Selection on time to parasite transmission shapes the host *Anopheles gambiae* transcriptional response and suggests immune evasion

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Abstract

Understanding host-parasite interactions is of the utmost importance for the correct disease prediction, prevention and management. Hence, this study assessed the transcriptional response of the primary malaria vector, *Anopheles gambiae*, to infection with several lines of the prominent vector-control parasite, the microsporidian *Vavraia culicis*. These parasitic lines have been selected for early or late transmission within this host. Previous studies extensively described them phenotypically, differing in their virulence, infection dynamics and host exploitation. Using RNA sequencing, gene expression profiles were analyzed in mosquitoes infected with early-selected, late-selected, unselected (reference) *V. culicis* lines and uninfected controls. The results revealed distinct transcriptional changes associated with each parasite line. Early-selected parasites induced a broader immune response than late-selected ones. Differential expression of immune-related genes, including *Toll-interacting protein* and *Protein ERGIC-53*, suggests enhanced immune evasion in late-selected parasites. Additionally, significant changes were observed in pathways related to Golgi membrane function and oxidative stress response, particularly in response to early-selected parasites. These findings highlight the evolutionary pressures shaping host-parasite coevolution and provide insights into how parasite transmission traits can influence mosquito immune response and regulation. This work offers a foundation for future studies on mosquito-microsporidia dynamics and potential applications for vector control strategies, particularly *Plasmodium*.

Background

As with any host, mosquitoes are exposed to and harbour numerous parasites [1–6], including microsporidia [7, 8]. Microsporidia are intracellular parasites that infect various hosts and occupy different ecological niches [7, 9–11]. Still, those infecting mosquitoes are predominantly aquatic, as they typically parasitize the aquatic stages of the mosquito life cycle [2]. These parasites have gained attention in the field of vector-borne diseases due to their potential use as biological agents for controlling *Plasmodium* and its mosquito host, *A. gambiae* [12–15]. Studies in both wild and laboratory settings have demonstrated that microsporidians possess *Plasmodium*-inhibitory potential when co-infecting mosquitoes with this parasite [8, 16].

Vavraia culicis is one of the most extensively studied microsporidians infecting mosquitoes and in the context of malaria research [15–18]. Although *A. gambiae* infections by *V. culicis* tend to result in low costs to adult survival, fecundity and developmental time [18, 19], little is known about the molecular basis of the host response to this microsporidian. In contrast, infections by *V. culicis* in the mosquito *Aedes aegypti*, the vector for dengue, are characterized by a weak immune response but significant investment in lectins and CLIP-domain serine proteases, both effectors involved in innate immunity and its signalling [20, 21]. Understanding how *A. gambiae* responds to microsporidian infection at the molecular level can provide valuable insights into host-parasite dynamics, immunity evolution, and the evolutionary pressures exerted by the parasite. Recent research has highlighted the role of host gene expression in response to pathogens, revealing that parasites can modulate or evade host immune responses to facilitate their transmission and survival [22–25]. In mosquitos, immune defences are mediated mainly by innate immunity, with critical pathways such as the Toll, Imd and JAK-STAT pathways involved in response to parasite recognition and elimination [26, 27] — however, the specific role of these pathways in response to microsporidian infections, like *V. culicis*, remains poorly understood. Therefore, this study sought to start by characterizing the *A. gambiae* host response to *V. culicis* at the molecular level.

Despite their small genome [11, 28, 29], microsporidia evolve rapidly [19]. Recent studies have demonstrated that artificial selection for early or late transmission can shape their virulence and several life history traits [19, 30, 31]. In particular, selection for early or late transmission of *V. culicis* in *A. gambiae* has been shown to influence parasite replication, survival outside the host, and virulence [19, 30]. For example, hosts infected with parasites selected for late transmission experienced the most virulent infections, resulting in shortened life cycles for both the host and parasite, increased host exploitation, and greater parasite growth [19]. Interestingly, late-selected parasites did not survive outside the host as well as the early-selected parasites, which could endure the environment for longer [30]. This trade-off is likely due to a better adaptation to the within-host

environment [32]. Thus, these selected lines of *V. culicis* differ significantly in their performance and virulence, particularly within the host and potentially offer variability in parasite strategies that can be examined further.

Considering this, the current study aimed to expand knowledge on the drivers of virulence and transmission evolution. In addition to the infection by the reference parasite, changes specific to selection for early or late transmission were explored through RNA sequencing (RNA-seq) of infected hosts. Through the inclusion of these selected lines, variability in parasite treatments was increased, which may provide insights into which expression patterns are more readily available or likely to evolve in response to different parasite challenges.

Hence, in this study, the transcriptional response of *A. gambiae* to infection by different lines of *V. culicis* - one selected for early transmission, one selected for late transmission and one unselected reference - was characterized. RNA-seq was used to analyze the differential expression of genes in mosquitoes infected or not (Fig. 1). The findings of this study provide the first insights into the *A. gambiae* transcriptional response to infection by *V. culicis*, while also demonstrating how parasite evolution drives changes in gene expression, impacting the mosquito's ability to respond to infection. This study complements previous work on these parasite lines, providing a multidimensional perspective of this particular host-parasite interaction. Moreover, the data presented here have important implications for parasite fitness and vector competence. Understanding the molecular basis of these interactions is crucial for designing effective vector control strategies that exploit the natural evolutionary pressures acting on mosquito-parasite systems [33].

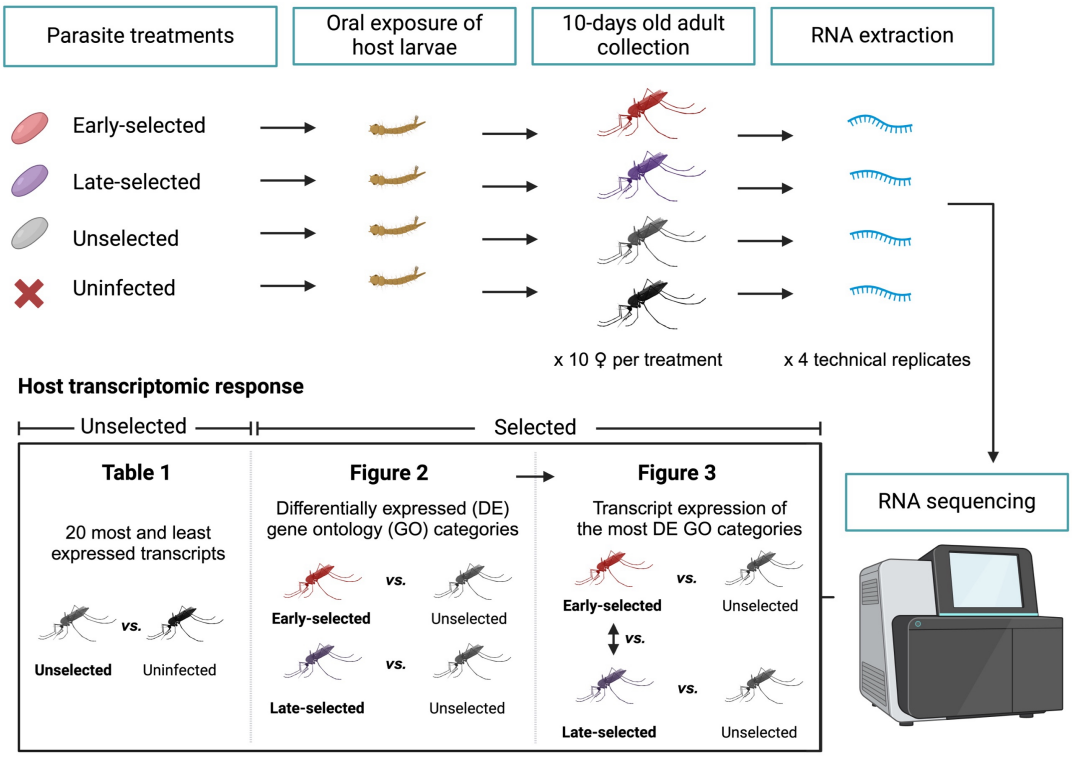


Figure 1. Illustration of the experimental workflow and results. Naive *Anopheles gambiae* larvae were infected, or not, with one of three lines of the parasite *Vavraia culicis,* totalizing in the following treatments: 1) infection with spores selected for early transmission; 2) infection with spores selected for late transmission; 3) infection with unselected reference spores; 4) uninfected. Adult mosquitoes were collected at day 10 in pools of 10 individuals per selection replicate for RNA extraction (10 females x 5 selection replicates). We then assessed differences in host response to the presence/absence of the parasite. First, we compared the 20 most and least expressed transcripts in reference-infected mosquitoes, comparatively to the uninfected ones (Table 1). Second, we categorized all DE transcripts according to their respective gene ontology (GO) biological process. We then presented which GO categories are upregulated/downregulated in infection by the Early- and Late-selected parasite, comparatively to the reference parasite (Fig. 2). Third, we selected candidate GO categories from Figure 2 that differ the most between early- and late-selected and we compared the expression of the transcripts in for each of the categories (Fig. 3) and if they differ between selected regimes.

Table 1. Most up-and down-regulated genes in mosquitoes are infected with the reference parasite compared to those uninfected. The host transcriptomic profile concerning an unselected parasite during infection was compared to the uninfected host profile. The 20 transcripts with the highest and lowest expression, compared to the control, are presented. Fold change and *p*-value were calculated using the transcriptional expression of four technical replicates per treatment. See materials

135 and methods for more details. The *p*-value was adjusted using the Benjamini-Hochberg correction
136 method for each comparison, controlling for a false discovery rate ($p < 0.05$).
137

#	Gene ID	Gene name	Fold change	Adj. *p*
1	LOC3291958	Putative uncharacterized protein DDB_G0271606	-1680.95	< 0.001
2	LOC133393242	Uncharacterized	-306.13	< 0.001
3	LOC1281554	Protein CIMAP1D	-250.60	< 0.001
4	LOC1271479	Uncharacterized	-246.11	< 0.001
5	LOC133393316	Uncharacterized	-195.71	< 0.001
6	LOC1281083	Arginine kinase 1	-191.24	< 0.001
7	LOC1273786	Serine/arginine-rich splicing factor 2	-190.72	< 0.001
8	LOC133394019	Nucleosome assembly protein 1-like 1	-164.62	< 0.001
9	LOC133391046	Putative cyclin-dependent serine/threonine-protein kinase DDB_G0272797/DDB_G0274007	-161.63	< 0.001
10	LOC1271272	Uncharacterized	-149.47	< 0.001
11	LOC5667228	Uncharacterized	-133.19	< 0.001
12	LOC1269550	Uncharacterized	-126.11	< 0.001
13	LOC1270351	Uncharacterized	-123.92	< 0.001
14	LOC133391930	Uncharacterized	-121.19	< 0.001
15	LOC1277465	Uncharacterized	-119.77	< 0.001
16	LOC133391419	Uncharacterized	-107.89	< 0.001
17	LOC11175695	Uncharacterized	-107.77	< 0.001
18	LOC1278052	Cytosol aminopeptidase	-103.75	< 0.001
19	LOC1267823	Uncharacterized	-95.91	< 0.001
20	LOC4576455	Dynein regulatory complex subunit 4	-89.14	< 0.001
1	LOC1281665	Uncharacterized	13.30	< 0.001
2	LOC1269260	Stress-activated protein kinase JNK	4.89	< 0.001
3	LOC1280522	Neuropeptide FF receptor 2	4.40	0.039
4	LOC4576208	Protein obstructor-E	4.15	< 0.001
5	LOC1271120	Protein regulator of cytokinesis 1	4.10	< 0.001
6	LOC1279262	CLIP domain-containing serine protease B15	4.10	< 0.001
7	LOC1269964	Chymotrypsin-2-like	3.99	0.007
8	LOC1270108	Uncharacterized	3.97	0.020
9	LOC1281383	Ninjurin-2	3.96	0.002
10	LOC1279267	CLIP domain-containing serine protease B15-like	3.89	0.002
11	LOC1277042	Uncharacterized	3.76	< 0.001
12	LOC133392142	Uncharacterized	3.62	0.030
13	LOC1271472	Uncharacterized	3.62	< 0.001
14	LOC4577422	Uncharacterized	3.43	< 0.001
15	LOC133393335	Uncharacterized	3.39	0.005
16	LOC133393411	Uncharacterized	3.33	0.001
17	LOC1280050	Annulin	3.27	< 0.001
18	LOC1269628	Monocarboxylate transporter 12	3.27	0.011
19	LOC1271676	Uncharacterized	3.18	< 0.001
20	LOC1277959	UDP-glucosyltransferase 2	3.12	< 0.001

138

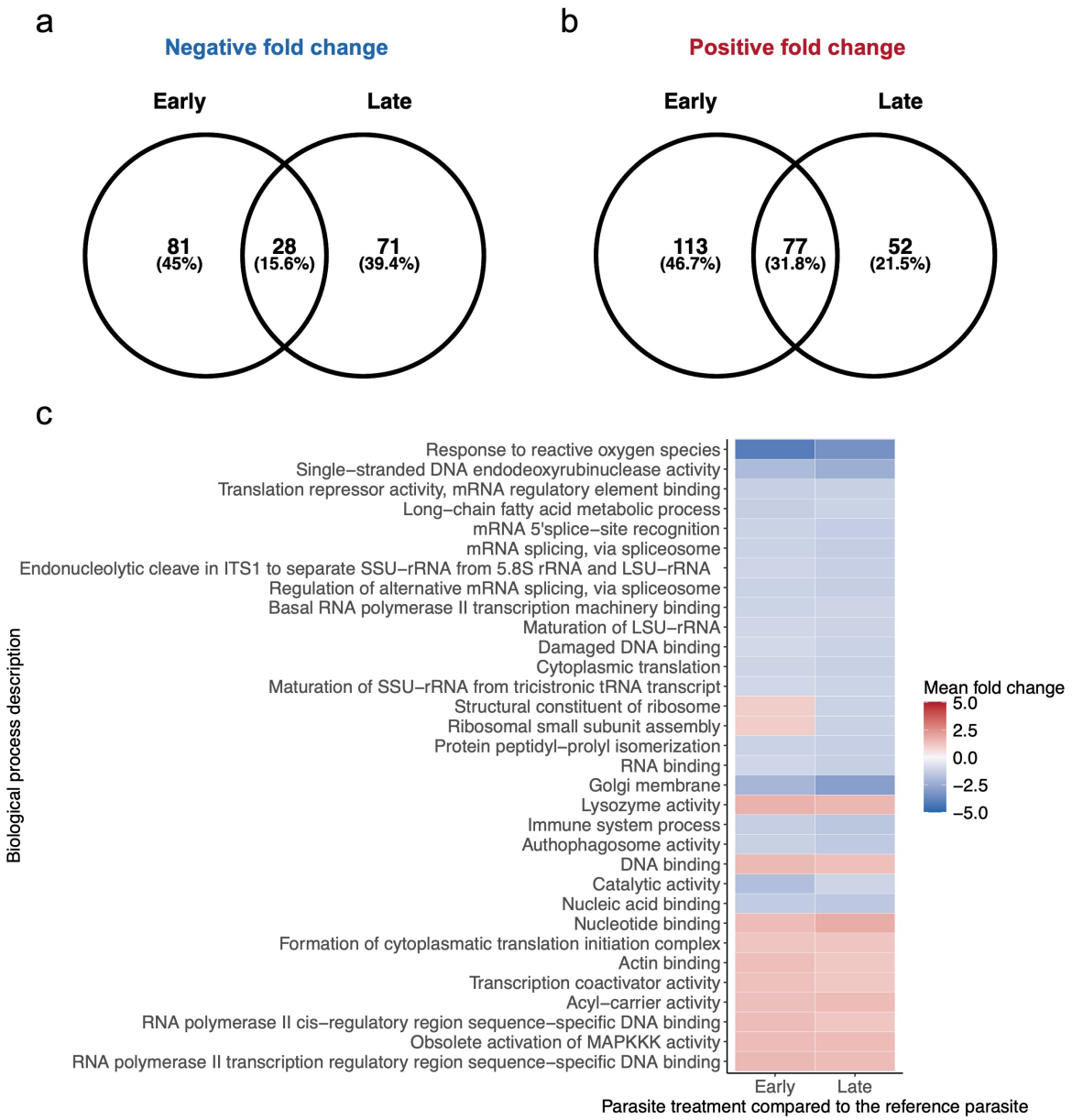
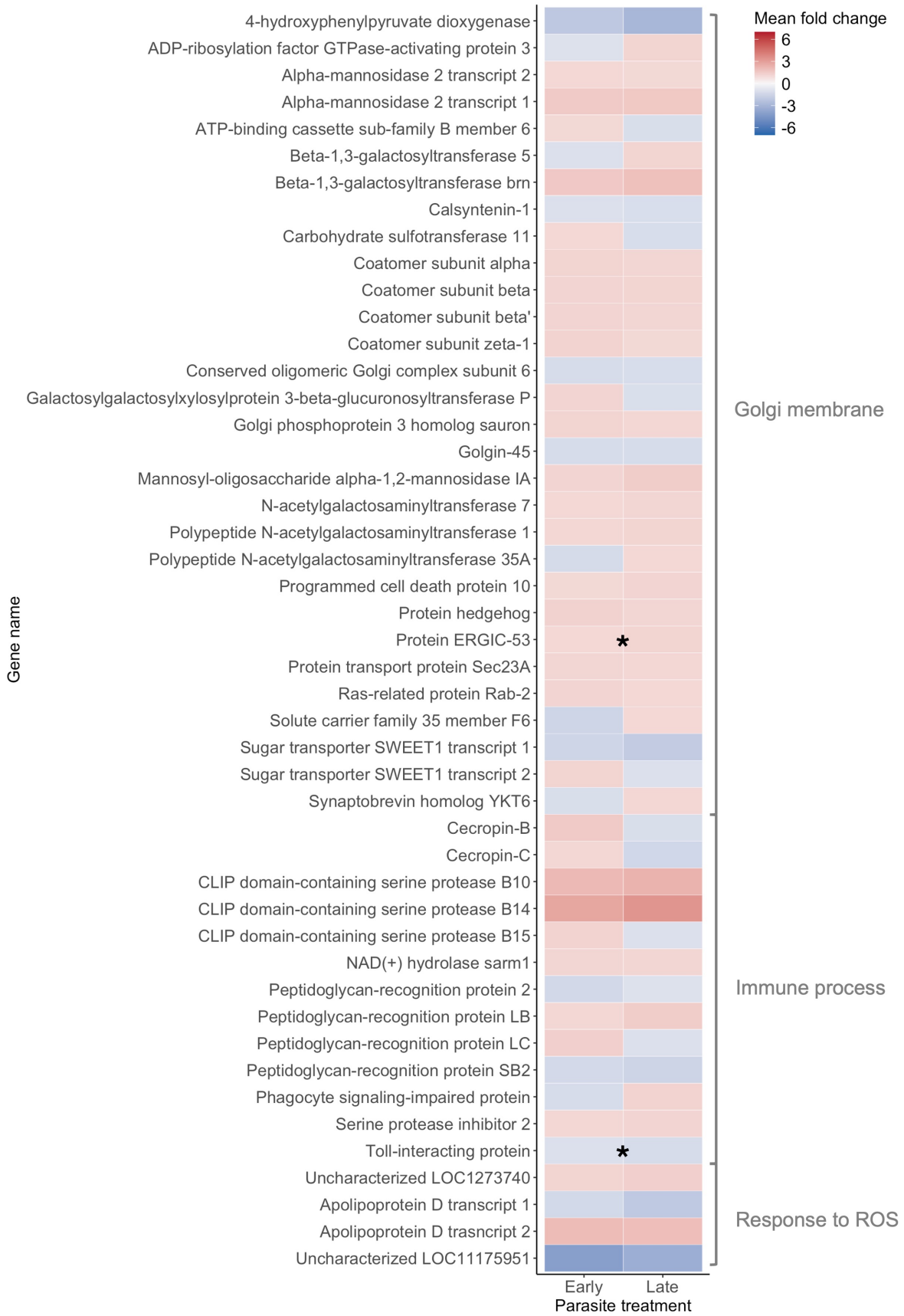


Figure 2. Host transcriptional signatures of infection by selected parasites compared to unselected parasites. Venn diagrams showing the number of differentially expressed (DE) genes (adjusted *p*-value < 0.05) exclusively and commonly found between hosts infected with Early- or Late-selected parasites, in comparison to the unselected reference parasite. Negative (a) and positive (b) differential expression are presented. (c) Heatmap illustrating mean fold change in expression per biological processes families of significantly DE transcripts across all replicates of the same selection regime. Gene ontology annotation was performed using PANTHER 19.0 and VectorBase.


Mean fold change
6
3
0
-3
-6
4-hydroxyphenylpyruvate dioxygenase
ADP-ribosylation factor GTPase-activating protein 3
Alpha-mannosidase 2 transcript 2
Alpha-mannosidase 2 transcript 1
ATP-binding cassette sub-family B member 6
Beta-1,3-galactosyltransferase 5
Beta-1,3-galactosyltransferase brn
Calsyntenin-1
Carbohydrate sulfotransferase 11
Coatomer subunit alpha
Coatomer subunit beta
Coatomer subunit beta'
Coatomer subunit zeta-1
Conserved oligomeric Golgi complex subunit 6
Galactosylgalactosylxylosylprotein 3-beta-glucuronosyltransferase P
Golgi phosphoprotein 3 homolog sauron
Golgin-45
Mannosyl-oligosaccharide alpha-1,2-mannosidase IA
N-acetylgalactosaminyltransferase 7
Polypeptide N-acetylgalactosaminyltransferase 1
Polypeptide N-acetylgalactosaminyltransferase 35A
Programmed cell death protein 10
Protein hedgehog
Protein ERGIC-53
Protein transport protein Sec23A
Ras-related protein Rab-2
Solute carrier family 35 member F6
Sugar transporter SWEET1 transcript 1
Sugar transporter SWEET1 transcript 2
Synaptobrevin homolog YKT6
Cecropin-B
Cecropin-C
CLIP domain-containing serine protease B10
CLIP domain-containing serine protease B14
CLIP domain-containing serine protease B15
NAD(+) hydrolase sarm1
Peptidoglycan-recognition protein 2
Peptidoglycan-recognition protein LB
Peptidoglycan-recognition protein LC
Peptidoglycan-recognition protein SB2
Phagocyte signaling-impaired protein
Serine protease inhibitor 2
Toll-interacting protein
Uncharacterized LOC1273740
Apolipoprotein D transcript 1
Apolipoprotein D trasncript 2
Uncharacterized LOC11175951
Gene name
Golgi membrane
Immune process
Response to ROS
Early
Late
Parasite treatment

147
148

149 **Figure 3. Differentially host expressed candidate genes during infection with selected parasites.**
150 Fold difference in host expression of Golgi membrane (GO:0000139), response to reactive oxygen
151 species (GO:0000302) and immune system process (GO:0002376) markers when compared to
152 infection with the unselected control. Early- and late-selected fold change parasites was then
153 compared to each other using a linear model (see materials and methods for details). Although the
154 expression of all genes presented differed from the unselected parasite, only the Toll-interacting and
155 ERGIC-53 transcripts' fold change significantly differed between early and late-selected parasites.
156 Late-selected had a lower expression of *Toll-interacting protein* ($df = 1$, $F = 9.70$, $p = 0.01$) and
157 *protein ERGIC-53* ($df = 1$, $F = 7.69$, $p = 0.02$) than early parasites, represented by an asterisk.
158

159 # Results

160 This study aimed to characterize the transcriptional response of *A. gambiae* to different lines of the
161 microsporidian parasite *V. culicis*. Infections were performed using an unselected reference parasite
162 and two treatment groups, each with five selection replicates, which had been artificially selected for
163 early or late transmission within this mosquito host [19]. Our findings suggest infection by each
164 parasite treatment resulted in distinct transcriptomic profiles, which differed in their host immune
165 response to each parasite. Moreover, we also observed the involvement of several biological
166 processes, namely Golgi apparatus-associated signalling and response to reactive oxygen species
167 (ROS). The following sections describe and discuss these and other findings in detail.
168
169 Transcriptional profiles were measured on day 10 of adulthood. Across the twelve samples, 20 to 38
170 million reads were obtained, covering over 9,900 genes. The reads were successfully aligned to the *A.*
171 *gambiae* genome (SI Fig. S1), and quality control confirmed their validity (SI Fig. S2).
172
173 First, the host transcriptional profile of *A. gambiae* infected with the reference parasite was compared
174 to uninfected controls (Table 1). Out of the 9,900 annotated genes, 863 (8.7%) were significantly
175 down-regulated, 1104 (11.1%) were significantly up-regulated, and 7953 (80.2%) showed no
176 differential expression (DE). A complete list of the transcripts for this comparison can be found in SI
177 Files S1-S11. Compared to uninfected controls, the 20 most up- and down-regulated transcripts were
178 then focused on (Table 1).
179
180 For this comparison, although a higher number of up-regulated genes was found, the down-regulated
181 genes exhibited higher fold changes. For instance, the most down-regulated gene was the *Putative*
182 *uncharacterized protein DDB_G0271606*, with a negative fold change of 1680.95. In contrast, the
183 most up-regulated gene was an uncharacterized gene with a positive fold change of 13.30.
184

185 Next, the DE profiles of *A. gambiae* infected by early—and late-selected parasite lines were analyzed
186 and compared to the reference parasite (Fig. 2). After comparing transcript expression to the reference
187 control, transcripts that were uniquely DE in each selected treatment and those shared between
188 treatments were identified (Fig. 2ab).
189
190 For down-regulated genes, 81 genes were suppressed by early-selected parasites. In comparison, 71
191 genes were down-regulated by late-selected parasites, with 28 genes commonly down-regulated in
192 both treatments (Fig. 2a). For up-regulated genes, 113 genes were up-regulated in early-selected
193 parasites, compared to 52 in late-selected parasites, with 77 genes commonly up-regulated in both
194 groups (Fig. 2b). These findings suggest that a higher number of DE genes are driven by early-
195 selected parasites compared to late-selected ones, at least during the 10th day of host adulthood.
196 However, a notable overlap exists between both treatments (Fig. 2ab).
197
198 After categorizing the DE genes into their respective biological processes using Gene Ontology (GO)
199 categories, a heatmap was plotted to show the mean fold-change for each GO category across parasite
200 treatments (Fig. 2c). Most categories exhibited similar fold changes between early- and late-selected
201 parasites. However, three categories stood out as potential candidates for explaining differences
202 between the two selected treatments due to their distinct expression patterns and biological relevance:
203 response to reactive oxygen species (ROS), Golgi membrane, and immune system processes.
204
205 Genes with DE and their respective fold-change relative to the reference control were plotted for each
206 candidate GO category (Fig. 3). The Golgi membrane category had the highest number of DE genes
207 and showed the greatest variation between early- and late-selected parasites, followed by the immune
208 process and response to ROS categories. Fold-change differences between the selected treatments
209 were also compared. Only two genes showed significant differences: *Protein ERGIC-53* ($df = 1$, $F =$
210 7.69, $p = 0.02$; Golgi membrane) and *Toll-interacting protein* ($df = 1$, $F = 9.70$, $p = 0.01$; Immune
211 process). Both genes had a higher fold change in late-selected parasites than in early-selected ones.
212 However, while *Protein ERGIC-53* was up-regulated, *Toll-interacting protein* was down-regulated
213 (Fig. 3).
214

215 # Discussion

216 Our study highlights distinct transcriptional changes when infected with different lines of this parasite
217 (Fig. 1). The findings discussed below provide key insights into this host-parasite interaction and
218 might have consequences for this vector's competence.
219

220 ***1. Mosquito response to reference V. culicis infection***

221 When the host's transcriptomic response to infection was compared (Table 1), a large number of
222 differentially expressed (DE) genes were observed (see File S2 for the complete list of transcripts and
223 respective expression). Such a broad response has been reported in the mosquito *Aedes aegypti* when
224 infected with *V. culicis* [20]. From this response, only a few DE immune markers were identified,
225 with two being notably over-expressed: *Cecropin-B*, an antimicrobial peptide known for its fungicidal
226 properties *in vitro* [34, 35], and *Ninjurin-2*, a non-apoptotic cell death protein induced by the Toll
227 pathway and which plays a key role in the dipteran response to microbial infections [36, 37].
228
229 Significant over-expression of regeneration markers was also detected. For instance, *Annulin* was
230 found to be responsible for peritrophic matrix formation in the gut and early resilience to infection
231 [38], and *Obstructor-E*, a member of a multigene family, was shown to regulate insect cuticle
232 development [39]. Additionally, *Rab11*, essential for circulatory iron internalization, was over-
233 expressed [40], further validating previous findings on the role of iron during infection with *V. culicis*,
234 where iron sequestration by the parasite has been hypothesized [31].
235
236 An overall down-regulation of sensory genes was observed, including *Prestin*, which increases gut pH
237 [41] and *CIMAP1D*, involved in sound-sensory function [42]. *Arginine kinase 1*, known to participate
238 in ATP-to-ADP conversion and consequent energy release in certain bacteria [43], was found to be
239 severely down-regulated in infected mosquitoes (Table 1). Interestingly, most Mucin proteins were
240 up-regulated, except for *Mucin-6*, which was down-regulated. Mucin proteins are recognized for their
241 role in epithelial lubrification, immune modulation and inflammation [44, 45]. Notably, *Mucin-6* is
242 exclusive to the *A. gambiae* gut, which has a protective function against parasites such as *Plasmodium*
243 *spp* [46].
244
245 ***2. Mosquito response to selected lines of V. culicis***
246 Next, the focus is turned to infections caused by parasite lines that have undergone selection for early
247 or late transmission within *A. gambiae* [19] (Fig. 2 and 3). As expected, fewer genes were responsible
248 for differences between selected and unselected parasites, i.e., 299 for early-selected and 228 for late-
249 selected (Fig. 2ab), than unselected and uninfected, i.e., 1967 genes. Notably, a broader
250 transcriptional response was induced by early-selected parasites than by late-selected ones (when they
251 are compared to the reference control), as indicated by the high number of DE genes in both selection
252 treatments (Fig. 2ab). Despite this, a core set of genes was found to be commonly up- or down-
253 regulated across both parasite treatments (Fig. 2ab).
254
255 To better understand the differences in transcriptomic profiles from infections with selected parasites,
256 DE genes were categorized into biological processes using GO analysis (Fig. 2c). Most of the
257 differences between early- and late-selected parasites were grouped into three GO biological

258 processes: Golgi membrane, immune processes, and response to reactive oxygen species (ROS).
259 These findings are unsurprising, given the biological relevance of these categories for infection [47–
260 49], particularly from the host's perspective. Equally interestingly, the distinct host transcriptomic
261 profile observed in response to infection between the early and late selected parasites indicates that
262 selection did affect not only phenotypic traits, as shown in previous studies [19, 30, 31], but also
263 drove significant parasite evolution, causing a substantial shift in the host's response to the selected
264 parasites (Fig. 2 and 3).
265
266 The three GO categories were treated as candidate categories, along with their associated genes,
267 which are believed to play crucial roles in modulating host-parasite dynamics and therefore warrant
268 further study (Fig. 3). First, the Golgi membrane was considered, as it has recently gained attention
269 for its critical role in innate immune signalling, preceding other organelles such as mitochondria and
270 the endoplasmic reticulum (ER) during infection [47]. As a result, the apparent difference observed in
271 the host response to early- or late-selected parasite treatments was unsurprising (Fig. 3). The genes
272 associated with the Golgi membrane include coatomers, galactosyltransferases, and mannosidases,
273 which are involved in vesicle transport and carbohydrate metabolism with the ER. The richness and
274 differential expression of Golgi membrane-associated genes suggest that some upregulation of
275 effectors involved in glycosylation and trafficking processes in the Golgi apparatus occurs across both
276 parasite treatments (Fig. 3). One gene, *ERGIC-53*, stood out due to its significantly different fold-
277 change between host responses to infections by early-selected and late-selected parasites. The protein
278 encoded by *ERGIC-53* is involved in glycoprotein trafficking. Early transmission-selected parasites
279 seem to induce higher production of ERGIC-53 and, consequently, increased investment in
280 glycoprotein trafficking compared to late-selected parasites (Fig. 3). Although the precise role of
281 ERGIC-53 in the immune response of *A. gambiae* remains unclear, it can be hypothesized, based on
282 existing literature, that glycoproteins (e.g., peptidoglycan recognition proteins or PGRPs) serve as
283 essential information markers for the host, vital for accurate recognition and elimination of parasites
284 [50, 51].
285
286 Second, variation in the expression of immune-related genes was analyzed, including *peptidoglycan-*
287 *recognition proteins* (PGRPs), *CLIP domain serine proteases* and *cecropins*, among others (Fig. 3). A
288 notable difference was observed between the host response to early- and late-selected parasite
289 treatments, with many of the DE immune related-genes being downregulated upon infection with late-
290 selected parasites (Fig. 3). Interestingly, both *cecropin-B* and *cecropin-C* were overexpressed in
291 response to early-selected parasites compared to the unselected reference, suggesting a more robust
292 antimicrobial response. However, this pattern was not observed in response to late-selected parasites,
293 but rather the opposite. In particular, *CLIP domain serine proteases* have been associated with
294 mosquito late resistance to *Plasmodium* via melanisation [52, 53] (Table 1, Fig. 3). In our study, we

295 noticed all lines of *V. culicis* induced an up-regulation of these immune effectors, suggesting that
296 infection by this microsporidian might elicit an immune response that has collateral damage to
297 *Plasmodium* development.
298
299 Although it was hypothesized that the immune response to late-selected parasites would be stronger
300 due to their higher virulence, time and replication within the host, the data suggests otherwise. Two
301 hypotheses are proposed to explain this finding. The first hypothesis involves the recognition of *V.*
302 *culicis* as a virulent parasite. As discussed in the manuscript, early-selected parasites induce a larger
303 Golgi membrane response, potentially leading to better recognition of PGRPs than infection by late-
304 selected parasites. This could explain the rapid up-regulation of immune markers in response to early-
305 selected parasites. The second hypothesis concerns the balance between the costs and benefits of the
306 immune response and its association with the presence/absence of damage-associated molecular
307 patterns (DAMPs) [54]. As with any other life history trait, investment in immunity is expected to
308 trade off with other physiological traits [55]. As suggested by Rolff and Lazzaro (2012), the type of
309 intensity of the immune response may result from the recognition of a combination of molecules,
310 alerting the host to the parasite (PGRPs) and/or to the damage inflicted by the infection (DAMPs). If
311 so, infection by the more virulent late-selected parasite could lead to a more costly and damaging
312 infection, potentially overwhelming the host. In this scenario, the host may prioritize reproductive
313 fitness over survival, which aligns with previous observations of increased reproductive investment
314 during infection by late-selected parasites [19]. This phenomenon has been documented in various
315 infections as predicted by life history theory [55]. In extreme cases, the investment shift to fecundity
316 can be lifelong due to a shortening of the host's survival. In this specific case, we refer to this
317 phenomenon as terminal investment, which may occur when a host invests its resources into fecundity
318 due to an inability to survive the infection [56–58]. It is unclear if this shift in fecundity and immunity
319 is temporary or observed throughout the host life, given that both fecundity and RNA-seq were
320 performed for the same infection time point and did not represent the whole infection process. It is
321 noteworthy to say the individuals in this study were not blood-fed, necessary for their reproduction.
322 Although we cannot confirm there is a trade-off with fecundity or other life history traits, we are
323 aware *A. gambiae* invests in its first clutch of eggs before having a blood meal, which would represent
324 at least a partial investment in fecundity. Moreover, from this category, the *Toll-interacting protein*
325 was found to have significantly different fold changes between the infection by each of the two
326 selection parasite treatments (Fig. 3). This gene, which produces a protein important for parasite
327 recognition [59–62], was slightly more up-regulated in response to early-selected parasites than to
328 late-selected ones (Fig. 3). This supports the idea that early-selected parasites may be more
329 recognizable by the host's immune system. However, the extent to which parasite selection has
330 affected the presence or absence of specific PGRPs that enable host recognition remains unclear.

331 Nonetheless, it is reasonable to assume that late-selected parasites might have evolved higher levels of
332 immune evasion within *A. gambiae*.
333
334 Third, only a few genes were found to be DE in response to ROS, showing mixed expression patterns
335 (Fig. 3). Overall, the early-selected parasites elicited a stronger host response to ROS than late-
336 selected ones, suggesting higher oxidative stress within the host. This is likely due to a heightened
337 immune response to this infection treatment.
338

339 **Conclusions**

340 In conclusion, the transcriptional response of *A. gambiae* to infection by *V. culicis* has revealed
341 curious and significant insights into this host-parasite interaction and its evolutionary history.
342 Moreover, the findings in this study further explain many of the results measured in previous studies
343 concerning this particular *V. culicis* selection lines for early and late transmission [19, 30, 31].
344 Differential expression of genes related to immune response, Golgi membrane function, and oxidative
345 stress were observed, with distinct patterns emerging between infections with reference to unselected,
346 early-selected and late-selected parasite lines. Overall, early-selected parasites elicited a broader and
347 more intense immune response, characterized by the upregulation of immune-related genes such as
348 cecropin and PGRPs and enhanced glycoprotein trafficking through the Golgi apparatus. In contrast,
349 late-selected parasites appeared to trigger a weaker immune response and, therefore, potentially
350 evolved immune evasion mechanisms.
351
352 Altogether, the results of this study demonstrate that selection pressures on *V. culicis* not only
353 influence its phenotypic traits but also drive significant changes in the host gene expression, revealing
354 complex host-parasite co-evolutionary dynamics. Further investigation is needed to elucidate, confirm
355 and quantify the specific molecular mechanisms involved, particularly in relation to immune evasion
356 strategies and the balance between host survival and reproductive fitness. Additionally, it is important
357 to position this study within the context of the microsporidian's ability to interfere with *Plasmodium*
358 development, as previously mentioned. Vector-borne parasites, such as *Plasmodium spp.*, transmitted
359 by this host, have long transmission cycles within their vectors. It is reasonable to expect that co-
360 infection of Plasmodium spp. and microsporidia may inadvertently select for late-transmitted
361 microsporidian parasites, which could have significant consequences for the spread of both parasites
362 within a population. Although the mechanism by which microsporidians suppress *Plasmodium*
363 development remains unclear, our study demonstrated that melanisation-based immunity is
364 upregulated, which might facilitate late collateral melanisation of *Plasmodium* oocysts. Nevertheless,
365 this hypothesis is still up for testing, as beside *CLIP proteases*, we observed a limited immune
366 response that could impact *Plasmodium spp.* and hence, resource competition between the parasites

367 might be a main source of this inhibitory relationship too. Moreover, the lack of immunity towards
368 late-transmitted *V. culicis* might highlight the potential of this parasite as a biological vector-control
369 as it suggests selection to reduce its prevalence in a population is low. Further research is required to
370 understand this interaction fully, but our study stands as a foundation for them. These findings
371 provide valuable insights that could inform future vector control strategies, particularly in the context
372 of the evolutionary pressures exerted by parasites on mosquito populations and the diseases they
373 vector.
374

375 # Materials and Methods

376 ***1. Experimental model***

377 The Kisumu strain of the mosquito host *Anopheles gambiae (s.s.)* and evolved lines of the
378 microsporidian parasite *Vavraia culicis floridensis* were used for these experiments. These parasite
379 lines had previously been selected for seven generations for: i) early transmission and shorter time
380 within the host; or for ii) late transmission and longer time within the host; or iii) they come from an
381 unselected reference, our laboratory stock [19]. Further details on the selection experiment and its
382 maintenance are described in Silva and Koella (2024). All experiments and rearing were conducted
383 under standard laboratory conditions (26 ± 1ºC, 70 ± 5% relative humidity, and 12 h light/dark).
384

385 ***2. Samples collection***

386 Freshly hatched *A. gambiae* larvae were reared in groups of 50 individuals per petri dish (120 mm
387 diameter × 17 mm) containing 80 ml of distilled water. Larvae were fed daily with Tetramin Baby
388 fish food according to their age: 0.04, 0.06, 0.08, 0.16, 0.32, and 0.6 mg/larva for 0, 1, 2, 3, 4, and 5
389 days or older, respectively. On the second day of larval development, individuals were exposed to
390 10,000 spores per larva of their respective *V. culicis* treatment, or not exposed in the case of the
391 uninfected treatment. At pupation, individuals were transferred into 50 ml falcon tubes with
392 approximately 10 ml of distilled water and were allowed to emerge as adults. Female mosquitoes
393 were transferred to individual cups with humid filter paper and given a constant 6% sucrose solution
394 until death. On day 10 of adulthood, all live female mosquitoes were collected, snap-frozen in liquid
395 nitrogen, and stored at -80ºC until RNA extraction. Transcriptomic profiles were assessed at day 10 of
396 adulthood, as previous studies on these lines have shown this to be when all hosts produce infective
397 spores [19]. Thus, a stronger host response and more pronounced signatures of parasite selection were
398 anticipated for this time point.
399

400 ***3. RNA extractions***

401 RNA extractions were performed using Qiagen's RNAeasy Universal Plus kit following the provided
402 protocol. Before beginning the protocol, ten female mosquitoes per treatment were pooled in a 1.5 mL

403 microcentrifuge tube with 1000 µl of Qiazol reagent (from the RNAeasy Universal Plus kit by
404 Qiagen) briefly mixed, and two stainless steel beads (Ø 2 mm) were added. Each tube was
405 homogenized using a Qiagen TissueLyser LT at a frequency of 30 Hz for three minutes. The Qiagen
406 kit protocol was then followed, resulting in a 50 µl eluted solution of RNA in nuclease-free water at a
407 concentration of approximately 300 ng/µl for each treatment. RNA samples were standardized to 10
408 ng/µl and delivered on dry ice to the Genomic Technologies Facility (University of Lausanne,
409 Switzerland) for further processing, as described below.
410
411 ***4. RNA sequencing***
412 RNA quality was assessed using a Fragment analyzer (Agilent Technologies). The poly-A tails and
413 adapters were removed from single-end reads, and quality was trimmed with Cutadapt version 4.8
414 [63]. Reads matching ribosomal RNA sequences were removed with fastq_screen version 0.11.1 [64].
415 Reads longer than 40nt were aligned against the concatenated genomes of *Anopheles gambiae* (NCBI:
416 GCA_943734735.2) and *Vavraia culicis floridensis* (NCBI: GCA_000192795.1) using STAR version
417 2.7.10a [65]. The number of read counts per gene locus was summarized with htseq-count version
418 0.11.2 [66] using gene annotation. RNA-seq libraries were prepared from 250 ng of total RNA with
419 the Illumina Stranded mRNA Prep reagents (Illumina) using a unique dual indexing strategy and
420 following the official protocol, which was automated on a Sciclone liquid handling robot
421 (PerkinElmer). Libraries were quantified by a fluorometric method (QubIT, Life Technologies), and
422 their quality was assessed on a Fragment Analyzer (Agilent Technologies). Sequencing was
423 performed on an Illumina NovaSeq 6000 for 150 cycles single read. Sequencing data were
424 demultiplexed using the bcl2fastq2 Conversion Software (version 2.20, Illumina).
425
426 ***5. Statistical analysis***
427 All the analyses were performed with R version 4.3.0. in RStudio version 3034.06.2+561. The
428 following packages were used for the statistical analysis and diagnosis, and for visualising and
429 plotting the data: "Glimma" [67], "cowplot" [68], "ggrepel" [69], "RColorBrewer" [70], "maptools"
430 [71], "sp" [72], "edgeR" [73], "MASS" [74], "limma" [75], "knitr" [76], "ggplot2" [77] and "dplyr"
431 [78]. Figure panels were created and edited with BioRender.com and Microsoft PowerPoint 16.16.27.
432
433 The high quality of the RNA-seq data alignment was verified using RSeQC version 5.0.1 [79].
434 Alignment rates to both genomes exceed 93.5% for all samples (SI Fig. S1a). A very few number of
435 reads aligned to *V. culicis*, ranging from 127 to 5,393 (SI Fig. S1b). *A. gambiae* samples exhibit good
436 sequencing depth (SI Fig. S1c) and diverse gene representation. The gene body coverage is biased
437 towards the 3' end, suggesting partial RNA degradation, but this pattern is consistent across all
438 samples.
439

Genes with low counts were filtered out according to the rule of 3 count(s) per million (cpm) in at least 4 sample(s). Library sizes were scaled using TMM normalization and log-transformed into counts per million or CPM (EdgeR package version 3.42.4) [73]. Statistical quality controls were performed through density gene expression distribution, clustering, and sample PCA (SI Fig. S2). Differential expression was computed with limma-trend approach [75] by fitting all samples into one linear model. Next, the following group of comparisons were performed:

- Infected with unselected reference *vs.* uninfected (1 comparison).
- Infected with selected parasite *vs.* infected with unselected reference (10 comparisons).

A moderated t-test was used for each contrast. The Benjamini-Hochberg method computed an adjusted p-value for each comparison, controlling for false discovery rate (FDR, Adj. p). Then, a host gene ontology (GO, biological processes) enrichment analysis was carried out on differentially expressed (DE) genes through Panther 19.0 [illegible] and "vectorbase.org" using the A. gambiae genome as a reference (Fig. 3 and 4).

Afterwards, a candidate gene analysis was performed (Fig. 4) taking into account the differently expressed biological processes in selected parasites, comparatively to the unselected reference (Fig. 3). For each DE transcript of the GO:0000139 (Golgi membrane), GO:0000302 (Response to reactive oxygen species) and GO:0002376 (Immune system process) classes, a linear model was run with fold-change as a dependent variable and parasite treatment as an explanatory variable. These comparisons allow us to test for differential fold change (compared to the unselected reference) for early- and late-selected parasites.

Author contributions

LMS designed and conducted the experiments, analyzed the data and wrote the manuscript.

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Competing interests

The author declare that they have no competing interests.

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657 ## Supplementary information

658 **Figure S1.** Host RNA-sequencing and parasite gene counts.

659 **Figure S2.** Quality control clustering and PCAs.

660 **File S1.** Transcript expression and statistical contrasts for reference infection *vs.* no infection.

661 **File S2.** Transcript expression and statistical contrasts for early-selected (selection replicate 1)
662 infection *vs.* reference infection.

663 **File S3.** Transcript expression and statistical contrasts for early-selected (selection replicate 2)
664 infection *vs.* reference infection.

665 **File S4.** Transcript expression and statistical contrasts for early-selected (selection replicate 3)
666 infection *vs.* reference infection.

667 **File S5.** Transcript expression and statistical contrasts for early-selected (selection replicate 4)
668 infection *vs.* reference infection.

669 **File S6.** Transcript expression and statistical contrasts for early-selected (selection replicate 5)
670 infection *vs.* reference infection.

671 **File S7.** Transcript expression and statistical contrasts for late-selected (selection replicate 1) infection
672 *vs.* reference infection.

673 **File S8.** Transcript expression and statistical contrasts for late-selected (selection replicate 2) infection
674 *vs.* reference infection.

675 **File S9.** Transcript expression and statistical contrasts for late-selected (selection replicate 3) infection
676 *vs.* reference infection.

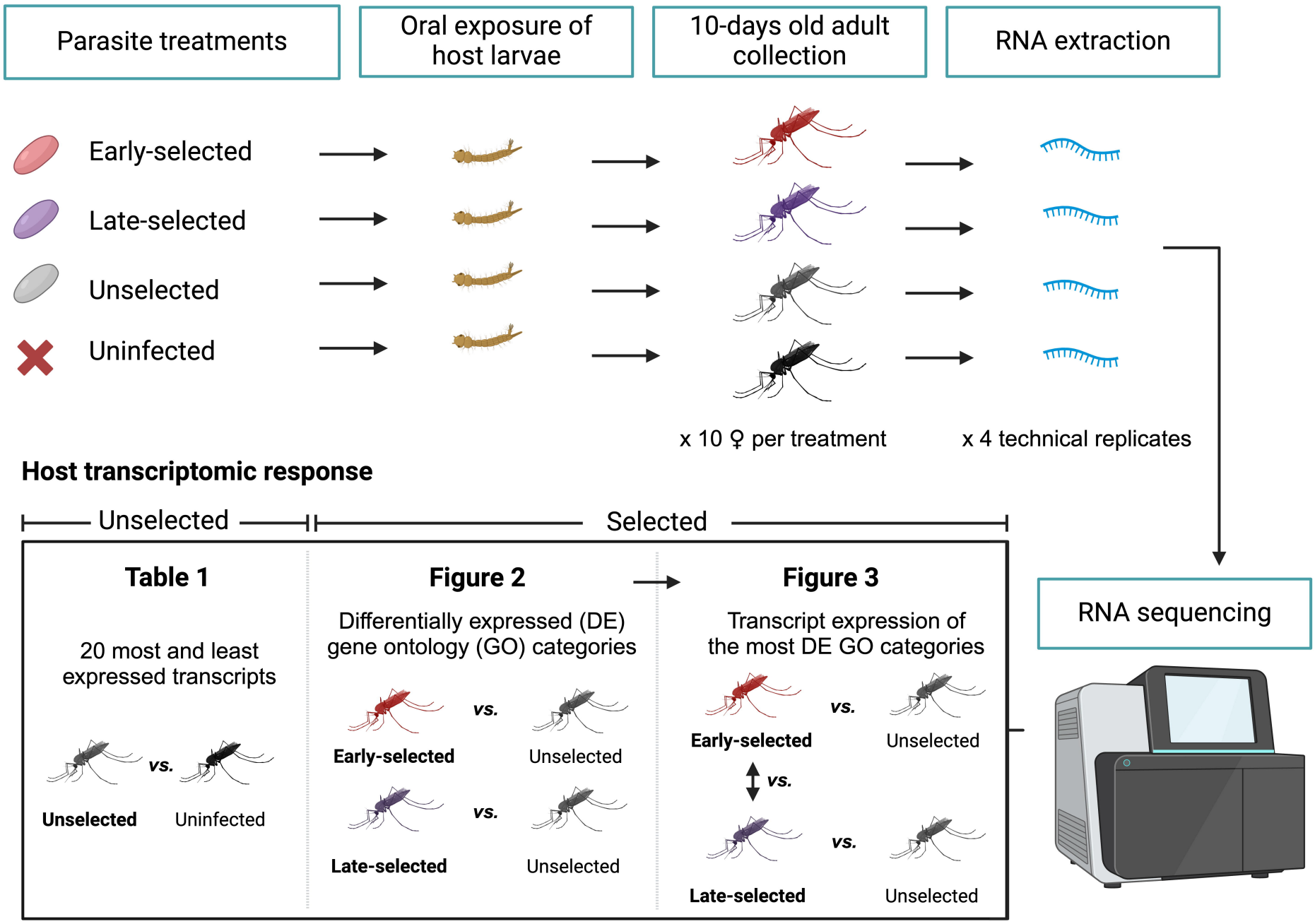
677 **File S10.** Transcript expression and statistical contrasts for late-selected (selection replicate 4)
678 infection *vs.* reference infection.

679 **File S11.** Transcript expression and statistical contrasts for late-selected (selection replicate 5)
680 infection *vs.* reference infection.

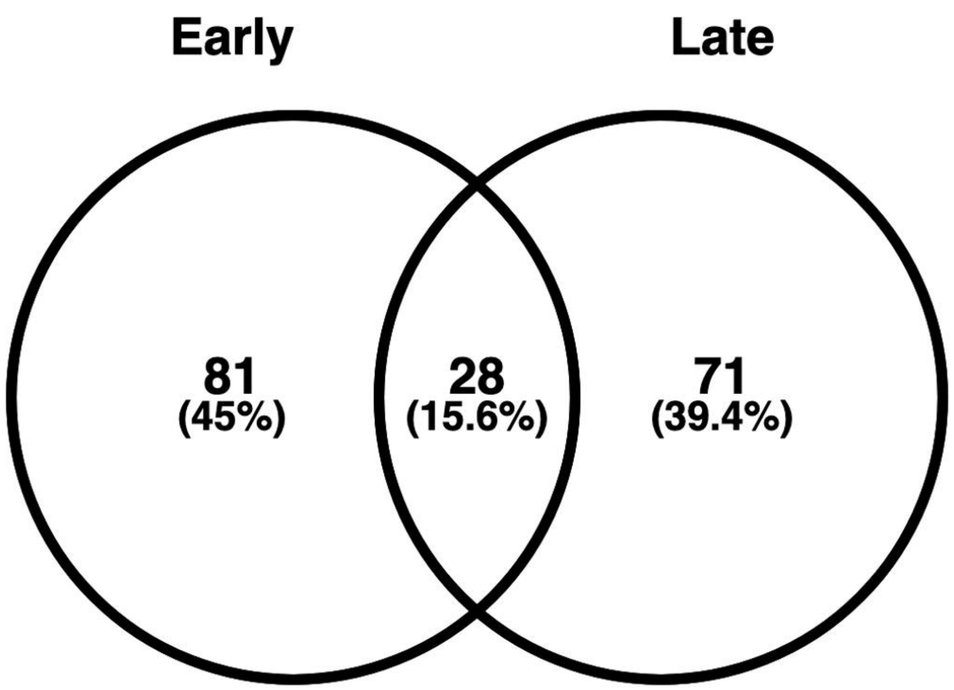
681

682

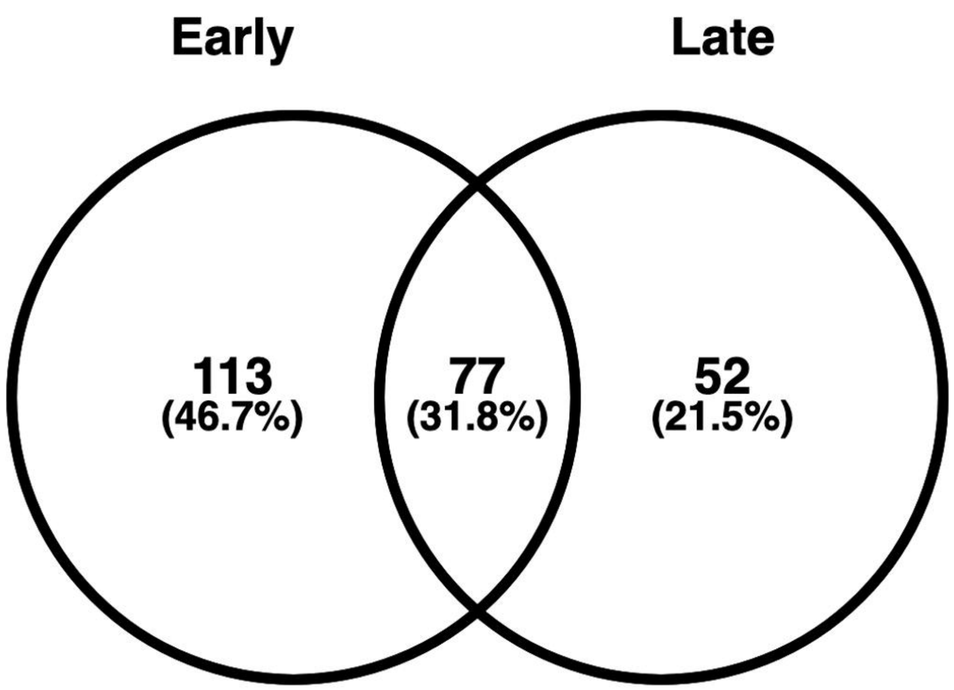
683


Parasite treatments
Oral exposure of host larvae
10-days old adult collection
RNA extraction
Early-selected
Late-selected
Unselected
Uninfected
x 10 ♀ per treatment
x 4 technical replicates
Host transcriptomic response
Unselected
Selected
Table 1
20 most and least expressed transcripts
vs.
Unselected
Uninfected
Figure 2
Differentially expressed (DE) gene ontology (GO) categories
vs.
Early-selected
Unselected
vs.
Late-selected
Unselected
Figure 3
Transcript expression of the most DE GO categories
vs.
Early-selected
Unselected
vs.
vs.
Late-selected
Unselected
RNA sequencing

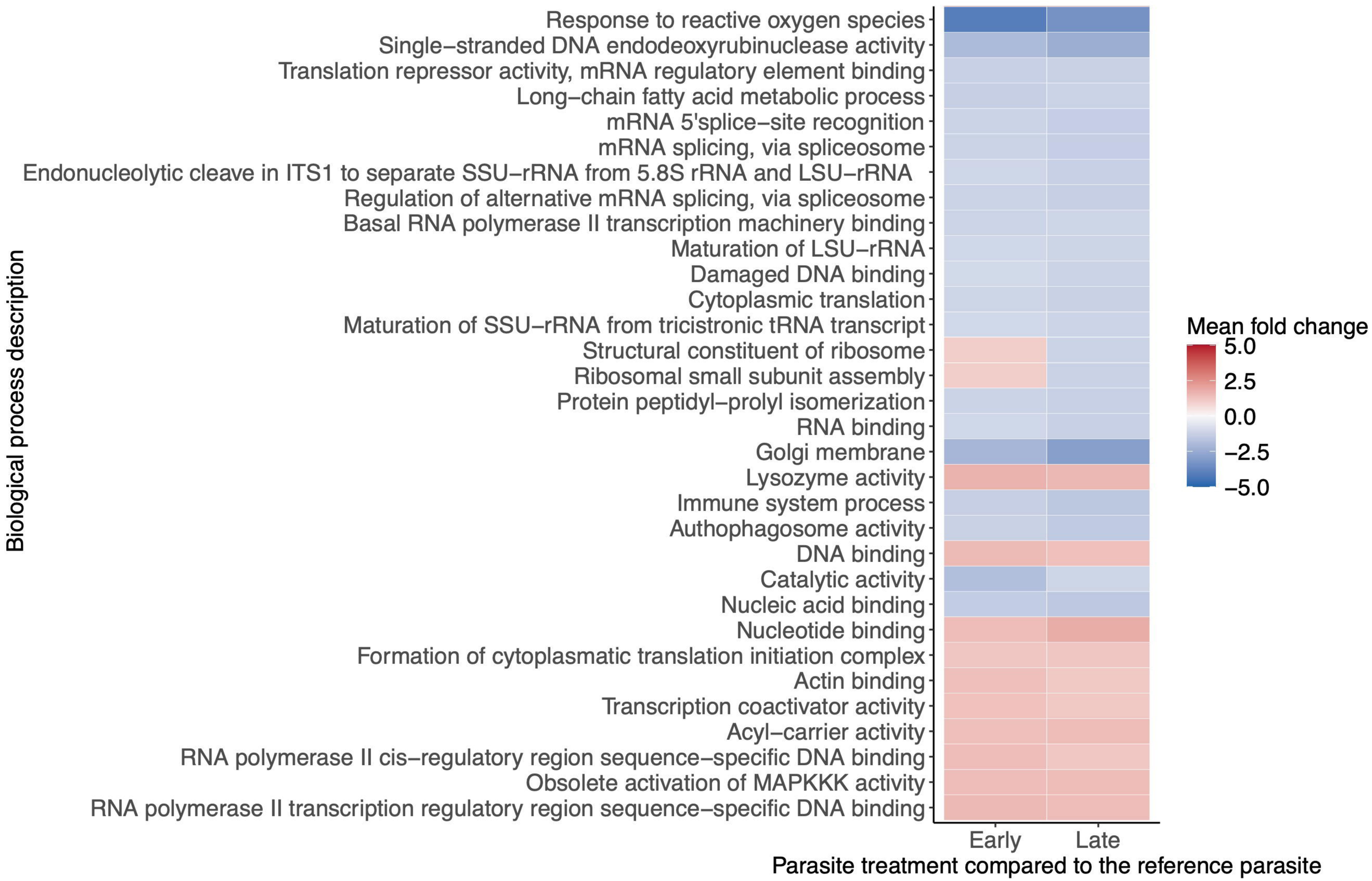
a


Negative fold change
Early
Late
81
(45%)
28
(15.6%)
71
(39.4%)

b


Positive fold change
Early
Late
113
(46.7%)
77
(31.8%)
52
(21.5%)

c


Response to reactive oxygen species
Single−stranded DNA endodeoxyrubinuclease activity
Translation repressor activity, mRNA regulatory element binding
Long−chain fatty acid metabolic process
mRNA 5'splice−site recognition
mRNA splicing, via spliceosome
Endonucleolytic cleave in ITS1 to separate SSU−rRNA from 5.8S rRNA and LSU−rRNA
Regulation of alternative mRNA splicing, via spliceosome
Basal RNA polymerase II transcription machinery binding
Maturation of LSU−rRNA
Damaged DNA binding
Cytoplasmic translation
Maturation of SSU−rRNA from tricistronic tRNA transcript
Structural constituent of ribosome
Ribosomal small subunit assembly
Protein peptidyl−prolyl isomerization
RNA binding
Golgi membrane
Lysozyme activity
Immune system process
Authophagosome activity
DNA binding
Catalytic activity
Nucleic acid binding
Nucleotide binding
Formation of cytoplasmatic translation initiation complex
Actin binding
Transcription coactivator activity
Acyl−carrier activity
RNA polymerase II cis−regulatory region sequence−specific DNA binding
Obsolete activation of MAPKKK activity
RNA polymerase II transcription regulatory region sequence−specific DNA binding
Biological process description
Early
Late
Parasite treatment compared to the reference parasite
Mean fold change
5.0
2.5
0.0
−2.5
−5.0

Mean fold change
6
3
0
-3
-6
4-hydroxyphenylpyruvate dioxygenase
ADP-ribosylation factor GTPase-activating protein 3
Alpha-mannosidase 2 transcript 2
Alpha-mannosidase 2 transcript 1
ATP-binding cassette sub-family B member 6
Beta-1,3-galactosyltransferase 5
Beta-1,3-galactosyltransferase brn
Calsyntenin-1
Carbohydrate sulfotransferase 11
Coatomer subunit alpha
Coatomer subunit beta
Coatomer subunit beta'
Coatomer subunit zeta-1
Conserved oligomeric Golgi complex subunit 6
Galactosylgalactosylxylosylprotein 3-beta-glucuronosyltransferase P
Golgi phosphoprotein 3 homolog sauron
Golgin-45
Mannosyl-oligosaccharide alpha-1,2-mannosidase IA
N-acetylgalactosaminyltransferase 7
Polypeptide N-acetylgalactosaminyltransferase 1
Polypeptide N-acetylgalactosaminyltransferase 35A
Programmed cell death protein 10
Protein hedgehog
Protein ERGIC-53
Protein transport protein Sec23A
Ras-related protein Rab-2
Solute carrier family 35 member F6
Sugar transporter SWEET1 transcript 1
Sugar transporter SWEET1 transcript 2
Synaptobrevin homolog YKT6
Cecropin-B
Cecropin-C
CLIP domain-containing serine protease B10
CLIP domain-containing serine protease B14
CLIP domain-containing serine protease B15
NAD(+) hydrolase sarm1
Peptidoglycan-recognition protein 2
Peptidoglycan-recognition protein LB
Peptidoglycan-recognition protein LC
Peptidoglycan-recognition protein SB2
Phagocyte signaling-impaired protein
Serine protease inhibitor 2
Toll-interacting protein
Uncharacterized LOC1273740
Apolipoprotein D transcript 1
Apolipoprotein D trasncript 2
Uncharacterized LOC11175951
Gene name
*
*
Golgi membrane
Immune process
Response to ROS
Early
Late
Parasite treatment