for the discovery of new members of RIP genes family. Using this approach, for the first time we reported
62 the presence of RIP genes in the metazoa kingdom (Lapadula et al. 2017; Lapadula et al. 2020b; Lapadula

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63 et al. 2013). Up to date, the taxonomic distribution of these genes in animals is very narrow, restricted to
64 mosquitoes from the Culicinae subfamily (Lapadula et al. 2017) (including *Aedes aegypti*) and whiteflies
65 from the Aleyrodidae family (including *Bemisia tabaci*) (Lapadula et al. 2020b). A combination of
66 phylogeny and synteny analyses revealed that both groups of genes are derived from two independent
67 HGT events, probably from bacterial and plant donors, respectively. Moreover, in both groups of insects,
68 we showed that the RIP open reading frames show signatures of evolution under purifying (negative)
69 selection, strongly suggesting that they positively impact the fitness of host organisms (Lapadula et al.
70 2017; Lapadula et al. 2020b). Recently, we have also demonstrated that two of the three RIP genes
71 present in the *A. aegypti* genome are transcribed, and that their transcripts are polyadenylated (Lapadula
72 et al. 2020a). Most importantly, the expression levels of these RIP genes are modulated across the
73 developmental stages of mosquitoes (Lapadula et al. 2020a). By using transcriptomic data, the expression
74 of these foreign genes could also be confirmed in *B. tabaci* (Lapadula et al. 2020b). Altogether, these data
75 support the hypothesis that RIP genes have a physiological role in insects.

76 Although several members of the RIP protein family have been extensively studied at the
77 biochemical level, their biological roles remain open to speculation. In some cases, activities against
78 viruses, microbes or parasites have been postulated for plant RIPs (Peumans et al. 2001; Stirpe 2013; Zhu
79 et al. 2018). Recently, RIPs from the symbiotic *Spiroplasma spp*. (class Mollicutes) have been suggested
80 to play a defensive role in Drosophila species by preventing the development of parasitic nematodes and
81 wasps (Ballinger and Perlman 2017; Ballinger and Perlman 2019; Hamilton et al. 2016). Based on these
82 reports, we consider that RIP genes fulfill an important functional niche in insects that would be filled
83 either from horizontally transferred genetic material or by a symbiotic interaction. The toxic nature of this
84 protein family makes it possible to postulate the hypothesis that RIP genes play a defensive role in
85 insects. In the present work we report the occurrence of a third independent HGT event supporting the
86 recurrent acquisition of RIP genes by this mechanism in insects. After analyzing public transcriptomic
87 data, we found a convergence in temporal and spatial expression of these independently acquired genes,
88 which is compatible with expression patterns of immune effectors. Moreover, we identified depurinated
89 sequences belonging to pathogens ribosomes that induce RIP expression in *A. aegypti*, strongly
90 suggesting the mechanism through which these ribotoxins could carry out the protective role. In

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91 summary, we provide new evidence supporting the hypothesis that these foreign genes are new immune
92 effector molecules of insects.

93 Results

94 Recurrent acquisition of RIP genes by Horizontal gene transfer in insects

95 Recent database searches focused on insects have led us to find new RIPs in the swede midge
96 *Contarinia nasturtii* (named RIPCn1 and RIPCn2) and in the fungus gnat *Bradysia odoriphaga* (named
97 RIPBo1 and RIPBo2). These flies of Diptera order belong to the Cecidomyiidae and Sciaridae sister
98 families, respectively. All sequences were recognized by Pfam as RIPs. Similar to whiteflies, fly RIPs
99 have a putative signal peptide in the N-terminal end and at least RIPCn2 harbor introns. Moreover, a
100 sequence alignment of predicted proteins revealed that the five residues responsible to form the active site
101 of RIPs were fully conserved for three of these toxins (**Supplementary fig. 1**). RIPBo2 showed a
102 premature stop codon encoding for a truncated protein lacking three of these key residues. BLAST
103 analyses of the genomics scaffolds (JAFDOW010000841, VYII01002082 and VYII01000852) harboring
104 these genes showed that most of the encoding protein sequences surrounding the RIP genes yielded
105 maximum scores with arthropod annotated proteins (**Supplementary Datafile 1**). In contrast, RIPs
106 showed maximum amino acid sequence identity to bacterial homologs (around 36%), and lower sequence
107 identity to the previously described RIPs from mosquitoes and whiteflies (around 24%).

108 The phylogenetic tree (**Fig. 1A, Supplementary fig. 2**) shows that new RIP encoding genes from
109 Sciaridae and Cecidomyiidae are monophyletic (Transfer Boostrap Expectation; TBE = 0.66) and are
110 embedded in a clade of bacterial sequences (TBE = 0.74). Moreover, metazoan (insect) RIP does not form
111 a clade. This result supports a common origin for fly RIPs belonging to these sister families, but
112 independent from whitefly and Diptera homologues. Furthermore, *C. nasturtii* and *B. odoriphaga* genes
113 form sister clades, revealing that the gene duplication events took place after divergence of these families,
114 yielding two different paralogues in each lineage. Moreover, the absence of RIP genes in other species
115 with fully sequenced genome of Sciaroidea superfamily (*e.g. Bradysia coprophila*, *Sitodiplosis*
116 *mosellana*, *Mayetiola destructor* and *Catotricha subobsoleta*) suggests the occurrence of gene loss events
117 (**Supplementary fig. 3**), a commonly observed pattern in this protein family (Lapadula et al. 2013).

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118 Hitherto, RIP genes have been found in three clades of insects (**Fig. 1B**). Previously, we proposed
119 that whiteflies and mosquitoes acquired RIP genes by two independent HGT events from plants and
120 bacteria, respectively (Lapadula et al. 2017; Lapadula et al. 2020b). In the case of whiteflies this event
121 took place before the divergence of *B. tabaci* and *T. vaporariorum* species in the range of 300 and 83
122 MYA (**Fig. 1B**). In mosquitoes these genes were acquired between the divergence of Anopheles and
123 Culex/Aedes lineages and before the separation of Aedes and Culex genus between 190 and 150 MYA
124 (**Fig. 1B**).

125 According to the phylogeny (**Fig. 1A**), fly RIPs share a common origin but their history is
126 independent from previously reported RIPs in insects. The homology searches using fly RIPs as queries
127 in complete genomes of insects other than Sciaridae and Cecidomyiidae families did not retrieve any new
128 hits. These results indicate that fly RIPs are not derived from vertical inheritance through the insect
129 lineage or any species previously reported to have RIP genes. Therefore, the most parsimonious
130 hypothesis explaining the presence of RIP genes in these sister families is a third HGT event. This
131 acquisition took place in a range of 190 and 108 MYA after Sciaridae and Cecidomyiidae cenancestor
132 diverged from the other families belonging to the Sciaroidea superfamily (**Fig. 1B**). The fact that fly RIPs
133 are embedded in a clade of bacterial homologues (**Fig. 1A**) indicates that the most likely donor is a
134 prokaryotic organism.

135 In summary, we found evidence of a third HGT event for RIP genes in insects. The recurrent
136 acquisition by this evolutionary mechanism supports the hypothesis that members of the RIP family have
137 found a functional niche in these organisms. In the following sections we show evidence that supports a
138 convergence in transcription profiles of different insect lineages that independently acquired these
139 ribotoxins encoding genes.

140 **RIPs genes show higher expression levels in early stage of insect ontogeny**

141 Recently, we reported that two of the three RIP encoding genes present in *A. aegypti* are
142 transcribed and their expression is modulated across the developmental stage (Lapadula et al. 2020a). In
143 this work we found that RIPae2 expression was higher for L4 and pupal stages while RIPAe3 showed the
144 highest expression values at L3 and L4 stages. From the analysis of transcriptome information available

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145 in BioProject PRJNA419241 (Matthews et al. 2018), we observed a similar expression pattern of RIPAe2
146 achieving maximal values for early pupal stage and RIPAe3 in L4 stage (**Fig. 2A**). The abundance of
147 transcript in sister species *A. albopictus*, (harboring seven RIP genes) indicated that different paralogous
148 genes have different expression profiles throughout their development (**Fig. 2B**). Genes RIPAl1 and
149 RIPAl2 are expressed in adult males while RIPAl3 and RIPAl6 transcripts are found between L1 and
150 pupal stages. As it was the case for *A. aegypti* in this species the highest expression levels for RIPs genes
151 as a whole are found in early stages of ontogeny.

152 In the case of *C. nasturtii* the abundance of transcripts obtained from BioProject PRJNA565761
153 showed that both RIPCn1 and RIPCn2 are expressed. According to this experiment we found the highest
154 expression level for L1 and L2 while in other stages (embryos, L3, pupal and adults of both sexes) no
155 transcripts of these genes were detected (**Fig. 2C**). In *B. odoriphaga* the absence of reference
156 transcriptome prevented the building of the index to determine the abundance of transcripts. However, we
157 performed an estimation by BLASTn searches. For this, we counted the number of retrieved hits after
158 performing searches against Sequences Read Archives (SRA) files of BioProjects PRJNA388516 and
159 PRJNA304774 using RIPBos as queries. This analysis indicated that the highest number of retrieved hits
160 for RIPBo1 were between L2 and L4, followed by pupal stages (**Table 1**). Interestingly, RIPBo2 that
161 encodes for a truncated variant showed no significant transcription in any stage. Thus, these results
162 indicate that RIP encoding genes are modulated during insect ontogeny with a trend to the transcription
163 during early development such as larval and pupal stages.

164 **RIPs are expressed in body parts involved in immune response**

165 In the previous section we described the expression profile of RIP genes along the
166 ontogeny of insects. In order to determine those tissues and body parts where transcripts of these foreign
167 genes are present, we performed a similar analysis in different transcriptomic experiments available in
168 databases. If these genes are involved in immune response, it is expected that their transcript will be
169 present in body parts like thorax or abdomen, where immune tissues such as fat body, gut or hemocytes
170 are located. According to previously observed for adults (**Fig. 2A**), RIPAe2 is slightly expressed in the
171 whole body of female *A. aegypti*. Despite this, from the analysis of Aegypti-Atlas, (Hixson et al. 2022)

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172 we found that their transcripts are mostly present in thorax and at a lower level in head and abdomen (**Fig.**
173 **3A**). Other body parts such as ovaries, Malpighian tubules and gut do not show the presence of these
174 transcripts. The analysis of BioProject PRJNA236239 (Matthews et al. 2016) indicated that RIPAe1 is
175 mostly expressed in maxillary palp of adult females, while in males, their expression is the highest in the
176 abdominal tip (**Fig. 3B**). On the other hand, transcripts of RIPAe2 have similar expression levels for both
177 sexes in body parts like rostrum, abdominal tip and brain (**Fig. 3B-C**). RIPAe3 is not detected in any body
178 part for both sexes of adult insects. BioProjects PRJNA687261 (Romoli et al. 2021) and PRJNA548563
179 (Filosa et al. 2019) contain transcriptomic information of midgut and hindgut obtained from L3 and adult
180 stages, respectively. In the L3 stage we observe that RIPAe2 and RIPAe3 transcripts are present in whole
181 larva samples. However, in midgut these genes are not expressed indicating their absence in this tissue
182 (**Supplementary fig. 4A**). On the other hand, RIPAe1 and RIPae2 were expressed in hindgut of adult
183 individuals. Interestingly, their expression level is in the top quartile (**Supplementary fig. 4B-C**). Finally,
184 in transcriptomes of Malpighi tubules obtained from BioProjects PRJNA246607 and PRJNA595990 no
185 RIPs transcripts were detected.

186 Consistent with previously reported data of whiteflies *B. tabaci* (Lapadula et al. 2020b)
187 transcripts of both RIP genes, RIPBt1 and RIPBt2, were found in the whole body of the adult stage, being
188 the expression level of RIPBt2 higher than RIPBt1. Interestingly, transcripts of these genes are mostly
189 found in thorax and abdomen while in salivary glands only RIPBt1 was expressed (**Fig. 3D**).

190 These results indicate that RIP transcripts are present in different body parts of insects. However,
191 their presence is mostly located in abdomen and thorax of mosquitoes and whiteflies. Moreover, it was
192 possible to identify expression signals in the hindgut of adult mosquitoes. Once again, we observed a
193 convergence in expression profiles of these foreign genes in different lineages of insects. Although
194 additional studies are needed to determine the exact location of RIP transcripts in abdomen and thorax,
195 these analyses constitute a piece of evidence that support the presence of mRNA in body parts involved in
196 the immune system.

197 **RIPs genes expression is increased after the infection with pathogens**

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198 If these foreign genes are immune effector molecules of insects, their transcription would
199 be expected to be triggered after the infection with different pathogens. In order to find evidence
200 supporting this hypothesis we analyzed information derived from bibliography for *A. aegypti*. In this
201 species only RIPAe2 (AAEL008050) encoding gene has been annotated in VectorBase biasing
202 bibliographic analysis. For this gene we found increased expression after the infection with several
203 pathogens (bacteria, nematodes, and fungi) in adult mosquitoes. The most striking examples found was its
204 upregulation post-infection with *Wolbachia pipientis* wMelPop (Kambris et al. 2009) and the
205 Microsporidia *Edhazardia aedis* (Desjardins et al. 2015).

206 The analysis of RNAseq experiments after the infection with the nematode *Brugia malayi*
207 (Choi et al. 2014; Juneja et al. 2015) in different refractory and susceptible strains of *A aegypti* support
208 the potential of these genes as immune effector molecules in insects. Susceptible strains of mosquitoes
209 support the development of nematode. On the contrary, in refractory strains parasites fail to develop and
210 die within a few days. The transcriptome of whole body obtained from BioProject PRJNA255467 (Juneja
211 et al. 2015) showed the upregulation of RIPAe2 for both strain (LVP-IB12^R and LVP-FR3^S) of
212 mosquitoes after the infection with the nematode (**Fig. 4A-B**). Moreover, the expression level of RIPAe2
213 increases along time, its highest being at 48 hours post infection. In this experiment, RIPAe1 showed no
214 difference in its expression after the infection with the pathogen and their TPM values were always lower
215 than RIPAe2. The RNA-seq experiment in thorax of *A. aegypti* obtained from BioProject PRJNA232599
216 (Choi et al. 2014) was consistent with the ones observed in the whole body. In blackeyed Liverpool
217 (BEY-LVP) susceptible strain RIPae2 was significantly upregulated only at day 1 and 8 post infection
218 with means of TPM values of 258 and 207, respectively (**Fig. 4F**). On the other hand, RIPae1 had lower
219 expression level in this strain and it never showed differences between infected and uninfected conditions
220 for the evaluated days (**Fig. 4E**). In the refractory *A. aegypti* (RED) strain both genes RIPAe1 and RIPae2
221 always showed higher levels of transcription after the treatment with the nematode (**Fig. 4C-D**).
222 Moreover, the TPM values obtained for both genes in this strain were higher than the values observed in
223 BEY-LVP strain, suggesting that RIPs might be involved in the resistance against the parasite. The
224 highest TPM values observed in thorax for both genes support the hypothesis that their expression is
225 enriched in this mosquito´s body part, which is consistent with data presented in **Fig. 3A**. In these

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226 experiments, no reads were detected for RIPae3 in any conditions, suggesting that its expression is not
227 induced by pathogen infection in the adult stage. However, the transcriptome of L3 after the infection
228 with *E. coli* (Romoli et al. 2021) indicated that RIPAe3 has an increase in level of transcription 20 hours
229 after the infection (**Supplementary fig. 4A**). From the analysis of transcriptome experiments where virus
230 infection is evaluated, no modulation in RIP expression was observed. Therefore, here we show evidence
231 that RIPs expression is upregulated in *A. aegypti* after the infection with different pathogens like fungus,
232 bacteria or nematodes. Moreover, the results presented suggest that RIP genes may contribute in defense
233 against the nematode *B. malayi* in refractory strains of mosquitoes.

234 **Evidence of RNA *N*-glycosidase activity of *A. aegypti* RIPs**

235 The toxicity of this family of proteins is presumably a consequence of their enzymatic
236 activity. These toxins are RNA *N*-glycosidases that irreversibly modify ribosomes through the
237 depurination of the first adenine residue in the G**A**GA motif present in the conserved SRL of rRNA. After
238 the retrotranscription process, the reverse transcriptase preferentially inserts a dAMP opposite to the
239 abasic site, which will result in a complementary dTMP after the first round of amplification step (**Fig.
240 5A**). Therefore, we searched for evidence of depurination in ribosomes of *B. malayi* from the
241 PRJNA232599 transcriptomic experiment. For this we performed BLASTn searches against SRA files
242 using a region of 61 bp from the 28S rRNA of nematode as query, including the SRL (**Fig. 5A**).
243 Consistent with the report by (Choi et al. 2014), we observed that in susceptible BEY-LVP strain, the
244 number of total reads increased during the course of infection, as expected from the nematode growth.
245 Interestingly, we found few reads with a depurinated site between 4th and 8th days after the infection (**Fig.
246 5B**). In the refractory RED strain, the number of reads post infection in all samples is similar suggesting
247 that the number of nematodes did not increase over time. In addition, in contrast with BEY-LVP, we
248 detected reads with depurination signals in all the SRA files belonging to infected samples. Although in
249 this strain the number of total retrieved reads was lower than for the BEY-LVP strain, the percentage of
250 depurinated reads was higher, achieving 43% of retrieved sequences at day 4 (**Fig. 5B**). Thus, our results
251 are the first piece of evidence supporting that these horizontally acquired genes of insects encode

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252 functional enzymes. On the other hand, these results support RNA *N*-glycosidases activity to be involved
253 in defense response.

254
Discussion

255 RIPs have been largely described in plant and bacterial lineages (Bolognesi et al. 2016; Di Maro
256 et al. 2014; Stirpe 2004). In recent years, we have demonstrated the presence of RIP genes in fungi and
257 metazoa (Lapadula and Juri Ayub 2017; Lapadula et al. 2020b; Lapadula et al. 2013). In animals, the
258 presence of these ribotoxins is restricted to a few species of insects, being this narrow and patchy
259 taxonomic distribution a hallmark of metazoan RIPs. The first RIP that we reported in insects belong to
260 mosquitoes from the Culicinae subfamily (Lapadula et al. 2017; Lapadula et al. 2013). Then, we
261 confirmed the presence of these genes in a second group belonging to whiteflies from the Aleyrodidae
262 family (Lapadula et al. 2020b). Here, we found evidence of RIP encoding genes in a third lineage of
263 insects belonging to the Sciaroidea superfamily. In this case these genes are present in two species of flies
264 (*C. nasturtii* and *B. odoriphaga*). From the primary structure of genes, it was evident that all RIP
265 encoding genes found in insects showed low sequence identity among them. Furthermore, features such
266 as the paralogues number, the presence of signal peptides or introns (**Table 2**) differ for each lineage.
267 Phylogenetic analysis (**Fig. 1**) indicated that metazoan RIPs does not form a monophyletic group. All
268 these pieces of evidence support the idea that metazoan RIP genes have independent origins. Previously,
269 we have reported that ribotoxins encoding genes were horizontally acquired by mosquitoes (Lapadula et
270 al. 2017) and whiteflies (Lapadula et al. 2020b) from bacterial and plant donors, respectively. Here, we
271 propose that flies RIPs were acquired by a third HGT event from a bacterial organism. The sister clade is
272 shaped by sequences from the entomopathogen genera *Photorhabdus* and phytopathogens genus
273 *Xanthomona* and *Brenneria* (**Supplementary fig. 2**). Across their ontogeny, organisms which belong to
274 the Sciaroidea superfamily live in soil or host like fungus and plants. This superfamily includes
275 fungivorous organism such as mycetophilids (Jakovlev 2012), and others like the Sciaridae family which
276 live as larvae primarily in soil litter feeding on plant roots (Binns 1981) and Cecidomyiidae whose larvae
277 produce secretions that dissolve the waxy cuticle and liquefy the underlying cells of the surrounding leaf
278 surface (Readshaw 1966). This last indicated that the cenancestor of these flies were likely exposed to a

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279 large number of bacteria present in their habitats. It has been reported that HGT could be facilitated in
280 early developmental stages of insects by the weakness of the Weisman barrier in these moments of their
281 lifecycle (Huang 2013). The most likely donor are organisms sharing the same ecological niche such as
282 symbiont (Dunning Hotopp et al. 2007; Kondo et al. 2002) or like -we previously proposed- plant and
283 microbe-feeding insects (Lapadula et al. 2017; Lapadula et al. 2020b). This intimate association between
284 recipient and donor organisms may facilitate the HGT.

285 Although horizontally transferred genes have been related to diet adaptation in several organism
286 like arthropods (Wybouw et al. 2016) and insect (Prasad et al. 2021) there is also evidence that foreign
287 genes could be involved in host defense (Di Lelio et al. 2019; Husnik and McCutcheon 2018). In insects
288 some examples of HGT acquired genes involved in defense include bacterial lysozymes acquired by pea
289 aphid, *Acyrthosiphon pisum* (Nikoh et al. 2010) and by *Halyomorpha halys* (Ioannidis et al. 2014).
290 Recently, the acquisition by HGT of five toxin genes in *C. nasturtii* species was reported, which plays a
291 nontrivial new role in insect immune function against eukaryotic enemies (Verster et al. 2021). In line
292 with our model, the authors postulated that most likely donors are microbes that share the same
293 environment with swede midge. The recurrent acquisition of RIP encoding genes by insects, the
294 maintenance of these genes in host genomes by effects of natural selection and the occurrence of
295 transcription are pieces of evidence that strongly support a functional role for these foreign genes in these
296 organisms. The toxic nature of this protein family and the defensive roles that RIPs from *Spiroplasma*
297 play in *Drosophila* species that lack these genes (Ballinger and Perlman 2017; Ballinger and Perlman
298 2019) further suggests that these ribotoxins could be immune molecules effectors.

299 Insect innate immunity includes both cellular and humoral responses (Ali Mohammadie Kojour et
300 al. 2020; Hoffmann et al. 1996). The first is performed by hemocytes and consists predominantly of
301 phagocytosis, nodulation and encapsulation of invading microorganisms (Ali Mohammadie Kojour et al.
302 2020; Hoffmann et al. 1996). The humoral response, mediated by soluble plasma proteins or fat body,
303 implies clotting, melanin synthesis, and a rapid synthesis of a battery of antimicrobial peptides (AMPs)
304 (Ali Mohammadie Kojour et al. 2020; Hoffmann et al. 1996). These molecules exhibit a broad spectrum
305 of activity directed against bacteria and/or fungi. RIPAe2 is upregulated in response to infectious

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306 challenges caused by bacteria and fungi in *A. aegypti*, we also observed that the expression was triggered
307 after the treatment with nematode *B. malayi,* we even observed depurination in nematode ribosome (**Fig.s**
308 **4 and 5**). Interestingly, (Choi et al. 2014) determined the host response profile comparing infected *vs*.
309 uninfected *A. aegypti* BEY-LVP and found that genes with rRNA *N*-glycosylase activity (GO:0030598)
310 are over-represented among the group of transcripts after the infection with *B. malayi*. In LVP-IB12^R and
311 LVP-FR3^S strains (Juneja et al. 2015) reported a striking concordance between the transcriptional
312 response of immune genes. Interestingly, they showed that RIPAe2 is one of the group of genes whose
313 expression is induced in both strains after the infection (Juneja et al. 2015). *In vitro* evidence indicates
314 that cecropin, an AMP from insect haemolymph, attenuates the motility of microfilariae of *Brugia*
315 *pahangi* (Chalk et al. 1995). Thus, we postulate that RIP encoding genes could be immune effector
316 molecules with regulation patterns comparable to AMPs.

317 The synthesis of AMPs is a hallmark of the host defense of higher insect orders like the
318 Holometabola (essentially the Lepidoptera, Diptera, Hymenoptera and Coleoptera) and some
319 Heterometabola (*e.g*. Hemiptera) (Hoffmann et al. 1996), organisms in which the presence of RIP
320 encoding genes has been reported (**Table 2**). On the other hand, the presence of antibacterial activity and
321 the expression of AMPs in early stages of insect ontogeny has been reported in some species. In the case
322 of *Drosophila melanogaster,* a decrease in the number of bacteria at late pupal stage was reported,
323 indicating antibacterial activity in this stage (Bakula 1969). Larvae of Black Soldier Fly, *H. illucens*,
324 produced several AMPs which protect the insect from pathogens such as *E. coli* and *S. enterica* (Erickson
325 et al. 2004). In a similar way the larvae of *Anopheles gambiae* mosquito, which live in a microbe-rich
326 aquatic environment, exhibit higher levels of immune gene expression than adults (League et al. 2017). In
327 *Bombix mori* at spinning and prepupa stages, a large increase in the expression of some AMPs was
328 detected in the gut (Wu et al. 2010). The upregulation in the expression of AMPs in early stages of insect
329 ontogeny is a consequence of hormonal regulations. 20-hydroxyecdysone is a steroid hormone produced
330 by prothoracic glands prior to each moult. This hormone promotes humoral immunity by increasing the
331 expression of AMP genes after immune challenge either by direct regulation or through interaction with
332 other players of the immune response (Nunes et al. 2021). Transcriptomic experiments carried out in *A.*
333 *aegypti*, *A. albopictus*, *B. odoriphaga* and *C. nasturtii* showed that the highest expression levels for RIPs

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334 genes are found in early stages of development (**Fig. 2 and Table 2**). These analyses were consistent with
335 results previously reported in two strains of *A. aegypti* (Lapadula et al. 2020a). All this evidence supports
336 a convergent expression profile across the ontogeny of insects.

337 In general, AMPs are produced after an immune challenge by the fat body that releases them into
338 the haemolymph. These kinds of peptides are regulated at the transcriptional level, through the binding of
339 the nuclear factor kappa-light-chain-enhancer of activated B cell (NF-κβ) (Manniello et al. 2021). RIPAe2
340 is found amongst the group of genes that are upregulated post-infection with *Plasmodium gallinaceum* in
341 the fat body of transgenic *Ae. aegypti*, where the transcription factor NF-kB REL-2 is overexpressed (Zou
342 et al. 2011). REL-1 and REL-2 are transcription factors that regulate the activation of genes downstream
343 of the Toll and IMD pathways, the two main signaling cascades regulating insect immunity (Manniello et
344 al. 2021; Valanne et al. 2011). In line with that, results shown here indicate that RIP transcripts are
345 present in the thorax and abdomen of mosquitoes and whiteflies, body parts where the fat body is located
346 (**Fig. 3 and Table 2**). Even in *A. aegypti* the level of transcripts present in thorax after the infection with
347 *B. malayi* were higher than that found in the whole body (**Fig. 4**). These pieces of evidence indicate that
348 RIP transcripts are present in body parts involved in immune response.

349 In plants, the RIP family has been associated in defense against several kinds of pathogens like
350 fungus, bacteria, virus and insects (Zhu et al. 2018). In a similar way, the overexpression of RIPs reported
351 in *A. aegypti* after the infection with the Microsporidia *Edhazardia aedis* (Desjardins et al. 2015), which
352 allowed the authors to propose that rRNA *N*-glycosylase activity (GO:0030598) might play a role in the
353 immune response of *A. aegypti*. On the other hand, bacterial RIPs from Spiroplasma endosymbiont are
354 key in Drosophila defense against wasp (Ballinger and Perlman 2017; Ballinger and Perlman 2019;
355 Hamilton et al. 2016). Here, we identified a higher number of depurinated sites on the SRL region that
356 belong to a pathogen inducing RIP expression in *A. aegypti* refractory strain. Additional searches using
357 the homologous region of 28S rRNA from mosquitoes as query did not retrieve any depurinated reads,
358 suggesting that RIP genes do not have an effect on host ribosomes. The mechanisms proposed for
359 resistance in mosquitoes against *B. malayi* include reduced ingestion of parasites, physical killing of
360 parasites in the foregut, barriers to penetration of the midgut, and hemolymph factors that kill the parasite

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361 in the thoracic cavity and lead to melanotic encapsulation (Kobayashi et al. 1986). Therefore, the
362 confirmation of the presence of depurination in ribosomes of *B. malagy* (**Fig. 5**) supports the hypothesis
363 that these foreign genes have an impact on pathogen viability and contribute to immune response of
364 infected organisms. RNA *N*-glycosidases activity could be the main mechanism through which these
365 proteins play a defensive role in insects.

366 **Conclusion**

367 In conclusion, although additional studies are needed, similarity in spatial and temporal
368 expression profiles found in organisms where RIP encoding genes have been independently acquired
369 support a functional convergence. Data from this study, along with previous information, prompted us to
370 propose that RIPs are immune effector molecules in insects. This hypothesis is supported by the follow
371 points:

372 I) The highest expression levels for these genes are found in early developmental stages of insects.
373 II) Transcripts of these genes are present in body parts involved in humoral immune response.
374 III) Transcription of RIP genes in *A. aegyti* is upregulated after the infection with several pathogens.
375 IV) These foreign proteins conserve their toxicity as a consequence of their enzymatic activity.
376 V) In refractory *A. aegypti* strain, the number of depurinated ribosome from *B. malayi* is higher than
377 in samples of infected susceptible strains.

378

379 # Experimental Procedures

380 **Homology searches and sequence analyses**

381 BLASTp homology searches were performed under default parameters on insect databases
382 (excluding *Aedes*, *Culex*, *Bemisia*, and *Trialeurodes* genus) using a previously reported set of RIP
383 sequences (Lapadula et al. 2020b) as queries. Bacterial sequences retrieved automatically annotated
384 sequences from *Contarinia nasturtii* and *Bradysia odoriphaga* genome database. Then, these new
385 sequences were used as queries in tBLASTn searches and new not annotated homologues were found in
386 both species of flies. Pfam analysis was performed to confirm the presence of RIP domain (PF00161).

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387 The presence of signal peptide was predicted using SignalP 5.0
388 (https://services.healthtech.dtu.dk/service.php?SignalP-5.0). The presence of introns was analyzed, when
389 available, comparing predicted mRNA with genomic DNA using the splign tool
390 (https://www.ncbi.nlm.nih.gov/sutils/splign/splign.cgi). A full list of insect RIP encoding genes is
391 available in **Supplementary Table 1**.

392 **Multiple sequence alignment and phylogenetic inferences**

393 *C. nasturtii* and *B. odoriphaga* RIP amino acids sequences were added to our previously reported
394 dataset of RIP (Lapadula et al. 2017; Lapadula et al. 2020b; Lapadula et al. 2013). This dataset was used
395 for constructing a Multiple Sequences Alignment (MSA), as previously described (Lapadula et al. 2017;
396 Lapadula et al. 2020b). This MSA containing 168 sequences and 159 residues was used to perform
397 phylogenetic analysis by Maximum Likelihood in RAxML (version 8.2.10, available at
398 https://github.com/stamatak/standard-RAxML) (Stamatakis 2014). The WAG substitution matrix was
399 selected using ProtTest 3.4 (Darriba et al. 2011). To estimate the robustness of the phylogenetic inference,
400 500 rapid bootstrap (BS) were selected. Transfer bootstrap expectation was calculated in BOOSTER
401 (Lemoine et al. 2018). Phylogenetic relationships and divergence times among species were obtained
402 from TimeTree knowledge-base (Kumar et al. 2017). FigTree (version 1.4.2, available at
403 https://tree.bio.ed.ac.uk/software/figtree) was used to visualize and edit the trees.

404 **Transcriptomic data analysis**

405 BioProjects of transcriptome experiments carried out in *Aedes aegypti*, *Aedes albopictus, Bemisia*
406 *tabaci* and *Contarinia nasturtii* were selected from the National Center for Biotechnology Information
407 (NCBI) database (https://www.ncbi.nlm.nih.gov/). Sequence Read Archive (SRA) in FASTQ format were
408 downloaded for the datasets (codes of SRA files used in this work are indicated in **Supplementary**
409 **Tables 2-4**). The quality of each SRA file was evaluated using FastQC software
410 (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). The abundance of transcripts from RNA-
411 seq data was quantified using the Kallisto program (Bray et al. 2016) which estimated counts in
412 Transcripts Per Millions (TPM). Not annotated RIP sequences of different species were incorporated in
413 each reference transcriptomes obtained in FASTA format from the NCBI database. These files were used

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414 to build the index for each species in Kallisto. The TPM values obtained for RIP genes were represented
415 in bar plots using GraphPad Prism version 5.00 for Windows. In the case of mosquito species databases
416 such as VectorBase (https://vectorbase.org/vectorbase/app) and *Aegypti-Atlas*
417 (http://aegyptiatlas.buchonlab.com/) were also analyzed.

418

419

420 **Quantification of SRL depurination**

421 The depurination of the SRL by RIP RNA *N*-glycosidase activity yields an abasic site. Upon
422 conversion, in a retrotranscription process, the reverse transcriptase preferentially inserts a dAMP
423 opposite to the abasic site. Following the first round of amplification step, this yields a complementary
424 dTMP. This is the basis used by (Pierce et al. 2011) to determine this enzymatic activity by qPCR. SRA
425 files belonging to BioProject PRJNA232599 carried out in *Aedes aegypti* after the infection with *B.*
426 *malayi* were used to quantify the number of reads derived from depurinated SRL. For this analysis, a
427 region of 61 bp from the 28S rRNA of the pathogen including the adenine (not depurinated) and
428 thymidine (depurinated) residues were used as queries in BLASTn searches. The searching parameters
429 were set in order to exclude *A. aegypti* as a result. All retrieved reads for each SRA file were downloaded
430 and aligned with MAFFT online server. In each alignment, the number of adenine and thymine present in
431 the target position of depurination was counted and represented in a bar plot using GraphPad Prism
432 version 5.00 for Windows.

433 Acknowledgements

434 W.J.L and M.J.A. are members of the scientific career of CONICET. This work has been funded
435 by grants from CONICET (Consejo Nacional de Investigaciones Científicas y Técnicas) PIP 2732,
436 PIBAA 28720210100081CO to M.J.A and W.J.L and UNSL (Universidad Nacional de San Luis)
437 PROICO 02-1720 to M.J.A. We would also thank Carolina Mirallas from the GAECI (English Language
438 Writing Advise) for their linguistic corrections of an early version of this work. Finally, W.J.L thanks

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439 Pablo V. Lapadula, who died as a consequence of SARS-CoV-2, for always encouraging him to continue

440 this research.

441

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445

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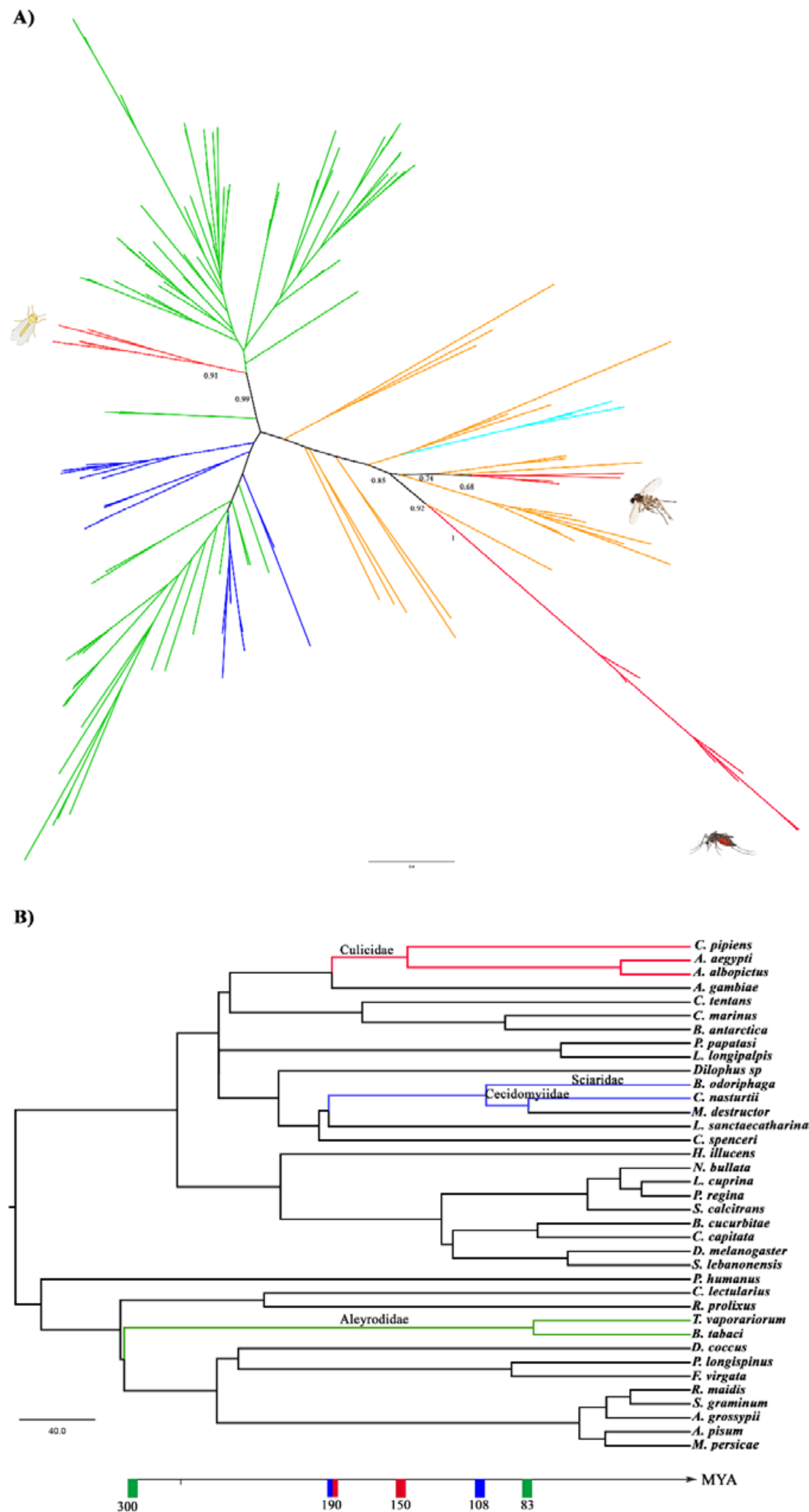
Figures



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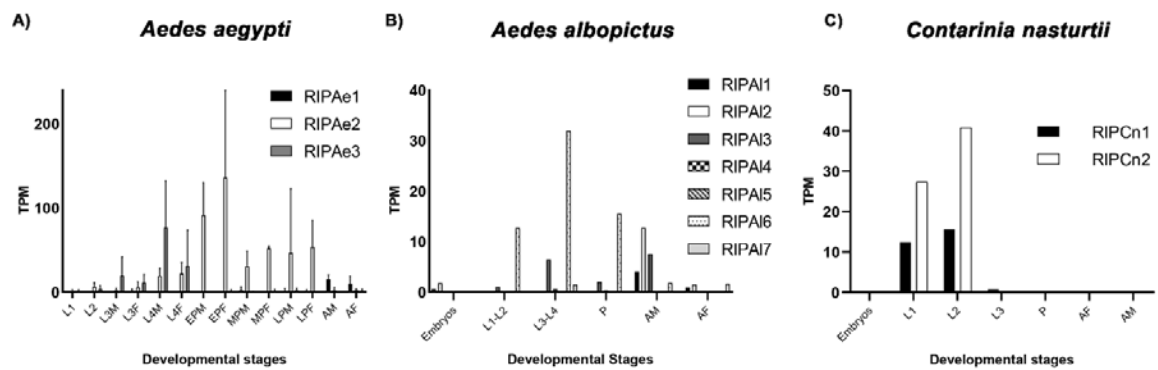
615 **Fig. 1 A)** Unrooted phylogeny of RIP genes. Branches are colored according to taxonomy: bacteria (orange),
616 Cyanobacteria (Cyan), plants (green), fungi (blue), metazoan (red). TBE support values of relevant divergences are
617 shown at nodes. Fly, mosquito and whitefly clades are marked with silhouettes. Fully annotated phylogeny is
618 available as Fig. Supplementary 2. **B)** Phylogeny of selected species from Neoptera orders. The tree including
619 species from Diptera (24), Hemiptera (12) and Psocodea (1) orders with fully sequenced genomes was constructed
620 with the TimeTree knowledge-base (Kumar et al. 2017). Insects harboring RIP genes are shown in red, blue and
621 green branches. The occurrence time of three independent HGT events are graphically represented with the
622 estimated time windows. Time in million years ago (MYA) is indicated at the bottom.



623

624 **Fig. 2 Expression of RIP genes across the developmental stages of insects as determined from transcriptomic**
625 **assays.** Expression of RIP genes was represented in transcript per millions (TPM). **A)** Larval 1-4 (L1-L4), early,
626 mid and late pupal (EP, MP, LP) and adult stages (A) of *A. aegypti*. In some stages both sexes female (F) and male
627 (M) were evaluated. Sequences Read Archives (SRA) files were taken from the BioProject PRJNA419241. **B)**
628 Embryos, larval 1-2 (L1-2), larval 3-4 (L3-4), pupal (P), male adult (AM) and female adult (AF) of *A. albopictus*.
629 SRA files were taken from BioProject PRJNA275727. **C)** Embryos, larval 1-3 (L1-L3), pupal and both sexes of
630 adult stage (AF and AM) in *C. nasturtii*. SRA files were taken from the BioProject PRJNA646761.

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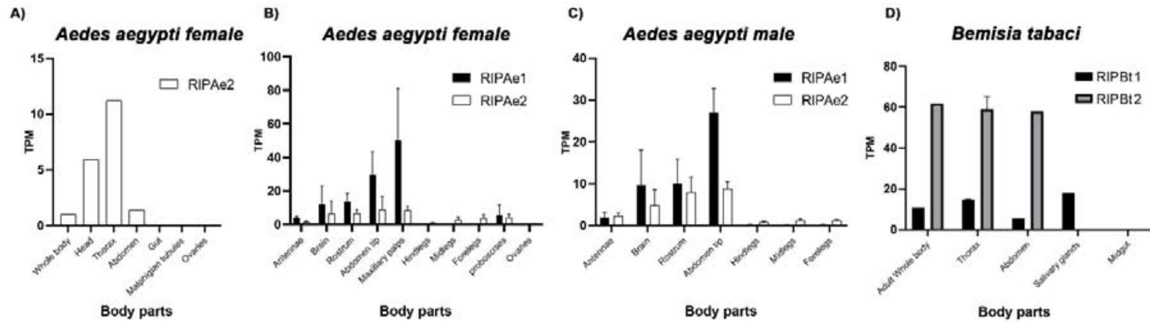

634

635 **Fig. 3. Expression of RIP genes in different body parts of insects. A)** Expression of RIPAe2 gene from *A. aegypti*
636 was represented in TPM for whole body, head, thorax, abdomen, gut, Malpighian tubules and ovaries of adult
637 female. These results were taken from Aegypti-Atlas (Hixson et al. 2022) **B-C)** Expression of RIP genes were
638 represented in TPM for adult females and males of *A. aegypti*, respectively. The expression was evaluated in
639 antennae, brain, rostrum, abdominal tip, hindlegs, midleg and forelegs for both sexes while in female maxillary palp,
640 proboscises and ovaries were included in the analysis. SRA files were taken from BioProject PRJNA236239. **D)**
641 Expression of RIP genes of *B. tabaci* was represented in TPM for whole body, thorax, abdomen, salivary glands and
642 midgut of adult individuals. SRA files were taken from the BioProject PRJEB26594.

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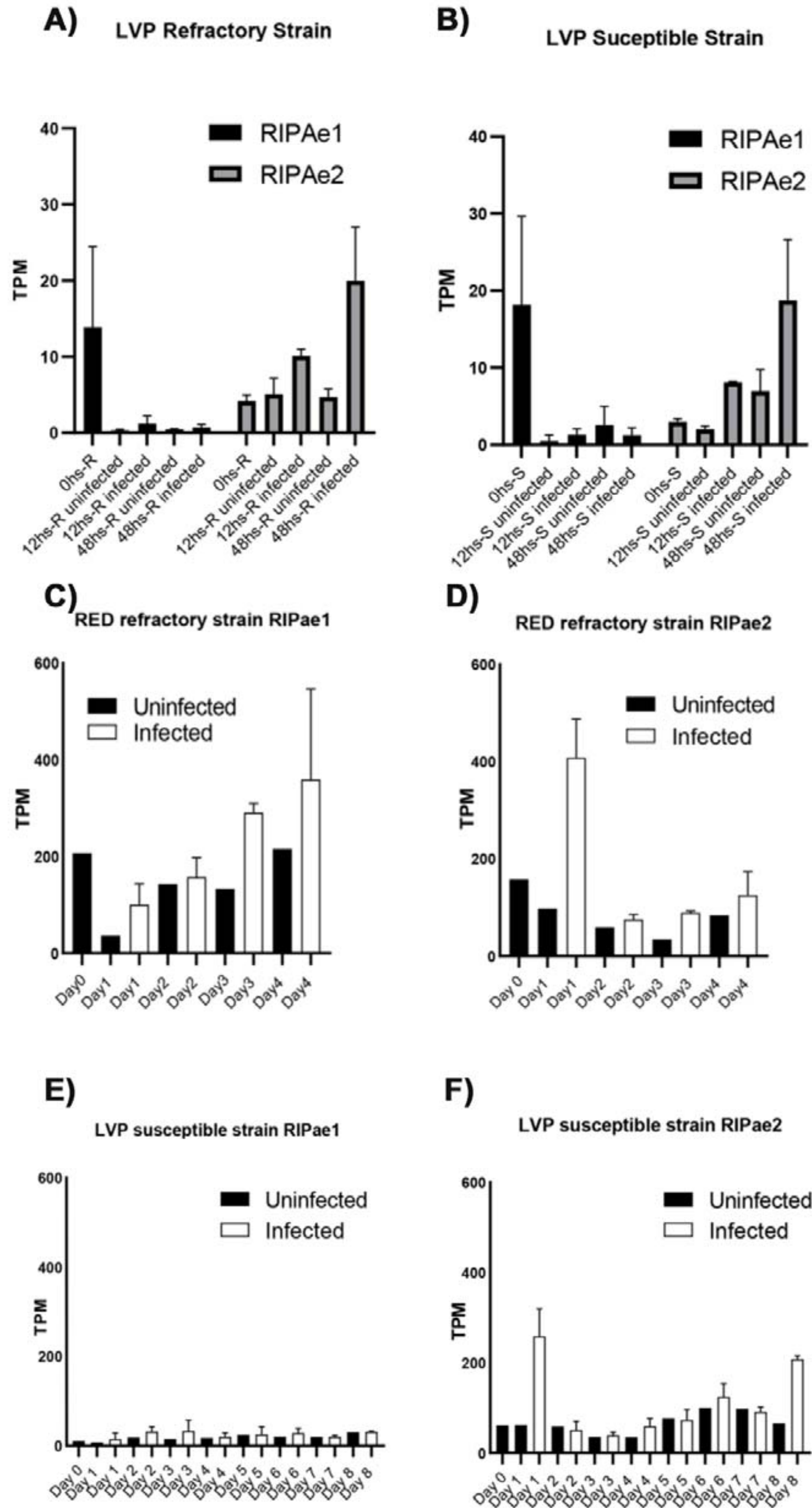

643

644 **Fig. 4 Expression of RIP genes in adults *A. aegypti* after the infection with the nematode *B. malayi*. A-B)** Reads

645 were taken from BioProject PRJNA255467 using the whole body of LVP-IB12^{R} and LVP-FR3^{S} strains,

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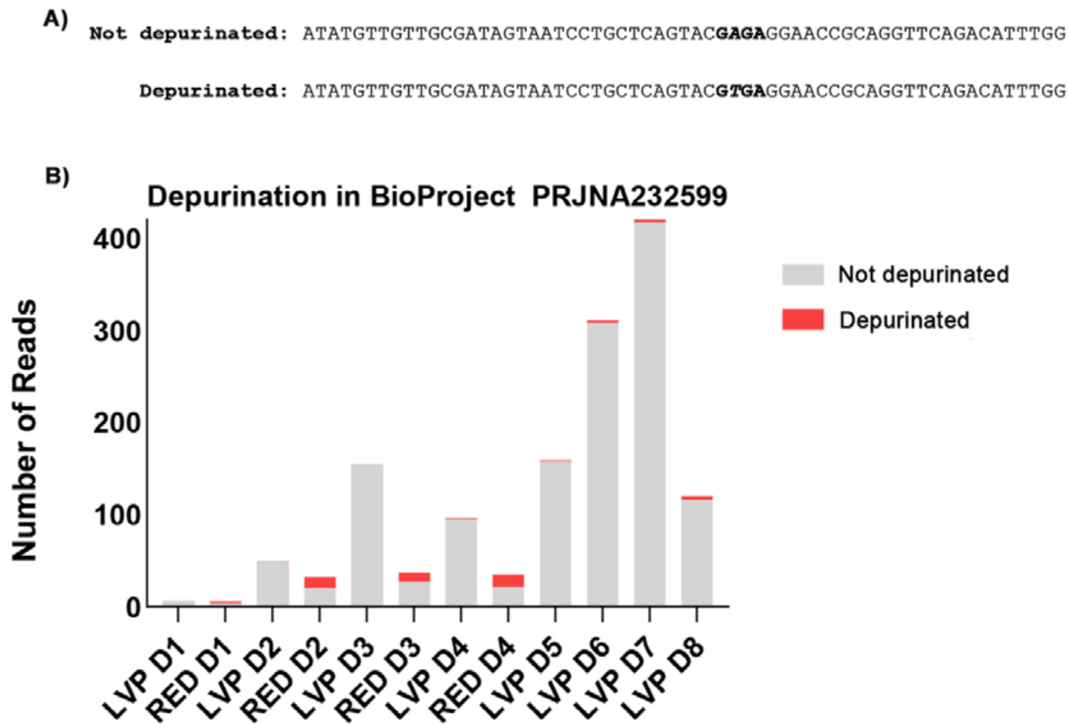
646 respectively. Expression of RIPAe1 and RIPAe2 genes was represented in TPM for different times after the
647 infection. **C-F)** Reads were taken from BioProject PRJNA232599 corresponding to thorax tissue of refractory
648 BEY-LVP (**C-D**) and susceptible RED (**E-F**) strains, respectively. Expression of RIPAe1 and RIPAe2 genes was
649 represented in TPM for different times after the infection.



650

651 **Fig. 5. Evidence of RNA *N*-glycosidases activity. A)** Not depurinated and depurinated sequences of 28S rRNA of
652 *B. malayi* used to perform BLAST searches. The GAGA motif of the SRL is indicated with bold letters. The
653 position which is target of depurination, adenine and thymine, are indicated with italic bold letters. **B)** Number of
654 reads retrieved by BLAST searches in SRA files of infected samples from BioProject PRJNA232599. Not
655 depurinated and depurinated reads are indicated with grey and red colors, respectively.

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Tables

658 **Table 1. Hits retrieved by BLASTn searches for SRA files of BioProjects PRJNA388516 and PRJNA304774.**

659 Mean of hits obtained for RIPBo1 and RIPBo2 in SRA files belonging to egg (E), larval 2 (L2), larval 4 (L4), pupal

660 (P) and adult (A) stages are indicated in the second and third columns.

Stage and SRA files	Mean of retrieved hits of PRJNA388516 (RIPBo1-RIPBo2)	Retrieved hits of PRJNA304774 (RIPBo1-RIPBo2)
E (SRX2879613/SRX2879612/SRX2879610)	1-0	ND
L2 (SRX2879611/SRX2879609/SRX2879608)	1700-3	ND
L3 (SRX1459738)	ND	1753-4
L4 (SRX2879607/SRX2879606/SRX2879604) (SRX1473238)	1500-4	2220-10
P (SRX2879616/SRX2879615/ SRX2879605) (SRX1473242)	365-1	151-0
A (SRX2879618 /SRX2879614/SRX2879617)	5-0	ND

661 ND* non detected

662 **Table 2. Information of RIP encoding genes in insects.** Number of paralogues, presence of introns and signal

663 peptides, potential donor lineage, evidence of evolution under purifying selection, evidence of expression,

664 transcriptional information such as expression in developmental stages (DS), body parts (BP), post infection with

665 pathogens and evidence of enzymatic activity are indicated for each specie.

Host lineage	Species	Number of paralogues	Intron	Putative signal peptide	Donor lineage	Purifying selection	Expression	DS with higher expression level	BP with transcript	Upregulation after infection	Evidence of enzymatic activity
Subfamily Culicinae (mosquitoes)	*A. aegypti*	3	NO	NO	Bacterial	YES	YES	L4 and pu	Thorax	YES	YES
	A. albopictus	7	NO	NO	Bacterial	YES	YES	L4	ND	ND	ND
	C. quinquefasciatus	1	NO	NO	Bacterial	YES	YES	ND	ND	ND	ND
Family Aleyrodidae (whiteflies)	*B. tabaci*	2	YES	YES	Plant	YES	YES	ND	Thorax Abd	ND	ND
	T. vaporariorum	3	YES	YES	Plant	YES	YES	ND	ND	ND	ND

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Superfamily Sciaroidea (flies)	*B. odoriphaga*	2	ND	YES	Bacterial	ND	YES	L2	ND	ND	ND
	C. nasturtii	2	YES	YES	Bacterial	ND	YES	L2	ND	ND	ND

666 ND* non detected

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