

1 The Transcriptome of the Avian Malaria Parasite 2 *Plasmodium ashfordi* Displays Host-Specific Gene 3 Expression

8 **Running title**

9 The Transcriptome of *Plasmodium ashfordi*

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25 Abstract

26
27 Malaria parasites (*Plasmodium* spp.) include some of the world's most widespread and virulent
28 pathogens, infecting a wide array of vertebrates. Our knowledge of the molecular mechanisms these
29 parasites use to invade and exploit hosts other than mice and primates is, however, extremely limited.
30 How do *Plasmodium* adapt to individual hosts and to the immune response of hosts throughout an
31 infection? To better understand parasite plasticity, and identify genes that are conserved across the
32 phylogeny, it is imperative that we characterize transcriptome-wide gene expression from non-model
33 malaria parasites in multiple host individuals. Here, we used high-throughput Illumina RNA-
34 sequencing on blood from wild-caught Eurasian siskins experimentally infected with a clonal strain of
35 the avian malaria parasite, *Plasmodium ashfordi* (lineage GRW2). By using a multi-step approach to
36 filter out all host transcripts, we successfully assembled the blood-stage transcriptome of *P. ashfordi*.
37 A total of 11 954 expressed parasite transcripts were identified, and 7 860 were annotated with protein
38 information. We further quantified gene expression levels of all parasite transcripts across three hosts
39 during two infection stages – peak and decreasing parasitemia. Interestingly, parasites from the same
40 host during different infection stages displayed remarkably similar expression profiles, but show large
41 differences across hosts. This indicates that *P. ashfordi* adjusts its gene expression to specific host
42 individuals, but contrary to expectation does not markedly change expression across different stages
43 of infection. Finally, we examined genome-wide sequence similarity between *P. ashfordi* and other
44 apicomplexan species, and searched for candidate genes involved in red blood cell invasion. The
45 majority of transcripts were most similar to the human parasite *Plasmodium falciparum*, and a large
46 number of invasion genes were discovered, suggesting conserved red blood cell invasion strategies
47 between mammalian and avian *Plasmodium* spp. The transcriptome of *P. ashfordi* and its host-
48 specific gene expression over two infection stages advances our understanding of *Plasmodium*
49 plasticity and will become a valuable resource as it allows for further studies analyzing gene evolution
50 and comparisons of parasite gene expression.

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54 Keywords

55 *Plasmodium*, transcriptome, RNA-seq, malaria, gene expression, host-parasite interaction

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58 Introduction

59

60 The apicomplexan parasites of the genus *Plasmodium* (malaria parasites) encompass a worldwide
61 distribution and infect a multitude of vertebrate hosts, including reptiles, birds and mammals
62 (Garnham, 1966). Their virulence can be highly variable between different strains and species. Some
63 induce mild pathogenic effects on hosts and some cause severe disease, leading to high mortality rates
64 (Palinauskas et al., 2008). Host individuals and host species also differ in their resistance and
65 tolerance to malaria, and this interaction between host and parasite ultimately determines disease
66 severity. Furthermore, the molecular response of hosts changes during the course of infection and
67 creates a dynamic environment in which the parasites need to accommodate. Nevertheless, our
68 understanding of how malaria parasites respond molecularly to different host individuals and to
69 changes in the host immune defense over time is very limited.

70

71 Parasites of two clades in *Plasmodium* have been extensively studied from a molecular perspective,
72 murine and primate parasites. We have learned a great deal of how malaria parasites of humans
73 evolved and function by studying their rodent-infecting relatives. The majority of studies investigating
74 gene expression in malaria parasites have been conducted using artificially selected cell lines (*in*
75 *vitro*) or tissue cultures (*ex vivo*). This has provided tremendous insight into the biology of
76 *Plasmodium* life stages (e.g. Bozdech et al., 2003; Hall et al., 2005; Otto et al., 2010; Siegel et al.,
77 2014). However, major discrepancies in parasite expression between cultures and live animals (*in*
78 *vivo*) have been documented (Lapp et al., 2015). A wide range of host environmental factors are
79 absent in the *in vitro* systems, for example temperature fluctuations, inflammatory and immune
80 effector molecules, hormones, metabolites, microenvironments, and varying levels of oxygen, pH, and
81 glucose (LeRoux et al., 2009). Parasites cultured outside hosts reflect this with different expression
82 patterns, and markedly downregulate important vaccine candidate genes such as cell surface antigens
83 (Daily et al., 2005; Siau et al., 2008). The natural host environment, including genotypic and
84 immunological cues, may therefore strongly affect the transcriptional responses of malaria parasites.
85 To obtain representative transcriptional information from *Plasmodium* parasites, it is therefore
86 valuable to utilize a system of natural host animals.

87

88 Alas, almost nothing is known about underlying molecular mechanisms in malaria parasites of hosts
89 other than mice and primates. Which genes are conserved across *Plasmodium* and how do virulence,
90 immune evasion, and host-specificity vary in species infecting non-mammalian animals? In order to
91 investigate these questions, it will be necessary to assemble and characterize genome-wide expression
92 information from malaria parasites throughout their phylogenetic host range. With the recent
93 development of high-throughput sequencing techniques, it has now become possible to generate
94 genomic sequences of non-model parasites (Martinsen and Perkins, 2013). Dual RNA-sequencing of
95 both host and parasite opens up fantastic possibilities of simultaneously studying host-parasite
96 interactions and describing transcriptome expression in both actors. Great care must be taken,
97 however, when assembling parasite sequences *de novo*. Transcripts from the host and/or other sources
98 of contamination may remain even after annotation and blast searches have taken place. Meticulous

99 filtering of assemblies using bioinformatics is therefore crucial to avoid erroneous conclusions (see
100 e.g. Koutsovoulos et al. 2016). After constructing filtered high-quality parasite transcriptome data, we
101 can start to investigate the aforementioned questions about *Plasmodium*.

102
103 In this study, we sequenced and assembled the blood transcriptome of the avian malaria parasite
104 *Plasmodium ashfordi*. Malaria parasites infecting birds are highly suitable for studying transcriptional
105 parasite responses due to their enormous diversity and large variation in host specificity and virulence
106 (Bensch et al., 2004; Križanauskienė et al., 2006; Lachish et al., 2011). Some avian *Plasmodium* are
107 extreme host generalists, successfully infecting birds over several orders, while other parasites are
108 host specialists infecting one species (Drovetski et al., 2014; Pérez-Tris et al., 2007). The avian
109 malaria system allows for the possibility of capturing wild birds in a natural setting, and evaluate their
110 status of malaria infection. Additionally, infection experiments have in the last couple of years
111 showed great potential to study *Plasmodium* in passerines under controlled conditions in laboratories
112 (Cornet et al., 2014; Dimitrov et al., 2015; Ellis et al., 2015; Palinauskas et al., 2008, 2011;
113 Zehtindjiev et al., 2008). We used our assembly of *P. ashfordi* to evaluate transcriptome
114 characteristics, genome-wide sequence similarity, and investigate genes known to be involved in the
115 *Plasmodium* red blood cell invasion process. We analyzed expression levels of parasite genes in three
116 experimentally infected birds during both peak and decreasing parasitemia, two infection stages where
117 the hosts exhibit different transcriptome responses (Videvall et al., 2015). This allowed us for the first
118 time to follow and describe the dynamic transcriptome of an avian malaria parasite over time in
119 individual hosts.

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121

122

123 **Results**

124

125 **The *Plasmodium ashfordi* transcriptome assembly**

126 We sequenced blood collected from three experimentally infected Eurasian siskins (*Carduelis spinus*)
127 during peak and decreasing parasitemia levels with Illumina dual RNA-sequencing (see Methods for
128 details). The transcriptome of *P. ashfordi* was assembled into two versions in order to make it
129 transparent and as useful as possible for other researchers. The first assembly version, which we refer
130 to as the annotated assembly, contains the transcripts with annotation information from apicomplexa
131 proteins (n = 7 860) (Figure 1; Supplementary Table S1). The second version that we refer to as the
132 total assembly, contains the unannotated contigs (n = 4 094) that were strictly filtered to remove
133 contigs from the avian host (Figure 1) together with the annotated contigs, resulting in a total of 11
134 954 representative transcripts (Table 1).

135 The size of the total transcriptome assembly is 9.0 Mbp and the annotated version of the
136 assembly comprise 7.3 Mbp (81.20%) (Table 1). We calculated assembly statistics using the
137 transcriptome-specific measurement E90N50, which represents the contig N50 value based on the set
138 of transcripts representing 90% of the expression data, and is preferable over the original N50
139 measurement when evaluating transcriptome assemblies (Haas, 2016). The assembly E90N50 is 1 988
140 bp and the mean transcript length of the annotated transcripts is 930.8 bp (Table 1, Supplementary
141 Figure S1). In comparison, the length of coding sequences in the *Haemoproteus tartakovskyi* (sister
142 parasite to *Plasmodium*) genome is mean = 1 206 and median = 1 809 bp (Bensch et al., 2016). The *P.*
143 *falciparum* transcriptome contains transcripts of median length 1 320 and mean length 2 197 bp
144 (Gardner et al., 2002). The longest contig in the *P. ashfordi* assembly, consisting of 26 773 bp, is
145 transcribed from the extremely long ubiquitin transferase gene (AK88_05171), which has a transcript
146 length around 27 400 bp in other *Plasmodium* species. The annotated transcriptome has an
147 exceptionally low mean GC content of 21.22% (Figure 1B), which is even lower than the already GC-
148 biased transcriptome of *P. falciparum* (23.80%).

149

150 **Biological functions of genes expressed in *P. ashfordi***

151 To evaluate biological and molecular functions of all expressed transcripts in *P. ashfordi*, we analysed
152 gene ontology annotation. Genes were found to primarily belong to the two major molecular groups:
153 binding and catalytic activity, as well as the biological functions: metabolic and cellular processes
154 (Supplementary Figure S5). To further investigate the metabolic processes of *P. ashfordi* in detail, a
155 subsequent analysis of metabolic pathway enrichment compared to *P. falciparum* yielded 19
156 significant pathways after correcting for multiple testing with the Benjamini and Hochberg false
157 discovery rate (q-value < 0.1). Glycolysis, purine metabolism, and methane metabolism were among
158 the top significant metabolic pathways (Figure 2). An investigation of annotated genes belonging to
159 the gene ontology category “kinase activity” resulted in a total of 95 genes (Supplementary Table S2).

160

161 **Gene expression is similar across different stages of infection**

162 The *P. ashfordi* transcripts were next analyzed for expression levels within individual hosts across the
163 two parasitemia stages. We accounted for differences in parasitemia levels between hosts and time
164 points, and any variation in sequencing depth between samples, by normalizing overall expression

165 values according to the DESeq method (Anders and Huber, 2010). We found that the parasites
166 displayed very similar gene expression patterns during peak and decreasing parasitemia stages (Figure
167 3). No genes were significantly differentially expressed between the time points (q-value > 0.99), and
168 the correlation in gene expression was extremely high (Pearson's product-moment correlation =
169 0.9983, $t = 1\ 905.2$, $df = 11\ 952$, p-value < 2.2e-16) (Figure 3A; Supplementary Table S3). Annotated
170 transcripts showing the highest expression fold change (non-significantly) between the two
171 parasitemia stages were derived from the following genes (in order of most observed change): rho-
172 GTPase-activating protein 1, 40S ribosomal protein S3a, two uncharacterized proteins, TATA-box-
173 binding protein, heat shock protein 90, and C50 peptidase (Supplementary Table S1, Supplementary
174 Figure S2).

175

176 **Gene expression is host-specific**

177 In contrast to the similarities in gene expression between parasitemia stages, the parasite
178 transcriptomes showed much larger differences in expression levels between the different host
179 individuals. A principal component analysis of expression variances clustered parasite samples
180 together within their respective hosts, which showed major similarities in expression profiles (Figure
181 3C). Samples derived from the same host individual did not separate until the third (15% variance)
182 and fourth (13% variance) principal component dimensions (Figure 3D). The parasite transcriptome
183 from host 4 during decreasing parasitemia showed the largest variation in parasite gene expression
184 among all samples, yet it was still most similar to the transcriptome from the same host during peak
185 parasitemia (Figure 3B; Figure 3C). In fact, all parasite transcriptomes during the decreasing
186 parasitemia stage demonstrated closest distance to the transcriptome sample within the same host ten
187 days earlier (Figure 3B).

188 To further evaluate if specific transcripts contributed to differences in parasite gene
189 expression levels between individual hosts, we performed a likelihood ratio test over all host
190 individuals while controlling for parasitemia stage. This resulted in 28 significant *P. ashfordi*
191 transcripts (q-value < 0.1) displaying very high expression variation between hosts (Figure 4;
192 Supplementary Table S4). The most significant transcripts were derived from the genes (in order of
193 most significance) cytochrome c oxidase subunit 1, 70 kd heat shock-like protein, M1 family
194 aminopeptidase, and metabolite/drug transporter (Supplementary Table S4).

195

196 **Transcriptome sequence similarities to other apicomplexan parasites**

197 Almost all annotated contigs (99.59%; n = 7 828) resulted in a best blast hit against a species within
198 the genus *Plasmodium* (Figure 5A). The remaining contigs had matches against species within the
199 genera of *Eimeria* (n = 12), *Cryptosporidium* (n = 6), *Neospora* (n = 5), *Babesia* (n = 4), *Hammondia*
200 (n = 2), *Ascogregarina* (n = 1), *Theileria* (n = 1), and *Toxoplasma* (n = 1) (Supplementary Table S5).
201 The great majority (73.59%) of the contig blast matches were proteins originating from primate
202 parasites, while 25.34% matched rodent parasites, and only 0.92% parasites of birds (Figure 5B).

203 At the species level, most contigs (29.91%) resulted in best blast hit against *P. falciparum*,
204 followed by *P. reichenowi* (16.88%) and *P. yoelii* (8.59%) (Figure 5C). The significant blast matches
205 to bird parasites consisted of the species *Plasmodium gallinaceum* (n = 56), *Eimeria acervulina* (n =
206 5), *Eimeria tenella* (n = 4), *Eimeria mitis* (n = 3), *Plasmodium relictum* (n = 3), and *Plasmodium lutzi*

207 (n = 1). The contigs giving matches to avian *Plasmodium* were primarily derived from commonly
208 sequenced apicomplexa genes and therefore available in public databases, for example cytochrome c
209 oxidase subunit 1 (COX1; *P. lutzi*), merozoite surface protein 1 (MSP1; *P. relictum*), thrombospondin
210 related anonymous protein (TRAP; *P. relictum*), and cytochrome b (CYTB; *P. gallinaceum*)
211 (Supplementary Table S1).

212 The five contigs with highest GC content in the *P. ashfordi* transcriptome (47.7% – 56.4%)
213 all had matches against the avian parasites *Eimeria*, despite them only comprising 0.15% (n = 12) of
214 the total annotation. *Eimeria* spp. have a very high transcriptome GC content (*E. acervulina*: 55.98%;
215 *E. mitis*: 57.30%), and the *P. ashfordi* transcripts matching this genus consist mostly of ribosomal and
216 transporter genes (Supplementary Table S1).

217 The *P. ashfordi* contigs with highest expression levels were primarily annotated by
218 uncharacterized protein matches to the rodent parasite *P. yoelii* (Table 2). In fact, the six most highly
219 expressed transcripts that were annotated, all gave significant blast matches to *P. yoelii*. Further
220 investigation revealed that these transcripts are most likely derived from ribosomal RNA.
221

222 Identification of conserved *Plasmodium* invasion genes

223 Finally, to assess molecularly conserved strategies in *P. ashfordi* compared to mammalian malaria
224 parasites, we searched for annotated genes known to be involved in the red blood cell invasion by
225 *Plasmodium* spp. (Beeson et al., 2016; Bozdech et al., 2003). We discovered successfully assembled
226 *P. ashfordi* transcripts from a whole suite of host cell invasion genes (Table 3). This includes for
227 example the genes merozoite surface protein 1 (MSP1), apical membrane antigen 1 (AMA1),
228 merozoite adhesive erythrocytic binding protein (MAEBL), GPI-anchored micronemal antigen
229 (GAMA), and the rhoptry neck binding proteins 2, 4, and 5 (RON2, RON4, and RON5). Interestingly,
230 the *P. ashfordi* RON genes in particular seemed to slightly decrease expression levels in all hosts over
231 the two time points (Figure 6). In general, however, the invasion genes showed a range of expression
232 patterns over time, going in various directions (Figure 6).

233 All genes known to be involved in the *Plasmodium* motor complex (Baum et al., 2006; Opitz
234 and Soldati, 2002), driving parasite gliding motion and enabling host cell invasion, were discovered in
235 *P. ashfordi*. These include: actin (ACT1), actin-like protein (ALP1), aldolase (FBPA), myosin A
236 (MyoA), myosin A tail interacting protein (MTIP), glideosome-associated protein 45 and 50 (GAP45
237 and GAP50), and thrombospondin related anonymous protein (TRAP). We also found the
238 bromodomain protein 1 (BDP1), which has been directly linked to erythrocyte invasion by binding to
239 chromatin at transcriptional start sites of invasion-related genes and controlling their expression
240 (Josling et al., 2015).

241 We found two transcripts matching the low molecular weight rhoptry-associated proteins 1
242 and 3 (RAP1 and RAP3) that are secreted from the rhoptry organelles during cell invasion. The
243 genomes of human malaria parasites contain a paralog gene called RAP2 as well, whereas rodent
244 malaria parasites contain a single gene copy that is a chimera of RAP2 and RAP3 (RAP2/3)
245 (Counihan et al., 2013). The *P. ashfordi* transcript in question (TR13305|c0_g1_i1) matches *P.*
246 *falciparum* RAP3 better than the rodent parasite version of RAP2/3. The three high molecular weight
247 rhoptry proteins (RhopH1, RhopH2, RhopH3) which bind to the erythrocyte plasma membrane and
248 transfer to the parasitophorous vacuole membrane upon invasion (Counihan et al., 2013; Vincensini et

249 al., 2008) were all identified in *P. ashfordi*. RhopH1 encompasses the multigene family of
250 cytoadherence linked asexual proteins (CLAGs), present in varying copy number across *Plasmodium*.

251 Other assembled *P. ashfordi* orthologs of genes involved in host cell invasion were the
252 rhoptry-associated leucine zipper-like protein 1 (RALP1), rhoptry-associated membrane antigen
253 (RAMA), armadillo-domain containing rhoptry protein (ARO), RH5 interacting protein (RIPR),
254 TRAP-like protein (TLP), merozoite TRAP-like protein (MTRAP), thrombospondin related apical
255 membrane protein (TRAMP), subtilisin proteases 1 and 2, (SUB1 and SUB2), and merozoite surface
256 proteins 8 and 9 (MSP8 and MSP9). MTRAP and TRAMP are proteins that belong to the TRAP-
257 family and are released from the microneme organelles during invasion (Cowman et al., 2012; Green
258 et al., 2006), and the subtilisin proteases SUB1 and SUB2 are heavily involved in the processing and
259 cleavage of immature merozoite antigens, for example MSP1 and AMA1 (Beeson et al., 2016). ARO
260 plays a crucial role in positioning the rhoptry organelles within the apical end of the parasite to enable
261 the release of rhoptry-associated molecules (Mueller et al., 2013) such as RAMA and RALP1, which
262 then bind to the erythrocyte surface.

263 We furthermore discovered transcripts of several reticulocyte binding proteins (RBP / RH)
264 thought to be absent in the genomes of avian malaria parasites (Lauron et al., 2015). These particular
265 transcripts, together with RAMA, interestingly showed much higher e-values than other invasion
266 genes (Table 3), indicating high differentiation between avian and mammalian *Plasmodium* RH
267 genes. Finally, two rhomboid proteases (ROM1 and ROM4) have been linked to host cell invasion in
268 *P. falciparum* via cleavage of transmembrane adhesins (Baker et al., 2006; Santos et al., 2012). We
269 found both of these genes, together with other rhomboid proteases (ROM2, ROM3, ROM6, ROM8,
270 and ROM10) expressed in *P. ashfordi*. More information about the assembled genes can be found in
271 Supplementary Table S1.

272

273

274 Discussion

275

276 Generating and describing genome-wide expression data of parasites from phylogenetically diverse
277 hosts is crucial to better understand the evolution of host-parasite interactions, virulence, and host-
278 specificity. In this study, we assembled and characterized the first transcriptome with quantified gene
279 expression of an avian malaria parasite, *P. ashfordi*. By developing a bioinformatic pipeline capable
280 of dealing with dual RNA-seq data, we successfully filtered away contigs originating from the host
281 and other sources of contamination in a multistep approach. This resulted in a transcriptome with
282 7 860 annotated transcripts, and an additional 4 094 species-specific unannotated transcripts. Parasite
283 gene expression displayed strikingly similar patterns during two infection stages and within individual
284 hosts. Furthermore, *P. ashfordi* shows large similarities to the human malaria parasite, *P. falciparum*,
285 and the assembly supports several important erythrocyte invasion genes (Table 3) indicating
286 evolutionary conserved invasion strategies across the phylogenetic host range of *Plasmodium*
287 parasites.

288

289 *P. ashfordi* displays host-specific gene expression

290 Interestingly, and contrary to our expectations, *P. ashfordi* showed exceptionally similar expression
291 profiles inside the same host, despite being sampled ten days apart during two different disease stages.
292 All birds were inoculated with the same clonal malaria strain, which means the parasite probably
293 regulates its gene expression levels to fit the different hosts, although direct modulation of parasite
294 gene expression by the host cannot be excluded. The mechanism behind this host-specific expression
295 pattern is unknown, but could be due to genotype by genotype interactions between the host and the
296 parasite, or plasticity of the parasite to varying host environments. This result has potentially
297 important implications for our understanding of the evolution of host-parasite interactions, and as a
298 result warrants further research extending the limited sample size to more hosts and more timepoints
299 throughout the infection.

300 Host genotype by parasite genotype interactions are not well documented in malaria parasite
301 systems. Studies with different genotypes of both host and parasite have found effects of host
302 genotype, but not parasite genotype, on factors such as host resistance and parasite virulence
303 (Mackinnon et al., 2002; de Roode et al., 2004; Grech et al., 2006; see also Idaghdour et al., 2012).
304 Even less is known about the transcriptome responses of malaria parasites to different hosts. Daily et
305 al. (2007) discovered host-specific distinct transcriptional states of *P. falciparum* in the blood of
306 Senegalese children, where parasite transcriptomes could be divided into three distinct clusters
307 associated with either starvation responses and invasion, glycolysis, or oxidative stress and heat shock
308 pathways (Daily et al., 2007).

309 The expression profiles of *P. ashfordi* did not exhibit any significant differences between
310 peak and decreasing parasitemia stages (day 21 and 31 postinfection). The hosts in our study
311 experienced relatively high parasitemia levels also during the decreasing parasitemia stage (see
312 Methods), so it is possible that these specific time points do not provide very different environmental
313 host cues to the parasites. However, the simultaneous transcriptomes of the avian hosts (analyzed in
314 Videvall et al. 2015) displayed large differences in gene expression between these two parasitemia
315 stages, notably with a reduced immune response during decreasing parasitemia. This is important

316 because it appears that *P. ashfordi* does not adjust its gene expression in response to the decreasing
317 immune levels of the hosts, but instead conforms to the specific environment of individual hosts.

318 *P. falciparum* evades the human immune defense via intracellularity, clonal antigenic
319 variation (Guizetti and Scherf, 2013), transcriptional antigenic switches (Recker et al., 2011), splenic
320 avoidance by erythrocytic adherence to the endothelium (Craig and Scherf, 2001) and sequestration in
321 organ microvasculature (Ashley et al., 2014), erythrocytic rosetting (Niang et al., 2014), host
322 immunosuppression (Hisaeda et al., 2004), and manipulation of host gene expression. It is possible
323 that *P. ashfordi* shares several of these evasion strategies with *P. falciparum*, although this remains
324 unknown. One example of immune evasion by manipulation of host gene expression is the parasite
325 gene macrophage migration inhibitory factor homologue (MIF), which contributes to *Plasmodium*
326 parasites' ability to modulate the host immune response by molecular mimicry (Cordery et al., 2007).
327 This gene was discovered transcribed in *P. ashfordi* as well (TR2046|c0_g1_i1), suggesting a similar
328 immune manipulation strategy.

329

330 **Similarities to *P. falciparum* and other malaria parasites**

331 The majority of all annotated contigs (73.59%) resulted in a best blast hit against primate parasite
332 species (Figure 5A). Curiously, the human malaria parasite *P. falciparum* comprised the majority of
333 all matches with almost a third of the transcripts (29.91%) (Figure 5C). This is likely because *P.*
334 *falciparum* currently constitutes the organism with most sequence similarities to *P. ashfordi* based on
335 publically available sequences (Bensch et al., 2016). The chimpanzee parasite *P. reichenowi* had the
336 second most blast matches to *P. ashfordi* (Figure 5C), and it is the closest living relative of *P.*
337 *falciparum* as far as we know (Otto et al., 2014). Furthermore, both parasites share the genome
338 characteristics of being extremely AT-biased, with *P. ashfordi* reaching a remarkably low
339 transcriptomic GC content of 21.22% (Table 1) compared to the already extremely AT-rich *P.*
340 *falciparum*, which has a transcriptomic GC content of 23.80%. Lastly, because of its role in human
341 disease, *P. falciparum* is the most sequenced *Plasmodium* species (172 official strains in the NCBI
342 taxonomy database as of May 2016) (Gardner et al., 2002), resulting in plenty of opportunities for
343 transcript sequences to find significant blast matches.

344 Less than one percent of all contigs resulted in a blast hit against avian parasites (0.92%).
345 This is due to the fact that almost no genomic resources are available for avian *Plasmodium*. Despite
346 their enormous diversity, world-wide distribution, and harmful effects on susceptible bird populations,
347 genomic studies of avian malaria parasites have been largely non-existent until now. The genome of
348 *P. gallinaceum*, the malaria parasite of chickens, has been sequenced but not published and is not yet
349 available (Ulrike Boehme, personal communication). A transcriptome assembly of *P. gallinaceum* is
350 available for download (Lauron et al., 2014, 2015), although still contains a large proportion of
351 contigs with significant blast matches to birds, making comparisons with *P. ashfordi* difficult
352 (Supplementary Figure S4). Dual RNA-sequencing of a more distantly related apicomplexan parasite,
353 *Leucocytozoon buteonis*, and its buzzard host was recently described (Pauli et al., 2015), though no
354 publically available transcriptome exists. Finally, 454 RNA-sequencing of the generalist avian malaria
355 parasite *P. relictum* (lineage SGS1) has been previously performed (Bensch et al., 2014; Hellgren et
356 al., 2013), but the low sequencing coverage does not allow for assembly nor any transcriptome

357 analyses. We hope that future versions of these avian parasite transcriptomes will enable genome-
358 wide comparisons.

359 The lack of genome-wide sequence data from malaria parasites of hosts other than mice and
360 primates, means that little is known about which genes across *Plasmodium* are conserved and which
361 are unique. As a first step to investigate this, we searched in the *P. ashfordi* assembly for genes known
362 to be involved in the merozoite invasion of host red blood cells. This particular group of genes has
363 shown the greatest diversity in mammalian *Plasmodium* and many are under strong positive selection
364 due to their role in host specificity (Hall et al., 2005; Otto et al., 2014). Previously, only a handful of
365 studies have sequenced candidate invasion genes in avian malaria parasites; these include MAEBL
366 (Martinez et al., 2013), AMA1 and RON2 (Lauron et al., 2014), MSP1 (Hellgren et al., 2013, 2015),
367 RIPR (Lauron et al., 2015), and TRAP (Farias et al., 2012; Templeton and Kaslow, 1997). Due to the
368 evolutionary distance between mammals and saurians, and their inherent blood cell differences
369 (birds/reptiles have erythrocytes with a nucleus and mitochondria while mammalian cells are
370 anucleated), we might expect to find few and highly differentiated gene orthologs. Remarkably, we
371 discovered a large number of red blood cell invasion genes expressed in *P. ashfordi* (Table 3),
372 indicating that most of these specific invasion genes are conserved across both mammalian and avian
373 *Plasmodium*.

374 The invasion genes that were most differentiated between birds and mammals were the
375 rhoptry-associated membrane antigen (RAMA) and the reticulocyte binding proteins (RBP/RH),
376 which had diverged almost beyond recognition. These RH genes, together with other erythrocyte
377 binding antigens (EBA), are missing in *P. vivax*, and have understandably been assumed absent in the
378 genomes of avian malaria parasites (Lauron et al., 2015). However, our result suggests that several are
379 not only present, but also transcribed, though with high sequence divergence. It is possible that
380 additional erythrocyte binding proteins are present in the *P. ashfordi* assembly, though ortholog
381 searches for these genes will become complicated if they have evolved under especially strong
382 selection pressure in avian *Plasmodium*. Genes that are orthologous between *P. falciparum* and avian
383 malaria parasites but absent from other mammalian parasites are important genomic markers of
384 maintained ancestral states (Bensch et al., 2016). We can therefore advance our understanding about
385 the evolution of *Plasmodium* species by analyzing genomes and transcriptomes of avian malaria
386 parasites.

387

388

389 Conclusion

390 In this study we have sequenced, assembled, and characterized the transcriptome of *Plasmodium*
391 *ashfordi*. The results presented here and the associated assembly will help improve our understanding
392 of host-parasite interactions, evolutionary conserved *Plasmodium* strategies, and the phylogenetic
393 relationships between apicomplexans. In addition, we have shown that *P. ashfordi* displays strikingly
394 similar expression profiles within individual hosts during two different stages of the infection – but
395 different expression patterns between individual hosts – indicating host specific parasite gene
396 regulation. The expression information of all transcripts in the *P. ashfordi* transcriptome will
397 ultimately assist researchers studying genes involved in e.g. immune evasion, host-specificity, and
398 parasite plasticity.

399

400

401 Methods

402

403 Experimental setup

404 We used four wild-caught juvenile Eurasian siskins (*Carduelis spinus*) in an infection experiment.
405 The procedure was carried out 2012 at the Biological Station of the Zoological Institute of the Russian
406 Academy of Sciences on the Curonian Spit in the Baltic Sea. All details regarding the experimental
407 setup have been outlined in Videvall et al. (2015). Briefly, three of the birds were inoculated with a
408 single injection of blood containing the erythrocytic stages of *Plasmodium ashfordi*, subgenus
409 *Novyella*, lineage GRW2. For a description of this parasite, see Valkiūnas et al. (2007). A control bird
410 (bird 1) was simultaneously inoculated with uninfected blood for the evaluation of host transcription
411 (Videvall et al., 2015). Blood samples for RNA-sequencing were taken from birds before infection
412 (day 0), during peak parasitemia (day 21 postinfection) and during the decreasing parasitemia stage
413 (day 31 postinfection).

414 All birds were thoroughly screened with both microscopic (Palinauskas et al., 2008) and
415 molecular (Hellgren et al., 2004) methods before the experiment to make sure they had no prior
416 haemosporidian infection. The parasitemia intensity varied substantially in infected birds, with bird 2,
417 bird 3, and bird 4 having 24.0%, 48.0%, and 71.3% of their red blood cells infected during peak
418 parasitemia, and later 8.2%, 21.8%, and 33.3%, respectively, during the decreasing parasitemia stage
419 (Videvall et al., 2015). The two parasitemia stages are sometimes referred to as different ‘infection
420 stages’ in the hosts, but we like to clarify that there is no evidence present suggesting that the parasites
421 have entered a different stage of their life cycle (e.g. tissue merogony). Experimental procedures were
422 approved by the International Research Co-operation Agreement between the Biological Station
423 Rybachy of the Zoological Institute of the Russian Academy of Sciences and Institute of Ecology of
424 Nature Research Centre (25 May 2010). Substantial efforts were made to minimize handling time and
425 potential suffering of birds.

426

427 RNA extraction and sequencing

428 From six infected samples (three treatment birds at days 21 and 31) and six uninfected samples (three
429 treatment birds at day 0 and one control bird at days 0, 21, and 31) total RNA was extracted from 20
430 µl whole blood. Detailed extraction procedures can be found in Videvall et al. (2015). Total extracted
431 RNA was sent to Beijing Genomics Institute (BGI), China, for RNA quality control, DNase
432 treatment, rRNA depletion, and amplification using the SMARTer Ultra Low kit (Clontech
433 Laboratories, Inc.). BGI performed library preparation, cDNA synthesis, and paired-end RNA-
434 sequencing using Illumina HiSeq 2000. The blood samples from bird 3 and bird 4 during peak
435 parasitemia were sequenced by BGI an additional time in order to generate more reads from the
436 parasite in preparation for this transcriptome assembly. These resequenced samples were regarded and
437 handled as technical replicates. We quality-screened all the demultiplexed RNA-seq reads using
438 FastQC (v. 0.10.1) (<http://www.bioinformatics.babraham.ac.uk/projects/fastqc/>).

439

440 De novo transcriptome assembly

441 Quality-filtered RNA-seq reads from all six infected bird samples together with the two re-sequenced
442 samples were used in a *de novo* assembly. This was performed using the transcriptome assembler

443 Trinity (v. 2.0.6) (Grabherr et al., 2011) with 489 million reads. Mapping of reads to available
444 genomes of human malaria parasites were employed simultaneously, but unfortunately these attempts
445 yielded few hits due to the evolutionary distance between avian *Plasmodium* and human *Plasmodium*,
446 so we continued annotating the assembly using blast searches.

447 The assembled transcripts were blasted against the NCBI non-redundant protein database
448 using the program DIAMOND BLASTX (v. 0.7.9) (Altschul et al., 1990; Buchfink et al., 2014) with
449 sensitive alignment mode. A total of 47 823 contigs generated significant hits against avian species
450 (Figure 1C). A large number of contigs ($n = 260\ 162$) did not produce any significant (e -value $< 1e-5$)
451 blastx hits (Figure 1D). This is because 1) the host species is a non-model organism without a genome
452 available, leading to a large number of host contigs without blast hits, 2) multiple contigs do not
453 necessarily contain coding sequences, but can be derived from noncoding RNAs, etc. and therefore
454 will not match protein sequences, 3) short, fragmented contigs may not yield sufficient blast hit
455 significance, and 4) an extreme underrepresentation of protein sequences from avian *Plasmodium*
456 species in the NCBI nr database will not result in any significant blast hits to genes unique in avian
457 malaria parasites.

458 We strictly filtered the initial assembly by only retaining a total of 9 015 transcripts
459 (isoforms) that produced significant blast matches against proteins from species in the Apicomplexa
460 phylum. A previous assembly using an earlier version of Trinity (v. r20140413p1) (Grabherr et al.,
461 2011) performed better when it came to assembling the longest contigs (> 6 kbp). Different versions
462 of assembler software may construct de Bruijn graphs somewhat differently, which is why it can be a
463 good idea to make several assemblies and later combine parts of them (Brian Haas, personal
464 communication). The previous assembly had been blasted and screened for apicomplexa in the same
465 way as described above. In order not to lose these important full-length transcripts, we therefore
466 included the longest contigs from the previous assembly that had 1) not assembled correctly in the
467 current assembly, and 2) significant blastx hits against apicomplexa ($n = 10$), resulting in a total of 9
468 025 transcripts. The fact that these contigs contained similar sequences already present in the
469 assembly was dealt with through downstream clustering of the sequences.
470

471 Transcriptome cleaning and filtering

472 Some contigs in the annotated assembly contained poly-A tails which complicated downstream
473 analyses and resulted in biased mapping estimates. We therefore removed all poly-A/T tails using
474 PRINSEQ (v. 0.20.4) (Schmieder and Edwards, 2011) with a minimum prerequisite of 12 continuous
475 A's or T's in the 5' or 3' end of the contigs. A total of 106 202 bases (1.18%) was trimmed and the
476 mean transcript length was reduced from 995.28 to 983.52 bases.

477 The unknown transcripts that failed to produce significant hits to any organism during the
478 blastx run ($n = 260\ 162$) were subsequently cleaned using the following procedure. First, we trimmed
479 them for poly-A tails, resulting in a total of 455 331 bases removed, and a slight decrease of the mean
480 length of the unknown sequences from 555.27 nt before trimming to 553.52 nt after trimming. The
481 majority of these unknown transcripts came from host mRNA, but their GC content displayed a clear
482 bimodal distribution (Figure 1D), where the contigs with very low GC were strongly suspected to
483 originate from the parasite. To avoid any host contigs, we strictly filtered the unknown transcripts to
484 only include sequences with a mean GC content lower than 23% ($n = 4\ 624$). This threshold was

485 based on the apicomplexa-matching transcripts which had a mean GC content of 21.22%, and all
486 poly-A trimmed contigs giving significant blast matches to birds (class: Aves) ($n = 47\ 823$), where the
487 mean GC content was 47.65%, and the avian contig with the absolute lowest GC content had 23.48%
488 GC (Figure 1C).

489

490 **Transcriptome clustering, further filtering, and validation**

491 To reduce redundancy of multiple isoforms and transcripts derived from the same gene, we first
492 merged together the annotated and the unknown transcripts with a GC content < 23% ($n = 13\ 649$).
493 We then clustered these sequences together in order to retain most transcripts but group the highly
494 similar ones based on 97% sequence similarity and a k-mer size of 10, using CD-HIT-EST (v. 4.6) (Li
495 and Godzik, 2006). The most representative (longest) contig in every cluster was selected to represent
496 those transcripts, resulting in 12 266 contigs/clusters. We further filtered all the short sequences (<
497 200 bases), to obtain a set of 12 182 representative transcripts.

498 A second blast filtering step with the trimmed representative contigs against the Refseq
499 genomic database was then employed using BLASTN+ (v. 2.2.29) (Altschul et al., 1990; Camacho et
500 al., 2009) to identify some ambiguous contigs suspected to contain non-coding RNA bird sequences.
501 We removed all contigs that gave significant matches (e-value: 1e-6) against all animals (kingdom
502 Metazoa), so we could be confident that the assembly only consisted of true parasite transcripts. This
503 last filtering step removed 228 contigs.

504 The unannotated transcripts ($n = 4\ 094$) of the final assembly were further validated to
505 originate from the parasite by using reads from the six uninfected samples of the same hosts sampled
506 before infection and the control bird. A total of 350 318 482 reads (65 bp and 90 bp) from all
507 uninfected samples were mapped to the unannotated transcripts using Bowtie2 (v. 2.2.5) (Langmead
508 and Salzberg, 2012), resulting in the alignment of 90 read pairs (0.000051%). This extremely low
509 mapping percentage from the uninfected samples greatly supported our conclusion that these
510 transcripts had indeed been transcribed by *P. ashfordi*. These 4 094 representative transcripts with
511 unknown function are referred to throughout the paper as unannotated transcripts.

512 The resulting final transcriptome assembly consists of 11 954 representative annotated and
513 unannotated transcripts. The genomes of *Plasmodium* parasites generally contain around 5-6 000
514 protein-coding genes (Kersey et al., 2015), making it reasonable to assume similar gene numbers for
515 the *P. ashfordi* genome. The larger number of transcripts in the assembly is therefore a result of
516 isoform varieties and fragmented contigs.

517

518 **Estimating expression levels**

519 Poly-A tails of 489 million RNA-seq reads from all samples were trimmed as well using PRINSEQ
520 (v. 0.20.4) (Schmieder and Edwards, 2011). A minimum prerequisite for trimming were 20
521 continuous A's or T's in the 5' or 3' end of each read. Only trimmed reads longer than 40 bp and still
522 in a pair were retained ($n = 451\ 684\ 626$) in order to confidently map high quality reads with good
523 minimum lengths. Bowtie2 (v. 2.2.5) (Langmead and Salzberg, 2012) was used to map the trimmed
524 RNA-seq reads of every sample ($n = 8$) (six biological and two technical replicates) back to the *P.*
525 *ashfordi* transcriptome consisting of the 11 954 representative sequences. We calculated expression

526 levels using RSEM (v. 1.2.21) (Li and Dewey, 2011), which produces expected read counts for every
527 contig.

528 The counts of the 11 954 transcripts were subsequently analyzed inside the R statistical
529 environment (v. 3.2.5) (R Core Team, 2015). We tested for expression differences in the malaria
530 parasites between the two time points, peak and decreasing parasitemia, using DESeq2 (v. 1.10.1)
531 (Love et al., 2014). The two resequenced samples (technical replicates) of bird 3 and bird 4 during
532 peak parasitemia were handled exactly the same as all other samples, and their respective read count
533 were added to their biological samples, according to the DESeq2 manual. Individual host was used as
534 a factor in the analysis, and counts were normalized to account for potential variation in sequencing
535 depth as well as the large differences in number of parasites present in the blood (parasitemia levels).
536 Regularized log transformation of counts was performed in order to represent the data without any
537 prior knowledge of sampling design in the principal component analysis and sample distance
538 calculations. This way of presenting counts without bias is preferred over variance stabilizing of
539 counts when normalization size factors varies greatly between the samples, as they naturally do in our
540 data.

541

542 **Transcriptome evaluation**

543 Transcriptome statistics such as GC content, contig length, and assembled bases were calculated using
544 bash scripts and in the R statistical environment (v. 3.2.5) (Pages et al., 2015; R Core Team, 2015). P-
545 values were corrected for multiple testing with the Benjamini and Hochberg false discovery rate
546 (Benjamini and Hochberg, 1995) and corrected values have been labeled as q-values throughout. We
547 calculated the GC content of two *Eimeria* transcriptomes downloaded from ToxoDB (v. 25) (Gajria et
548 al., 2007), initially sequenced by Reid et al. (2014). Transcriptome E90N50 was calculated using
549 RSEM (v. 1.2.21) and Trinity (v. 2.0.6) (Grabherr et al., 2011; Haas, 2016; Li and Dewey, 2011).
550 Plots were made with the R package ggplot2 (v. 2.1.0) (Wickham, 2009). Metabolic pathway and
551 gene ontology enrichment analyses were conducted in PlasmoDB (Aurrecoechea et al., 2009) using
552 orthologs of annotated gene names to compare against the gene ontology and metabolic pathway
553 background information from *P. falciparum* 3D7. The red blood cell invasion genes were searched for
554 in the transcriptome annotation of *P. ashfordi* (Supplementary Table S1). Only genes with
555 documented involvement in *Plasmodium* erythrocyte invasion were included.

556

557 **Data availability**

558 The sequence reads of both host and parasite have been deposited at the NCBI Sequence Read
559 Archive under the accession number PRJNA311546. The assembled *P. ashfordi* transcripts are
560 available for download at <http://mbio-serv2.mbioekol.lu.se/Malavi/Downloads>.

561

562

563 **Author Contributions**

564

565 The study design was initially conceived by OH, VP and GV, and further developed together with EV
566 and CKC. VP performed the experiments. OH performed the RNA extractions. Assembly and
567 bioinformatic and statistical analyses were performed by EV. DA advised in the trimming step of the
568 assembly and in the mapping of sequence reads. OH, CKC, and EV planned the paper. EV drafted the
569 paper with extensive input from all authors.

570

571

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578

579

580

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582

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- 830

831 **Tables**

832

833

834 **Table 1. Assembly statistics of the *Plasmodium ashfordi* transcriptome.**

835

	Annotated transcripts	Unannotated transcripts	Complete assembly
Number of contigs	7 860	4 094	11 954
Number of bases	7 316 007	1 694 373	9 010 380
Contig length min-max (bp)	200 – 26 773	200 – 4 171	200 – 26 773
Median contig length (bp)	681.0	321.0	498.0
Mean contig length (bp)	930.8	413.9	753.8
GC content (%)	21.22	17.26	20.48

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839 **Table 2. Most highly expressed transcripts in the *Plasmodium ashfordi* transcriptome.**

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<i>P. ashfordi</i> transcript	Protein description	Species match (blastx)	Mean FPKM ¹
TR71765 c7_g6_i1	Uncharacterized protein (PY04653)	<i>P. yoelii</i>	218 188.9
TR71765 c7_g7_i1	Uncharacterized protein (PY01927)	<i>P. yoelii</i>	189 356.5
TR87146 c0_g1_i1	Uncharacterized protein (PY04656)	<i>P. yoelii</i>	31 677.2
TR55310 c0_g1_i1	Unannotated <i>P. ashfordi</i> transcript	NA	13 753.1
TR24310 c0_g1_i1	70 kd Heat shock-like protein	<i>P. yoelii</i>	6 528.5
TR87393 c0_g5_i1	Histone H3	<i>P. yoelii</i>	5 917.4
TR52464 c0_g1_i1	Uncharacterized protein (PY00146)	<i>P. yoelii</i>	5 756.5
TR69136 c5_g1_i1	Uncharacterized protein (PFTANZ_01287)	<i>P. falciparum</i>	5 448.1
TR73260 c2_g1_i1	Uncharacterized protein (PFFCH_03197)	<i>P. falciparum</i>	4 980.4
TR162674 c0_g1_i1	Histone H4	<i>P. falciparum</i>	3 769.6
TR55306 c1_g1_i1	Heat shock protein 86	<i>P. vivax</i>	3 665.4
TR16018 c0_g1_i1	Histone H2B	<i>Babesia equi</i>	2 716.3
TR213315 c0_g1_i1	Cytochrome c oxidase subunit 1	<i>P. lutzi</i>	2 453.2
TR59776 c0_g2_i1	Uncharacterized protein (PY07298)	<i>P. yoelii</i>	2 236.1
TR113917 c0_g1_i1	Uncharacterized protein (PRCDC_1024200)	<i>P. reichenowi</i>	1 994.0

841

842 ¹Fragments Per Kilobase of transcript per Million mapped reads (FPKM) is a relative expression
843 measure normalized for library size and gene length.

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845

846 **Table 3. Assembled transcripts of genes involved in *Plasmodium* invasion of red blood cells.**

847

Gene name	Gene product	Represented by transcripts	Species match (blastx)	Bit score	e-value
ACT1	Actin 1	TR215622 c0_g1_i1	<i>P. vivax</i>	758.4	5.5E-216
ALP1	Actin-like protein 1	TR55613 c0_g1_i1	<i>P. vivax</i>	619	5.1E-174
AMA-1	Apical membrane antigen 1	TR118224 c0_g1_i1	<i>P. gallinaceum</i>	624	2.1E-175
ARO	Armadillo-domain containing rhoptry protein	TR18124 c0_g1_i1 TR18124 c0_g2_i1	<i>P. knowlesi</i>	520 501.9	1.9E-144 6.0E-139
BDP1	Bromodomain protein 1	TR66841 c0_g2_i1 TR66841 c0_g2_i2	<i>P. falciparum</i>	364.8 357.1	1.9E-97 3.9E-95
FBPA	Fructose-bisphosphate aldolase	TR12911 c0_g1_i1 TR12911 c0_g2_i1	<i>P. cynomolgi</i> <i>P. berghei</i>	223.8 353.2	2.5E-55 2.7E-94
GAMA	GPI-anchored micronemal antigen	TR16322 c0_g2_i1	<i>P. reichenowi</i>	567.4	2.5E-158
GAP45	Glideosome-associated protein 45	TR34884 c0_g1_i1	<i>P. vivax</i>	209.9	3.9E-51
GAP50	Glideosome-associated protein 50	TR144721 c0_g1_i1	<i>P. knowlesi</i>	577.8	1.2E-161
MAEBL	Merozoite adhesive erythrocytic binding protein	TR234951 c0_g1_i1 TR99718 c0_g1_i1 TR125315 c0_g1_i1	<i>P. yoelii</i> <i>P. gallinaceum</i> <i>P. gallinaceum</i>	171.8 146.4 84.3	6.3E-40 2.4E-32 7.2E-14
MCP1	Merozoite capping protein 1	TR122100 c0_g1_i1	<i>P. berghei</i>	164.5	1.7E-37
MSP1	Merozoite surface protein 1	TR112241 c0_g2_i1 TR176579 c0_g1_i1	<i>P. relictum</i>	368.6 134.4	4.2E-98 1.4E-28
MTIP (MLC1)	Myosin A tail domain interacting protein	TR8937 c0_g1_i1	<i>P. vivax</i>	295	8.6E-77
MTRAP	Merozoite TRAP-like protein	TR188691 c0_g2_i1	<i>P. vinckeii</i>	98.6	1.3E-17
MYOA	Myosin A	TR198550 c0_g1_i1	<i>Babesia microti</i>	199.1	1.5E-47
RAMA	Rhoptry-associated membrane antigen	TR144799 c0_g1_i1	<i>P. knowlesi</i>	70.1	5.0E-09
RALP1	Rhoptry-associated leucine zipper-like protein 1	TR7446 c0_g1_i1	<i>P. falciparum</i>	102.1	1.2E-18
RAP1	Rhoptry-associated protein 1	TR115690 c2_g1_i1	<i>P. coatneyi</i>	236.1	1.3E-58
RAP3	Rhoptry-associated protein 3	TR13305 c0_g1_i1	<i>P. falciparum</i>	202.6	9.9E-49
RBP1 (RH1)	Reticulocyte-binding protein 1	TR53756 c1_g1_i1	<i>P. falciparum</i>	84.7	8.0E-13
RBP2 (RH2)	Reticulocyte-binding protein 2	TR208311 c0_g1_i1 TR66282 c1_g2_i1	<i>P. vivax</i>	76.6 146.7	5.9E-11 2.1E-31
RBP2b (RH2b)	Reticulocyte-binding protein 2 homolog B	TR87235 c1_g1_i1 TR35437 c1_g1_i1	<i>P. vivax</i> <i>P. falciparum</i>	97.8 86.7	8.7E-18 1.9E-14
RHOPH1	High molecular weight rhoptry protein 1 (CLAG9)	TR7367 c0_g1_i1 TR200056 c0_g1_i1 TR233854 c0_g1_i1	<i>P. reichenowi</i> <i>P. reichenowi</i> <i>P. falciparum</i>	272.3 82.8: 61.6	7.9E-70 2.3E-13 4.8E-07
RHOPH2	High molecular weight rhoptry protein 2	TR25858 c0_g2_i1 TR25858 c0_g3_i1 TR175810 c0_g1_i1	<i>P. vivax</i> <i>P. fragile</i> <i>P. vivax</i>	542.3 335.9 180.6	7.8E-151 5.7E-89 3.4E-42
RHOPH3	High molecular weight rhoptry protein 3	TR83052 c6_g1_i1	<i>P. falciparum</i>	612.8	7.3E-172
RIPR	RH5 interacting protein	TR49727 c0_g2_i1 TR49727 c0_g2_i2	<i>P. reichenowi</i> <i>P. fragile</i>	502.3 299.3	6.4E-139 1.0E-77
ROM1	Rhomboid protease 1	TR178976 c0_g1_i1	<i>P. yoelii</i>	102.4	2.4E-19
ROM4	Rhomboid protease 4	TR92176 c1_g1_i1	<i>P. reichenowi</i>	783.5	2.1E-223

RON2	Rhoptry neck protein 2	TR62454 c0_g1_i1	<i>P. cynomolgi</i>	659.8	8.5E-186
RON4	Rhoptry neck protein 4	TR145809 c0_g1_i1	<i>P. reichenowi</i>	349.4	8.9E-93
RON5	Rhoptry neck protein 5	TR67313 c1_g1_i1	<i>P. reichenowi</i>	1105.9	0
SUB1	Subtilisin 1	TR164664 c0_g1_i1	<i>P. inui</i>	636.7	3.0E-179
SUB2	Subtilisin 2	TR169322 c0_g1_i1	<i>P. inui</i>	563.9	2.7E-157
TLP	TRAP-like protein	TR153597 c0_g1_i1	<i>P. falciparum</i>	440.7	3.2E-120
TRAMP	Thrombospondin related apical membrane protein	TR179194 c0_g1_i1	<i>P. falciparum</i>	231.1	2.1E-57
TRAP	Thrombospondin related anonymous protein	TR16495 c0_g1_i1	<i>P. relictum</i>	229.2	6.4E-57

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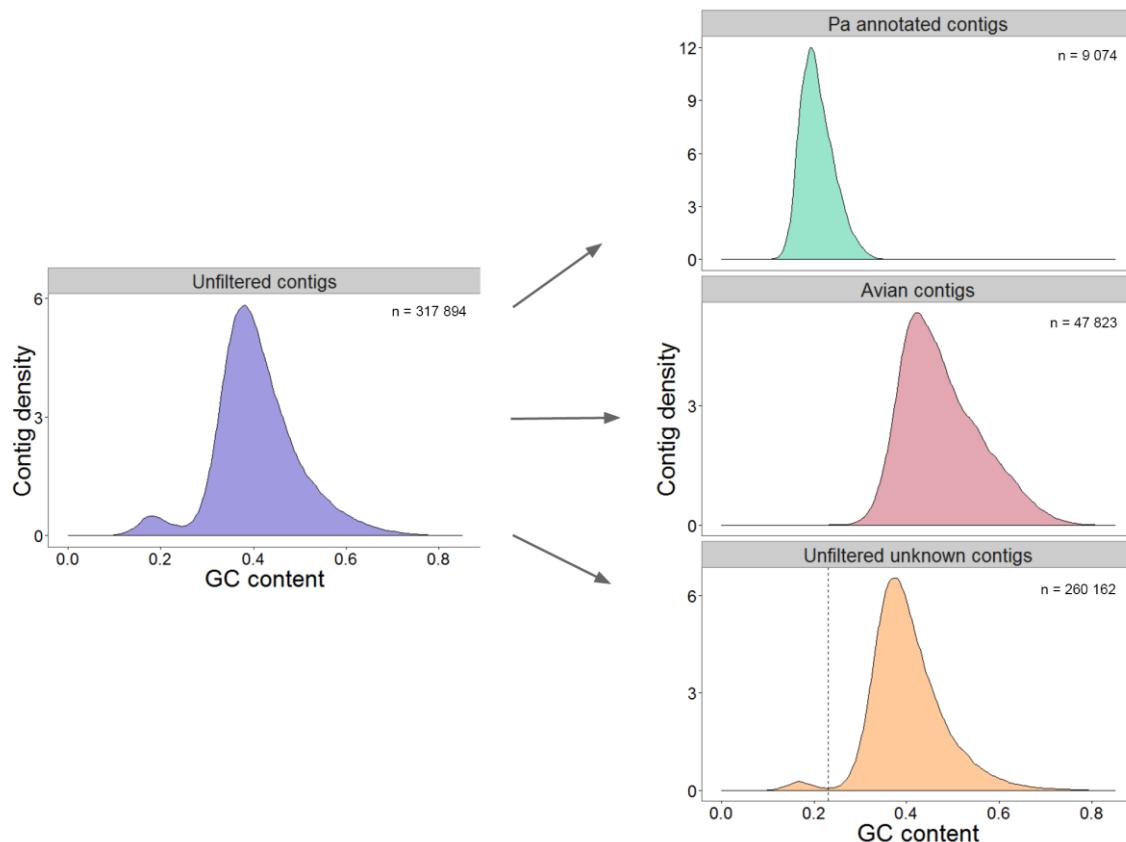
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852 **Figures**

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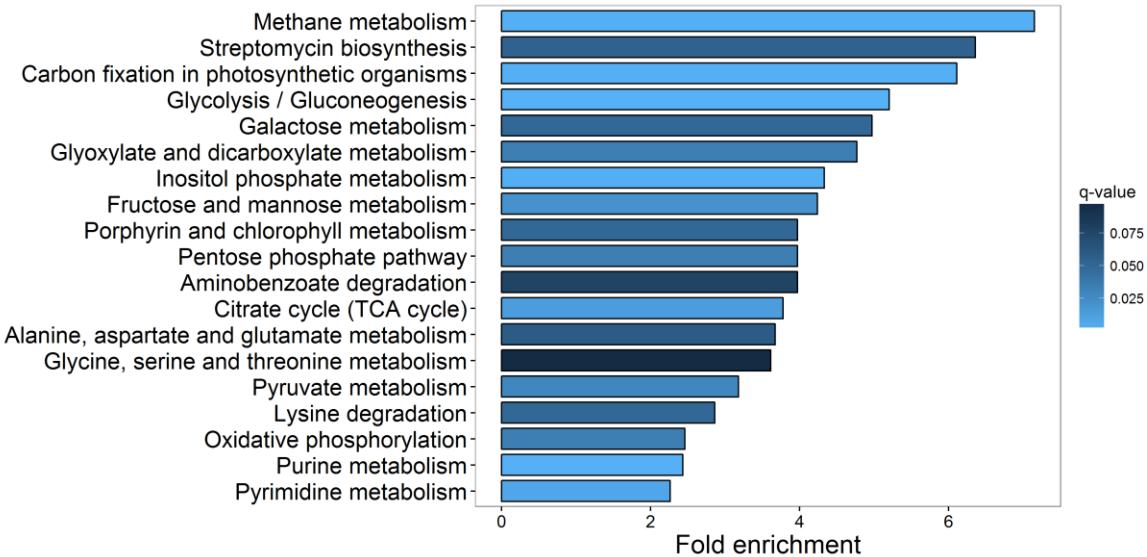
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855 **Figure 1. Density of contig GC content in the *Plasmodium ashfordi* transcriptome.** In (A) the
856 initial, unfiltered assembly, (B) the annotated *P. ashfordi* transcriptome assembly, (C) all contigs
857 giving significant blastx matches to birds, and (D) all unknown contigs before GC % filtering. The
858 arrows indicate assembly versions before and after initial filtering and cleaning steps. Both the initial,
859 unfiltered assembly and the assembly with unknown, unfiltered contigs display a bimodal curve,
860 incorporating both avian and *P. ashfordi* transcripts. The dashed straight line in (D) indicates the 23%
861 GC cut-off where the unknown transcripts with lower GC content were extracted, filtered, and later
862 included in the final *P. ashfordi* assembly as unannotated parasite transcripts.

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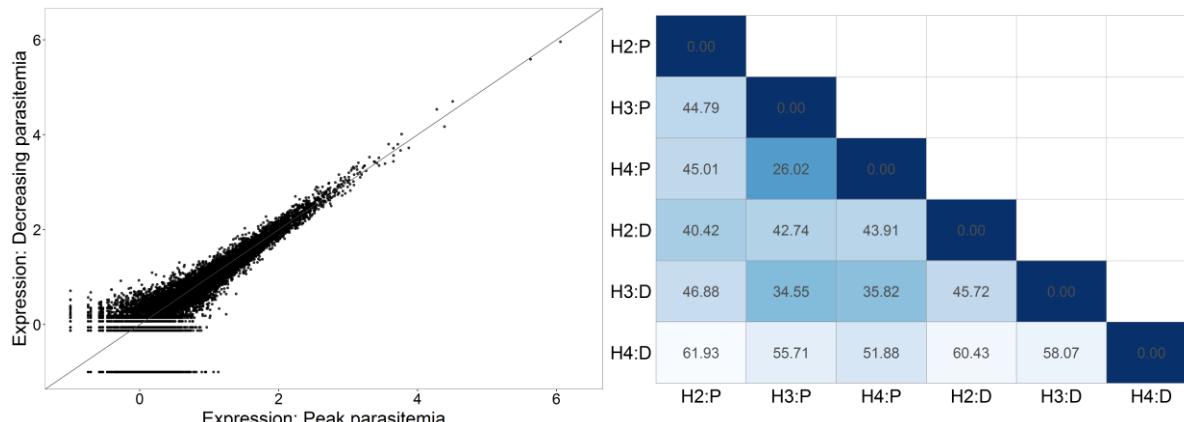


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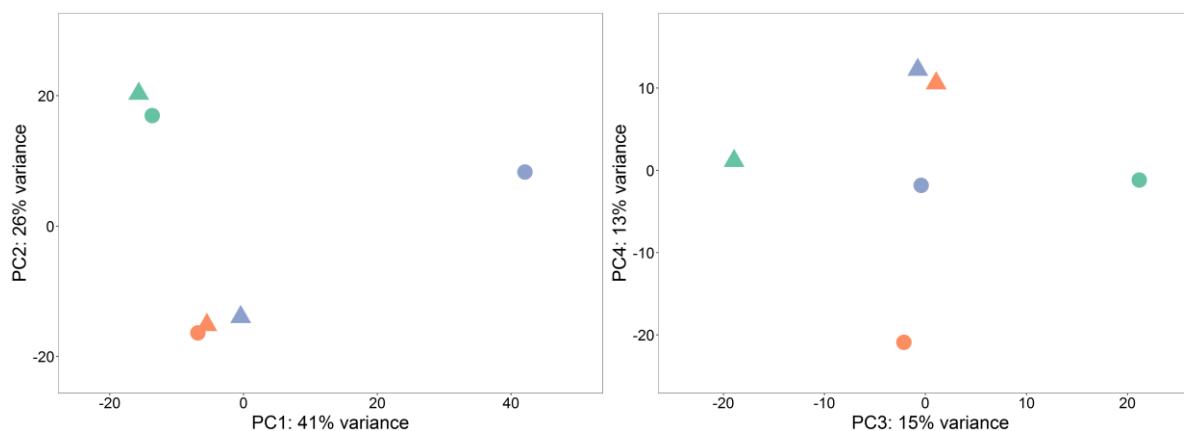
868 **Figure 2. Enriched metabolic pathways in the *Plasmodium ashfordi* transcriptome.** Significantly
869 enriched metabolic pathways based on all transcripts in the annotated assembly. The x-axis denotes
870 fold enrichment of genes in respective pathway compared to the annotation background of *P.*
871 *falciparum*. Color gradient indicates p-values after Benjamini and Hochberg correction (q-value),
872 where a lighter blue color signifies increased statistical significance.
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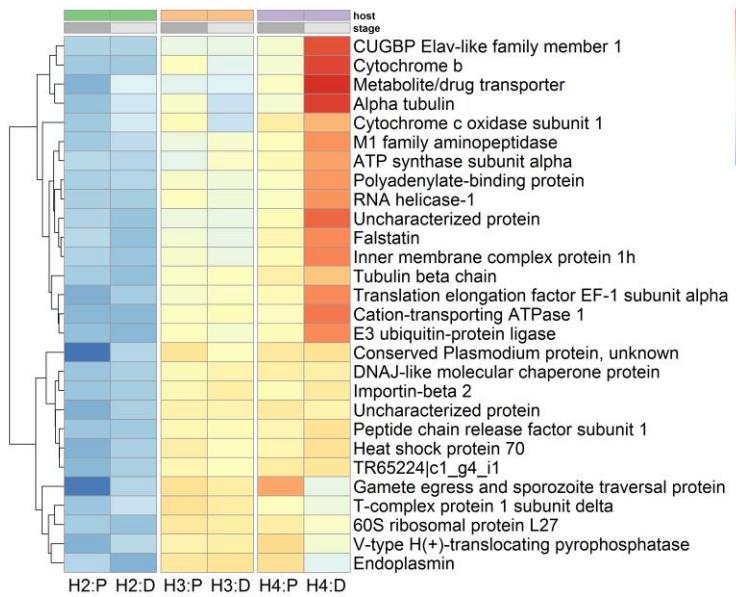
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880 **Figure 3. *Plasmodium ashfordi* gene expression in individual hosts during two infection stages.**

881 (A) Scatterplot displaying expression levels of all transcripts in the *P. ashfordi* transcriptome ($n = 11$
882 954). The axes show log-transformed normalized mean expression values + 0.1 during peak
883 parasitemia stage (x-axis; $n = 3$) and decreasing parasitemia stage (y-axis; $n = 3$). (B) Heatmap
884 portraying Euclidian distance measures between parasite expression profiles in different hosts during
885 two time points. Lighter color signifies greater distance. H = host, P = peak parasitemia, and D =
886 decreasing parasitemia. The distances between parasite transcriptomes in the decreasing parasitemia
887 stage and to the samples from same host sampled during peak parasitemia stage constitute the shortest
888 distances for all the samples during decreasing parasitemia. (C-D) Principal component analysis
889 (PCA) plots show clustering of samples based on variation in regularized log-transformed normalized
890 gene expression. (C) shows principal component 1 and 2, and (D) shows principal component 3 and 4.
891 Colors of parasite transcriptomes illustrate which host they are sampled from: host 2 = green, host 3 =
892 orange, host 4 = purple. Triangles and circles, respectively indicate transcriptomes sampled during
893 peak and decreasing parasitemia stages.

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898 **Figure 4. Expression of *Plasmodium ashfordi* transcripts differentially expressed between**
899 **individual hosts.** Heatmap of relative gene expression levels of 28 significant transcripts (rows)
900 between parasites in different hosts (columns). Warmer color signifies higher expression, and blue
901 color indicates lower transcript expression. Hierarchical clustering of genes is portrayed with a
902 dendrogram. H = host, P = peak parasitemia (dark grey), and D = decreasing parasitemia (light grey).
903 Same color scheme for hosts as in Figure 3C-D applies (host 2 = green, host 3 = orange, host 4 =
904 purple). To compare across genes, expression levels have been normalized with respect to library size,
905 regularized log-transformed, and scaled and centered around zero to give Z-scores.

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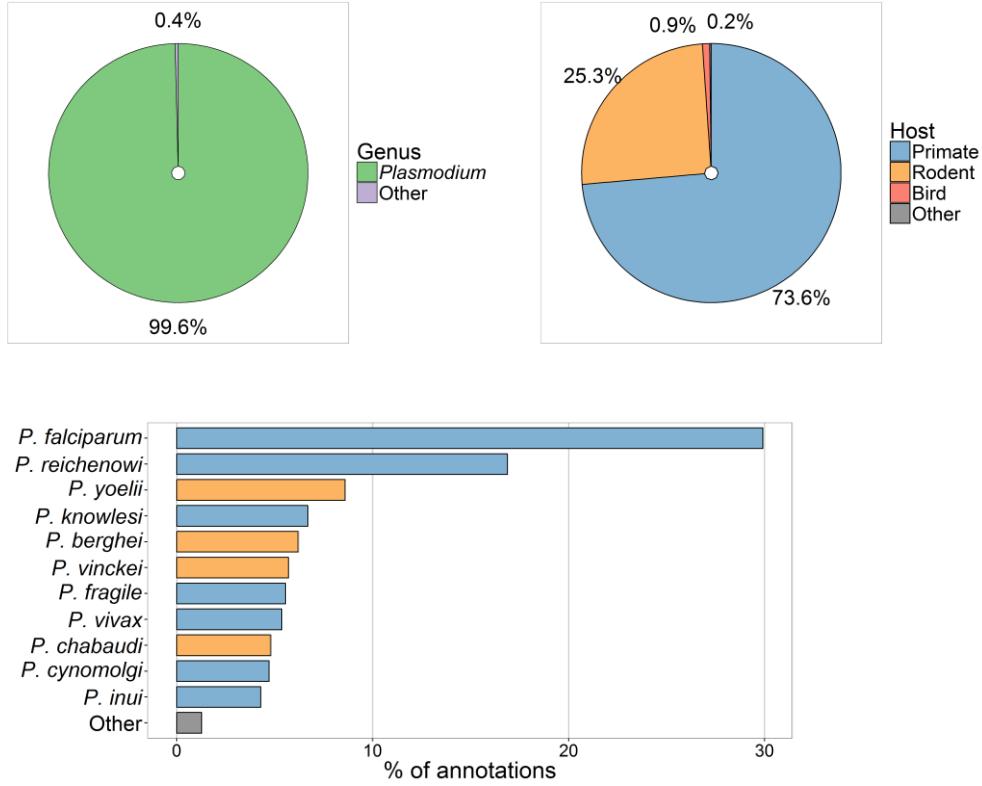
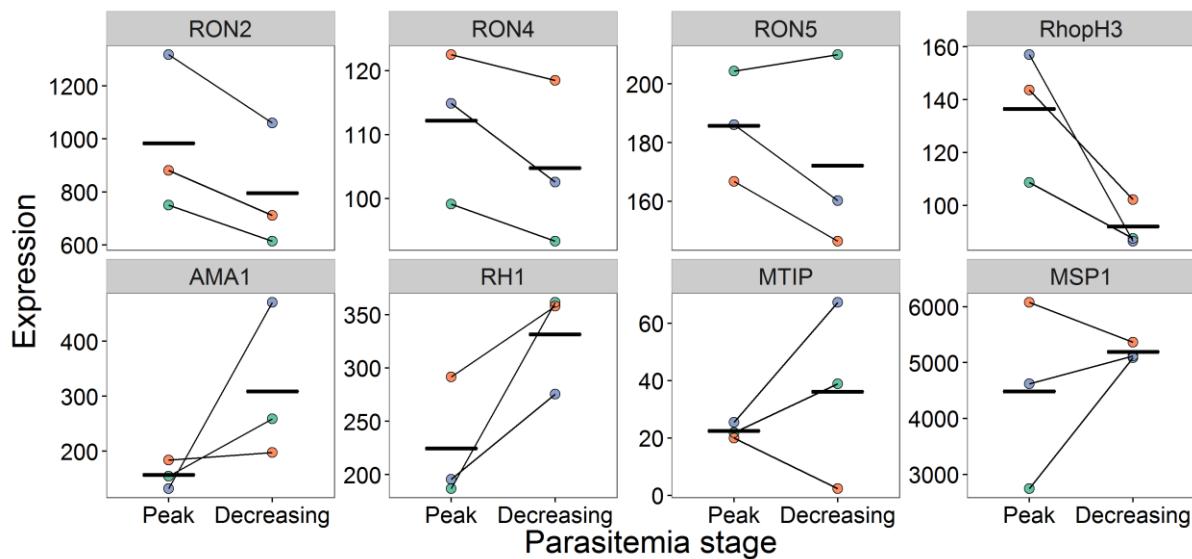


Figure 5. Distribution of apicomplexan parasites significantly matching the *Plasmodium ashfordi* transcripts. (A) Pie chart showing the distribution of *P. ashfordi* annotations giving significant blast matches to *Plasmodium* spp. and parasites of other genera. **(B)** Pie chart showing the distribution of annotations giving significant matches to parasites infecting primates, rodents, birds, and other hosts. **(C)** Bar plot displaying the percentage of annotated contigs giving significant matches to parasite species. Similar color scheme as in (B) applies, i.e. blue signifies a parasite of primates, orange a parasite of rodents. The “other” category contains here all other apicomplexan species, including bird parasites, comprising a total of 20 different species (complete list can be found in Supplementary Table S5).

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926

927 **Figure 6. Individual gene expression plots for some of the *Plasmodium ashfordi* transcripts**
928 **involved in host cell invasion.** Line plots displaying normalized parasite gene expression in each
929 individual host over the two sampled parasitemia stages. Host 2 is depicted in green, host 3 in orange,
930 and host 4 in purple. Thick horizontal lines indicate mean expression levels in each stage.

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932

933 **Supplementary Figures**

934

935 **Supplementary Figures S1 – S5.**

936 See attached supplementary pdf file.

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940 **Supplementary Tables**

941

942 Please contact EV for details regarding unpublished supplementary tables.

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944

945 **Supplementary Table S1.** Annotation information of all annotated *P. ashfordi* transcripts (n = 946 7 860).

947 [csv file]

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950 **Supplementary Table S2.** *P. ashfordi* genes annotated with the gene ontology term “kinase activity” 951 (n = 95).

952 [csv file]

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955 **Supplementary Table S3.** Normalized expression levels of all *P. ashfordi* transcripts (n = 11 954) in 956 individual hosts during peak and decreasing parasitemia stages.

957 [csv file]

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960 **Supplementary Table S4.** *P. ashfordi* transcripts that were significantly differentially expressed 961 between host individuals (n = 28).

962 [csv file]

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965 **Supplementary Table S5.** Species distribution matches from the annotated transcripts of *P. ashfordi*.

966 [csv file]

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