Host Transcriptional Responses to High- and Low-Virulent Avian Malaria Parasites

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Abstract

The transcriptional response of hosts to genetically similar pathogens can vary substantially, with important implications for disease severity and host fitness. A low pathogen load can theoretically elicit both high and low host responses, as the outcome depends on both the effectiveness of the host at suppressing the pathogen and the ability of the pathogen to evade the immune system. Here, we investigate the transcriptional response of Eurasian siskins (Spinus spinus) to two closely related lineages of the malaria parasite Plasmodium relictum. Birds were infected with either the high-virulent lineage P. relictum SGS1, the low-virulent sister lineage P. relictum GRW4, or sham-injected (controls). Blood samples for RNA sequencing were collected at four time points during the course of infection, totaling 76 transcriptomes from 19 birds. Hosts infected with SGS1 experienced up to 87% parasitemia and major transcriptome shifts throughout the infection, and multiple genes showed strong correlation with parasitemia. In contrast, GRW4-infected hosts displayed low parasitemia (maximum 0.7%) with a minor transcriptional response. We furthermore demonstrate that the baseline gene expression levels of hosts prior to infection were irrelevant as immunocompetence markers, as they could not predict future pathogen load. This study shows that the magnitude of the host transcriptional response can differ markedly from related parasites with different virulence, and it enables a better understanding of the molecular interactions taking place between hosts and parasites.

Keywords: Plasmodium, gene expression, transcriptome, parasitemia, immune response, virulence.

Introduction

The transcriptional responses of hosts to pathogens have important consequences for disease severity, infection clearance, and host fitness (Adams et al. 2009; Lee et al. 2018a), yet the response can differ to a large degree both across and within host species (De Vos et al. 2005; Jenner and Young 2005; Sarson et al. 2008). Some hosts seem to respond intensely with a high number of activated genes and biological pathways, while the responses of other hosts may stay relatively unchanged (Adams et al. 2009; Idaghdour et al. 2012). For example, the human malaria parasite Plasmodium falciparum can be highly virulent and induce high immune responses resulting in clinical symptoms such as fever, headache, vomiting, and diarrhea (Mibe et al. 2005; World Health Organization 2018). However, P. falciparum can also appear avirulent, resulting in asymptomatic infections (Dal-Bianco et al. 2007; Laishram et al. 2012). Transcriptome analyses of whole blood have shown that humans with febrile malaria infections can have gene expression levels that are remarkably similar to those of individuals with asymptomatic or pre-symptomatic infections (Ockenhouse et al. 2006; Tran et al. 2016). Unfortunately, it has proved difficult to identify the precise cause behind the magnitude of host transcriptional responses to infection because several mechanisms are possible (fig. 1). For example, a minor transcriptional response to an infection with low parasite load could in theory stem from either (1) a host immune response that is effective at suppressing the parasite even at lower levels, (2) an inability of the parasite to successfully proliferate, or (3) a deliberate parasite strategy of maintaining lower levels in order to evade host immunity (fig. 1). As a consequence, before we can start disentangling underlying causes, we first need to evaluate the quantitative and qualitative aspects of the host transcriptional response to pathogens of different virulence.

Malaria parasites of wildlife (Plasmodium spp.) are excellent model organisms for the study of virulence and host responses. These parasites infect a wide range of
vertebrates and some are associated with high virulence and high pathogen load (parasitemia), whereas others are more benign (Valkiūnas 2005; Palinauskas et al. 2008). While *P. falciparum* offers a well-studied human system, experimental malaria infections of birds provide the advantage of following the hosts throughout the course of the disease. Being able to measure hosts prior to infection is particularly valuable since condition and immunocompetence of hosts may play potential roles in the pathogenesis of malaria (Cornet et al. 2014; Ellis et al. 2015). For example, aspects such as dietary intake of antioxidants and hormone levels may have effects on the avian immune response (McGraw and Ardia 2003; Owen-Ashley et al. 2004). Importantly, by using birds, it is possible to control for confounding host factors prevalent in human malaria studies, such as medication, prior infection, age, and diet, together with the possibility of minimizing parasite genetic variation by infecting all host individuals with the same lineage (Palinauskas et al. 2008; Videvall et al. 2017).

*Plasmodium relictum* is a well-known parasite of birds and is considered to be one of the world’s top 100 most invasive species (Alowe et al. 2000; Valkiūnas et al. 2018). The morphologically defined species *P. relictum* consists of several cryptic lineages based on absolute differences in the mitochondrial gene cytochrome b, and the most prevalent lineage is SGS1 (Palinauskas et al. 2007; Hellgren et al. 2009, 2015). As of 2019, SGS1 has been found in 126 bird species comprising 11 orders (Bensch et al. 2009), yet the number of host species it can infect is continuously rising with increased sampling efforts. Interestingly, SGS1 is not only extremely prevalent but also often reaches high parasitemia and can cause morbidity and mortality in many bird species (Palinauskas et al. 2008; Cellier-Holzem et al. 2010; Ellis et al. 2015).

Closely related to SGS1 is the *P. relictum* lineage GRW4 (Valkiūnas et al. 2007, 2018), which also encompasses a broad host range of 80 bird species spanning five orders (Beadell et al. 2006; Bensch et al. 2009). When identified in the blood of wild birds, GRW4 normally displays low parasitemia and seems in general to be quite avirulent with little or no fitness consequences for the host (Bensch et al. 2007; Asghar et al. 2011; Dimitrov et al. 2015). However, naive bird species that have evolved on remote islands without malaria parasites seem to experience exceptionally high parasitemia and mortality when exposed to GRW4 (Atkinson et al. 1995, 2000; Beadell et al. 2006). Crucially, the two *P. relictum* lineages SGS1 and GRW4 are morphologically indistinguishable (Valkiūnas et al. 2018) and differ by only 1.8% nucleotides in cytochrome b (Bensch et al. 2009). It is possible that SGS1 and GRW4 should be considered separate (cryptic) species, as they have also been shown to harbor additional differences in their nuclear genes (Hellgren et al. 2013, 2015; Garcia-Longoria et al. 2014). However, the moderate mtDNA sequence similarity suggests a fairly recent evolutionary split.

In this study, we evaluate the host transcriptional response to malaria parasites of low and high virulence. We experimentally infected Eurasian siskins (*Spinus spinus*)
with either of the two closely related *P. relictum* lineages, SGS1 and GRW4, and followed the course of infection for 36 days. Siskins are susceptible to both lineages, although SGS1 can reach high parasitemia and is considered highly virulent, whereas GRW4 results in low parasitemia and has low virulence in this host. Parasitemia levels have previously been strongly associated with virulence in *Plasmodium* in general (Mackinnon and Read 1999), as well as for this particular host-pathogen combination (Palinauskas et al. 2008; Dimitrov et al. 2015). We sampled peripheral blood on day 0 (before infection) and 8, 20, and 36 days postinoculation and sequenced a total of 76 transcriptomes from 19 bird individuals using Illumina RNA sequencing. The transcriptional responses of both SGS1-infected and GRW4-infected birds were then analyzed against control birds. This setup allowed us to evaluate genomewide gene expression of an avian host, both qualitatively (which genes/functions) and quantitatively (how much; fig. 1), in response to a high-virulent and a low-virulent parasite. Specifically, we examined the questions that follow: (1) Do SGS1 and GRW4 elicit high or low transcriptional responses (fig. 1) in the hosts? (2) Which genes and biological processes are involved in the host response to each parasite lineage? (3) Do baseline gene expression levels in hosts prior to infection (initial state) predict their future parasitemia? (4) Finally, are there specific host genes that correlate their expression with parasitemia? We examine the answers to these questions in light of the patterns and potential mechanisms outlined in figure 1.

**Material and Methods**

**Experimental Setup**

The experiment was carried out in 2013 at the Biological Station Rybachy of the Zoological Institute of the Russian Academy of Sciences on the Curonian Spit in the Baltic Sea (55°05′N, 20°44′E). Experimental procedures were approved by the International Research Cooperation Agreement between the Biological Station Rybachy of the Zoological Institute of the Russian Academy of Sciences and Institute of Ecology of Nature Research Centre (permit 25-05-2010). Wild juvenile Eurasian siskins (*Spinus spinus*) were caught with mist nets in the summer and housed in aviaries. Siskins have proved to be excellent study organisms for avian malaria infection experiments (Palinauskas et al. 2008, 2011; Videvall et al. 2015, 2017; Weinberg et al. 2019). They are abundant at the study site, suitable to house in captivity, and susceptible to several *Plasmodium* species. In addition, when caught early in the summer, juvenile siskins are still uninfected due to the absence of susceptible mosquitoes. Nevertheless, to ensure no prior infection, all birds were screened for parasites with both molecular polymerase chain reaction screening (Hellgren et al. 2004) and microscopic blood smear examination (Valkiūnas et al. 2008).

We used two mitochondrial lineages of the ubiquitous avian malaria species *Plasmodium relictum*: SGS1, which is highly virulent in siskins, and GRW4, which has low virulence in siskins. Nine birds were inoculated with SGS1, five birds were inoculated with GRW4, and an additional five birds were injected with blood free from parasites to act as control birds. More of the SGS1-infected birds were included to make more accurate correlation tests with parasitemia levels. The full experiment incorporated additional birds, and a detailed description has been provided by Mukhin et al. (2016). The SGS1 strain was originally isolated from red crossbill (*Loxia curvirostra*) and GRW4 from garden warbler (*Sylvia borin*). Infected blood samples frozen in liquid nitrogen were thawed, and parasites were multiplied in crossbills and siskins (Palinauskas et al. 2008). A single subinoculation of 0.15 mL blood mixture (3.7% sodium citrate, 0.9% saline, and blood, 1:5:4) was made into the pectoral muscle of recipient birds (Palinauskas et al. 2008). The mixture contained blood from one siskin donor and one crossbill donor for each of the two experiments. Two donor birds were needed to obtain an amount of blood that was sufficient to infect all recipient birds at the same time, and the donors were selected based on having similar parasitemia levels. All inoculated birds were exposed to the same clonal strain and quantity of either SGS1 or GRW4. The intensity of mature meronts in the SGS1 inoculation was 0.075%, and the dose of inoculated meronts was ∼1.6e5 parasites per bird. For the GRW4 inoculate, the mature meront parasitemia was 0.09%, and ∼1.9e5 meronts were inoculated into each individual. The control birds were injected simultaneously with the inoculated birds but instead with blood mixture derived from a crossbill donor bird free of parasites. The procedure of injecting peripheral blood is likely to induce a minor immune response in both control and inoculated birds directly after transfusion. However, the survival time of foreign red blood cells after heterologous blood transfusion has been estimated to be only 0.1–2.6 days in birds (Sandmeier et al. 1994; Degernes et al. 1999). It is therefore highly unlikely that an immune response to blood cells would still be present 8 days later during the first postinjection RNA sampling. Nonetheless, if any genes would show extended differential expression 8 days later in response to blood injection alone, these genes would not become significant in our analyses because of the comparison against control birds.

Blood for quantitative estimations of parasitemia (%) infected red blood cells) was sampled throughout the experiment every fourth day in heparinized microcapillaries by puncturing the brachial vein and subsequently smeared
on glass slides for microscopic examinations (Godfrey et al. 1987). Blood for RNA sequencing was collected in empty tubes and immediately frozen in liquid nitrogen and stored at −80°C until extraction. The birds were observed continuously throughout the duration of the experiment and substantial efforts were made to minimize handling time. RNA samples were collected from all 19 birds on day 0 (before infection) and 8, 20, and 36 days postinoculation. Altogether, this resulted in a total of 190 data points of parasitemia levels and 76 blood samples for RNA sequencing.

**RNA Extraction and Sequencing**

Total RNA was extracted from 20 μL of peripheral blood cells using 1,000 μL Trizol LS reagent (Invitrogen Carlsbad, CA) and homogenized using a vortex. The samples were then incubated at room temperature for 5 min before 200 μL of chloroform (Merck, Darmstadt, Germany) was added. Following another room-temperature incubation for 3 min, the samples were centrifuged at 11,000 rpm for 15 min at 4°C. The supernatant was then transferred to new tubes, and using an RNeasy Mini kit (Qiagen, Hilden, Germany), we followed the manufacturer’s protocol starting at point 4 by adding one volume of 70% ethanol to the lysate. Total extracted RNA was shipped on dry ice to Beijing Genomics Institute (BGI) in China for RNA quality control, DNAse treatment, ribosomal RNA reduction, and amplification using the Smarter Ultra Low kit (Clontech). BGI performed library preparation, complementary DNA synthesis, and 100-bp paired-end sequencing using Illumina HiSeq 2000.

**Noncoding RNA Details**

RNA sequencing studies of eukaryotes do not often incorporate small noncoding RNAs in their data, because poly(A)-selection during library preparation is a common protocol that specifically targets protein-coding messenger RNA (mRNA) molecules. In this study and in our previous avian transcriptome study (Videvall et al. 2015), the library preparation protocol depleted ribosomal RNA in favor of poly(A)-selection, which is the reason we were able to retrieve noncoding RNA sequences. Additionally, while the mature microRNA (miRNA) molecule only consists of ~22 nt, the immature miRNAs are transcribed as long primary transcripts (pri-miRNAs), which can be several kilobases long (Lee et al. 2002). The pri-miRNAs are cropped into an intermediate ~70 nt precursor-miRNA (pre-miRNA) molecule by the enzyme Drosha, and then exported out of the nucleus to be cleaved by the enzyme Dicer into the mature short form (Lee et al. 2004). The miRNA sequences present in our data are therefore likely derived from pri-miRNA or pre-miRNA molecules.

**Data Analyses**

Raw reads were filtered with the Illumina chastity filter and quality-screened using FastQC (ver. 0.10.1; www.bioinformatics.babraham.ac.uk/projects/fastqc). A combined total of 1.11 billion paired 100-bp reads passed read quality control. High-quality reads were mapped with TopHat2 (ver. 2.0.9; Kim et al. 2013) to the unmasked zebra finch (Taeniopygia guttata) genome (Warren et al. 2010), obtained from Ensembl (ver. 3.2.4; Cunningham et al. 2015). Reads were allowed a maximum mismatch rate of 20% and guided in TopHat2 with the zebra finch transcriptome, an approach that has worked well with sikin reads previously (Videvall et al. 2015). In total, 678 million unique reads were successfully mapped unambiguously to the genome. Although Plasmodium genomes are highly differentiated from bird genomes, we avoided any potential crossmappings between the organisms by removing all reads that also aligned with the P. relictum genome (Böhme et al. 2018) from the data set. Filtered and mapped reads were then counted using HTSeq (ver. 0.11.2; Anders et al. 2015).

Differential gene expression analyses were performed with DESeq2 (ver. 1.18.1; Love et al. 2014) in R (ver. 3.4.4; R Core Team 2017). Counts were normalized for library size differences using the geometric mean and modeled with a negative binomial distribution. This normalization procedure effectively makes all host transcriptomes comparable, regardless of the birds’ parasitemia levels. Variance-stabilized transformation of counts was used to perform principal component analyses (PCAs) without bias (Anders and Huber 2010). Relative changes on the PC1 and PC2 dimensions over time were correlated with arcsine-square-root-transformed parasitemia levels. Variance-stabilized transformation of counts was used to perform principal component analyses (PCAs) with arcsine-square-root-transformed parasitemia levels. Variance-stabilized transformation of counts was used to perform principal component analyses (PCAs) using the Pearson correlation coefficient. We also performed the same tests with untransformed parasitemia values (%) using the ranked Spearman correlation and obtained highly similar results with slightly higher correlation coefficients and lower P values (data not shown). Differentially expressed genes were corrected for multiple testing using the Benjamini and Hochberg false discovery rate (FDR), and the corrected P values are reported throughout the article as Q values. Genes with Q < 0.1 were considered significantly differentially expressed for the test between GRW4-infected birds and controls; however, a stricter alpha level of significance (Q < 0.05) was used for the SGS1-infected birds to account for their higher sample size (n = 9).

Linear mixed effects (LME) models, incorporating bird identification (ID) as random effect and day of experiment...
as fixed effect, were used to calculate associations between log-transformed normalized expression levels and arcsine-square-root-transformed parasitemia levels of the SGS1-infected birds using the nlme R package (Pinheiro et al. 2016). LMEs between gene expression at day 0 and parasitemia at a later time point (day 8 or 20) were performed in a similar way, controlling for bird ID but without day in the model. P values from the LMEs were corrected with the FDR, and genes with $Q < 0.05$ were considered statistically significant. We analyzed functional annotations and overrepresentation of gene ontology (GO) terms in Cytoscape (ver. 3.7.1; Cline et al. 2007), using Bingo (ver. 3.0.3; Maere et al. 2005). The ontology file was obtained from http://www.geneontology.org (ver. 2019-01-14), and the GO analyses were performed using a hypergeometric test for overrepresentation and corrected for multiple testing with the FDR. Functional groups with at least two genes and $Q < 0.1$ were regarded as statistically significantly overrepresented. Plots were made with ggplot2 (Wickham 2009).

**Results**

**Parasitemia over the Course of Infection**

Parasitemia levels (% infected red blood cells) of lineage GRW4 differed markedly from those of SGS1 over the course of infection. While all GRW4-infected birds experienced low parasitemia (maximum = 0.7%), SGS1-infected birds suffered comparatively high parasitemia (maximum = 87%; fig. 2; table S1; tables S1–S17 are available online). Both infected groups showed large variation in parasitemia between host individuals and over time, as some birds experienced relatively low and some very high levels (mean ± SD on day 8; GRW4: 0.35% ± 0.32; SGS1: 31.27% ± 24.04). Three GRW4-infected birds experienced a peak in parasitemia around day 8 postinoculation, and two with lower levels peaked at day 12 (fig. 2). In comparison, peak of parasitemia in the SGS1-infected birds generally took place later, with two individuals reaching maximum parasitemia at day 8, one at day 12, and five at day 16 (fig. 2). While several of the birds experienced

![Figure 2](image-url)

**Figure 2:** Parasitemia (% infected red blood cells) over time in birds infected with a low-virulent lineage (GRW4; top) and a high-virulent lineage (SGS1; bottom) of the parasite *Plasmodium relictum*. Colors indicate different host individuals. Note the large discrepancy in the Y-axes between the two parasite lineages.
recrudescence of the infection at later time points, the individual with the highest initial parasitemia levels also experienced the most severe recrudescence (28%) at day 28. The five control birds remained uninfected (0% parasitemia) during the entire experiment (table S1).

**Differences in Transcriptional Responses between SGS1- and GRW4-Infected Hosts**

Large overall gene expression differences between the SGS1- and GRW4-infected birds were evident in the host transcriptomes. The majority of the variation in a combined PCA was explained by the SGS1-infected hosts (fig. 3A), in particular 8 days postinoculation. The first PC dimension was strongly associated with parasitemia in the early stage of infection, as parasitemia levels at day 8 were strongly correlated with the relative change of individuals from day 0 to day 8 in PC1 values ($r = 0.82$, df = 17, $P = 1.6e-05$; fig. 3B) but not with PC2 values ($r = 0.38$, df = 17, $P = .11$). Instead, the second PC dimension was strongly correlated with parasitemia at day 20 ($r = -0.58$, df = 17, $P = .009$), while the PC1 was not ($r = -0.07$, df = 17, $P = .77$). A separate PCA of the hosts infected with SGS1 showed a clear circular trajectory of gene expression over the course of infection. All birds started in the top left of the PCA pre-infection, with a large movement to the right on the PC1 dimension at day 8, a subsequent drop over the PC2 dimension at day 20, and later approached their original, uninfected state at day 36 (fig. 3C). In contrast, the GRW4-infected birds did not show any such circular trajectories in their own PCA (fig. 3D), but displayed instead a slight movement along PC1 after infection and a minor drop in the PC2 dimension from day 0 to day 8 postinoculation. The small variation in host transcriptome response for the GRW4-infected birds at days 20 and 36 corresponded with their extremely low parasitemia levels during these days (fig. 2).

**Major Transcriptome Response in SGS1-Infected Hosts**

Eight days after inoculation, the SGS1-infected birds showed a total of 437 differentially expressed genes compared with those of control birds, of which 273 were upregulated and 164 downregulated (fig. 4). The most significant genes during this time point were all upregulated in the infected birds and classified as various miRNA, uncharacterized, and heat-shock genes (table S2). GO analyses of the upregulated genes in SGS1-infected birds during day 8 revealed multiple overrepresented processes related to “response to virus,” “regulation of cell death,” and “response to stress.” More specific immune processes were also overrepresented, including “positive regulation of cytokine production,” “positive regulation of NF-kappaB transcription factor activity,” and “regulation of interferon-gamma-mediated signaling pathway” (table S3). Other GO categories overrepresented multiple times included those related to telomere maintenance, posttranscriptional regulation of gene expression, and protein folding (table S3). The most significant downregulated genes in the SGS1-infected birds during day 8 were ANK1, PRKD1, and a gene coding for glutamine synthetase (table S2). Biological processes that were overrepresented among the downregulated genes involved primarily around “vasodilation,” “gene silencing by miRNA,” and the “apoptotic signaling pathway.” Other overrepresented terms included molecular binding functions and immune-related processes such as “antigen receptor-mediated signaling pathway” and “leukocyte migration” (table S4).

At day 20, when the SGS1-infected birds had recently passed their peak in parasitemia and all but one exhibited highly reduced parasitemia levels (fig. 2), their transcriptomes showed a much larger number of genes ($n = 664$) as differentially expressed (fig. 4). Of those genes, 292 were upregulated and 372 were downregulated in infected birds. In contrast to day 8, however, the most significant protein-coding genes during day 20 were now downregulated, and these included, for example, heat-shock proteins, regulatory genes, DNA repair factors, and the pro-inflammatory cytokine interleukin-1β (table S5). GO analyses of the downregulated genes showed that overrepresented processes were almost exclusively related to “blood coagulation,” “interaction with symbiont,” and “response to stress” (table S6). The most significant upregulated genes in SGS1-infected birds on day 20 were RIPK1, PPA1, RREB1, and miRNA genes (table S5). The upregulated genes at day 20 showed significant overrepresentation within ontology terms primarily linked to the stress response, “positive regulation of wound healing,” “tRNA aminoacylation,” and various metabolic processes (table S7).

During the last time point, 36 days postinoculation, the transcriptional response in SGS1-infected birds had largely diminished, with 15 upregulated and 41 downregulated genes (fig. 4). The most significant upregulated genes at this stage of the infection primarily consisted of miRNA, transferases, and receptor proteins like the interleukin 21 receptor. The most significant downregulated genes were TLNRD1, IGF1R, and PGRMC2 (table S8). The 15 upregulated genes at day 36 showed only significant overrepresentation within the “cell cycle process” (table S9), while the most significant overrepresented GO terms for the downregulated genes included “positive regulation of B cell proliferation/activation,” “defense response to gram-negative bacterium,” and various signaling and binding functions (table S10).
Minor Transcriptome Response in GRW4-Infected Hosts

In contrast to the SGS1-infected birds, the GRW4-infected birds had very few genes differentially expressed during the entire course of infection (fig. 4). Eight days after inoculation, when most of the GRW4-infected birds experienced peak parasitemia, seven genes were upregulated (of which five were miRNAs) and six were downregulated compared with those of control birds (table S11). The differentially expressed annotated genes had functions primarily within “T cell extravasation,” “apoptotic signaling pathway,” and the “glutamine biosynthetic process” (table S11). Two miRNA genes were upregulated in GRW4-infected birds also during day 20, but no genes were differentially expressed at day 36 (fig. 4). To further evaluate how hosts responded to GRW4, we compared gene expression of SGS1-infected birds with that of the GRW4-infected birds. At day 8, we found 306 differentially expressed genes, at day 20 we found 435, and at day 36 we found 15 (tables S12–S14). These results revealed that the

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**Figure 3:** Principal component analyses (PCAs) of transcriptome variation in control birds and birds infected with malaria parasites of either high (SGS1) or low (GRW4) virulence. A, PCA of all transcriptome samples with geometrical shapes outlining the distribution of groups. B, Transcriptome changes over the PC1 dimension from day 0 to 8 (Y-axis) is strongly correlated with parasitemia at day 8 (arcsine-transformed values; X-axis). The blue line represents the linear regression, and the shaded area represents the 95% confidence interval. C, D, PCAs of individuals infected with the high-virulent parasite lineage SGS1 (C) and the low-virulent lineage GRW4 (D). Green illustrates individuals that had no infection (all birds at day 0 and control individuals throughout the experiment), the various shapes represent day of experiment, and gray lines illustrate the path of individuals over time in the PCA plots.
Figure 4: Gene expression levels comparing malaria-infected birds with control birds at three different time points during infection. The first row displays birds infected with the low-virulent lineage GRW4, while the second row displays birds infected with the high-virulent lineage SGS1. Expression levels have been normalized and log transformed (+0.1). Red circles illustrate significantly differentially expressed genes and black circles illustrate nonsignificant genes. The diagonal line shows the 1:1 relationship.
SGS1-infected birds showed similar but less pronounced gene expression differences from GRW4-infected birds as they did from control birds. In general, the birds infected with GRW4 changed the expression of their genes in the same direction as the SGS1-infected birds, as 85% (n = 232) of the genes that were upregulated in SGS1-infected birds at day 8 also showed higher expression in GRW4-infected birds relative to control birds (log, fold change > 0; 91% [n = 267] at day 20 and 100% [n = 15] at day 36). However, this response was quantitatively very small, which is why the GRW4-infected birds showed large significant differences from the SGS1-infected birds (tables S12–S14) but minor differences from the controls (table S11).

Dynamic Transcriptional Responses to SGS1 over the Course of Infection

Evaluating host responses to SGS1 across time points showed large dissimilarities in the genes that were differentially expressed. Of the 273 genes upregulated during day 8 postinoculation, 51 (19%) remained upregulated during day 20. Similarly, of the 164 downregulated genes during the eighth day, 30 (18%) were also downregulated at day 20 (tables S2, S5). A total of five genes were upregulated in SGS1-infected birds during all three time points of the infection (tables S2, S5, S8). These long-lasting highly expressed genes coded for CNP, MHCK-C, an uncharacterized protein, and two miRNA genes. Four genes were downregulated during the entire infection: VAV3, PRKD1, FGD6, and one gene belonging to the septin 11 family (tables S2, S5, S8). To visually depict gene expression over time, we plotted the expression levels of three highly significantly genes during the course of infection in figure 5.

To investigate whether host gene expression at day 0 (before infection) could predict subsequent parasitemia levels during infection, we performed two sets of analyses. A PCA of transcriptomes at day 0 showed that the birds’ relative position on the first two PC dimensions did not correlate with their parasitemia levels at day 8 (PC1: \( r = -0.62, P = .08 \); PC2: \( r = 0.12, P = .75 \)), nor day 20 (PC1: \( r = -0.44, P = .24 \); PC2: \( r = 0.32, P = .39 \)). Furthermore, no genes showed significant associations between expression levels at day 0 and subsequent parasitemia levels in the SGS1-infected birds (LME at day 8: \( F < 123.1, Q > 0.07 \); LME at day 20: \( F < 70.4, Q > 0.23 \)).

Regulatory Genes Show Positive Correlation with Parasitemia Levels

We identified 411 genes that showed a positive correlation between gene expression and parasitemia levels in the SGS1-infected birds (Q < 0.05; table S15). The most significantly correlated genes were comprised of miRNA genes, uncharacterized genes, and CCDC51. GO analyses of the genes positively correlated with parasitemia showed that they primarily belonged to regulatory processes, such as “positive regulation of transcription by RNA polymerase I,” “posttranscriptional regulation of gene expression,” “covalent chromatin modification,” and “histone modification” (table S16). Multiple molecular binding functions and cellular components related to gene regulation and processing of small noncoding RNAs were also significant, for example, “U5 and U6 snRNA binding,” “RNA-induced silencing complex (RISC),” “mRNA cleavage factor complex,” “CCAAT-binding factor complex,” and “RNA polymerase III transcription factor complex” (table S16). Other notable overrepresented processes among genes positively correlated with parasitemia were those related to telomerase, including “telomerase RNA stabilization” and “telomerase RNA binding,” and responses to the immune-related steroid hormones glucocorticoid and corticosteroid (table S16).

A total of 342 genes showed a negative correlation with parasitemia (Q < 0.05; table S15). Some of the most significant genes included transcription factor ELF1, kinase-related genes AK2 and PIK3R1, CCPG1, and RNF103. GO analyses revealed a broad involvement in “protein catabolic processes” and “protein polyubiquitination” (table S17). Another group of highly overrepresented functions were those related to oxygen, including “oxygen transport,” “oxygen binding,” “oxygen carrier activity,” and “hemoglobin complex.” Finally, “regulation of response to oxidative stress” was also found to be overrepresented among genes negatively correlated with parasitemia (table S17).

Discussion

In this study, we demonstrated that the avian transcriptome displays substantial differences in response to infection with a low-virulent and a high-virulent malaria parasite. We found that (1) the high-virulent Plasmodium relictum lineage SGS1 elicited a very high transcriptional response in the hosts compared with a minor response in the birds infected with the less virulent sister lineage GRW4 (the response to GRW4 was generally in a similar direction as the response to SGS1 but substantially lower with only a few genes differentially expressed during peak parasitemia); (2) genes involved in the transcriptional response to SGS1 had functions primarily within the immune system, stress response, cell death regulation, metabolic processes, vasodilation, and transcriptional regulation; and (3) the baseline gene expression levels in hosts prior to infection could not predict their future parasitemia levels. Finally, we identified hundreds of host genes
correlating expression levels with parasitemia. Together, these results indicate that the magnitude of the host transcriptional response is highly proportional to pathogen load (fig. 1A, 1D). As a result, malaria infections with low parasitemia are not necessarily a consequence of a high transcriptional response suppressing the parasite (fig. 1C), but instead it is likely that the parasite is unable to successfully proliferate, or has evolved low replication rates, or that the host is highly effective at controlling the infection even at low gene expression levels (fig. 1A).

The peak parasitemia levels of SGS1 (maximum 87%) were more than 100 times higher than those of GRW4, and the resulting infection was longer and involved more severe recrudescence. These high parasitemia levels are in

Figure 5: Gene expression levels of three highly significant genes during the course of infection in control birds, birds infected with a low-virulent malaria lineage (GRW4), and a high-virulent lineage (SGS1). A. The most significant differentially expressed gene (ENSTGUG00000018706) 8 days postinoculation. B. The most significant differentially expressed gene 20 days postinoculation (ENSTGUG00000010855). C. The most significant gene correlated with parasitemia levels (ENSTGUG00000018567). The first column shows control birds, the second shows GRW4-infected birds (low virulence), and the third shows SGS1-infected birds (high virulence). Colored lines illustrate host individuals, and expression levels have been normalized and log transformed (+1).
line with previous infection experiments of siskins, crossbills, and canaries, which have found that SGS1 is able to reach severe parasitemia in these birds (Palinauskas et al. 2008; Dimitrov et al. 2015; Ellis et al. 2015). While the low parasitemia of GRW4 in our study (maximum 0.7%) caused a minor transcriptome shift in the same direction as SGS1-infected birds, this was only evident during day 8 of infection, when 13 genes were differentially expressed. In contrast, the high parasite load in SGS1-infected birds elicited an elevated transcriptome response involving several hundred genes. The strong associations between parasitemia and host gene expression were first evident in the PCA (fig. 3) where the PC1 and PC2 primarily explained parasitemia levels 8 and 20 days postinoculation. Second, hundreds of genes were differentially expressed during SGS1 infection compared with only a few genes during GRW4 infection. And third, multiple host genes showed strong correlation with parasitemia levels. The circular PCA trajectories of all SGS1-infected birds during the course of infection strongly resemble the disease curves described by Schneider (2011), and just like in mice with malaria infection (Torres et al. 2016), the individuals in our study with higher parasitemia levels made larger loops over the PCA plot. While all individuals were resilient enough to recover from the infection (Schneider 2011), approaching a closure of the loops, the individuals making smaller loops through the PCA likely had higher resistance to the parasite. Additional health measurements in the future would be useful to further evaluate the path of tolerance in the hosts. Our results in birds also agree with studies of malaria infection in humans, where parasite load has shown to be a dominant determinant of human gene expression (Griffiths et al. 2005; Idaghdour et al. 2012; Lee et al. 2018b). Additional studies of transcriptional responses to malaria in nonmammalian hosts will be highly beneficial to draw more general conclusions about the effects of parasitemia on host responses.

Genes that were differentially expressed in the SGS1-infected birds during malaria infection had functions in the immune system, stress response, cell death regulation, metabolic processes, transcriptional regulation, and vasodilation. These functions broadly correspond with our previous study where we evaluated avian transcriptome responses to Plasmodium ashfordi (Videvall et al. 2015), albeit at different time points of the infection. Certain GO terms associated with "response to virus" and "response to bacterium" were overrepresented among differentially expressed genes 8 and 36 days, respectively, into the infection with SGS1. The genes falling under these annotations are primarily regulatory genes with broad involvement in the immune system. For example, NLRCS negatively regulates interferon and nuclear factor κ-light-chain-enhancer of activated B cells (NF-κB) activity and positively drives major histocompatibility complex (MHC) class I and β-2-microglobulin expression (Cui et al. 2010; Meissner et al. 2010), and MyD88 is considered a universal adapter protein as it is use by most toll-like receptors to activate NF-κB (Arancibia et al. 2007). It has been shown that the vast majority of genes responding to P. falciparum malaria are involved in the innate (nonspecific) immune system (Franklin et al. 2009; Yamagishi et al. 2014). Molecular pathways that are commonly activated include the NF-κB, mitogen-activated protein kinases, interferon-γ, and IL-1β (Ockenhouse et al. 2006; Tran et al. 2016; Lee et al. 2018a). Multiple genes related to these pathways were differentially expressed in this study as well, hence the viral and bacterial GO terms likely became statistically overrepresented as a by-product of the upregulated innate immunity. The MHC genes, although associated with malaria (Lee et al. 2018a), have not been possible to evaluate here because they are highly fragmented and not well annotated in the zebra finch genome (Balakrishnan et al. 2010; Warren et al. 2010).

We found multiple miRNA genes in the list of genes most strongly correlated with parasitemia and in the list of genes most upregulated in SGS1-infected birds. MicroRNA genes produce noncoding RNA molecules that have critically important regulatory functions in the vertebrate immune system (Lindsay 2008; Xiao and Rajewsky 2009). The dominant presence of these miRNAs demonstrates their significance in the avian immune response to malaria. This finding strongly agrees with our previous transcriptome analyses of siskins where miRNA genes comprised five of the 10 most significantly upregulated genes during malaria infection (Videvall et al. 2015). The same miRNA genes were again highly upregulated in SGS1-infected birds, either at day 8 or day 20, or during both days of infection. In addition to the miRNA genes themselves, multiple protein-coding genes with direct involvement in the miRNA gene silencing pathway were upregulated during infection. For example, the pri-miRNA cleaving protein Drosha was upregulated at day 20, the pre-miRNA cleaving protein Dicer was upregulated at day 8, and the Mov10 RISC complex RNA helicase protein, which is required for the miRNA gene silencing process, was upregulated at day 20 (tables S3, S5). GO analyses of the upregulated annotated genes also showed that processes related to "gene silencing by miRNA" were overrepresented during infection. Future studies using specific miRNA sequencing techniques (e.g., Jia et al. 2017) will be highly useful to better understand the role of these miRNAs in the avian immune response.

While the genes showing positive correlation with parasitemia were predominantly regulatory, some were involved in functions related to telomere maintenance and the glucocorticoid response. Birds with chronic malaria infection have been shown to have shorter telomeres (Asghar et al. 2015), and genes related to telomerase are
differentially expressed in birds during the disease (Videvall et al. 2015). Corticosterone is the primary glucocorticoid stress hormone in birds, and higher levels have been associated with increased telomere loss and disease-associated mortality (Owen et al. 2012; Herborn et al. 2014). Genes that showed negative correlation with parasitemia were, in contrast, primarily involved in oxygen transport and binding processes, such as hemoglobin and betaglobin. Birds with erythrocytic malaria infection often experience anemia with reduced hematocrit and hemoglobin concentrations (Palinauskas et al. 2008, 2011; Schoenle et al. 2017). The oxygen-related genes could therefore theoretically result in a negative correlation with parasitemia due to a reduced proportion of erythrocytes in the peripheral blood of the SGS1-infected birds (Ellis et al. 2015).

Finally, we found that gene expression levels of hosts prior to infection (day 0) were not associated with future parasitemia levels. This result demonstrates that having a higher baseline immune gene expression does not necessarily confer greater resistance to infection, supporting a highly reactive role of the avian transcriptional response to malaria. Previous studies measuring immunocompetence in birds have found conflicting results of host traits like body condition prior to an immune challenge, and different components of the immunity often show complex relationships (Råberg et al. 2003; Matson et al. 2006; Cornet et al. 2014). However, specific immune parameters and disease resistance seem primarily independent of baseline immunity in birds (Hegemann et al. 2013; Vermeulen et al. 2016; cf. Matson et al. 2012), further signifying the avian immune system’s responsive capacity.

In conclusion, our study provides important insights into the transcriptional activities of passerine birds during malaria infection, including a response that is highly proportional to parasitemia, involving genes of predominantly regulatory character. The results demonstrate that hosts react with high transcriptional responses to a virulent parasite with high parasitemia (fig. 1D) and with low transcriptional responses to a low-virulent parasite (fig. 1A). To further differentiate which mechanisms caused these particular outcomes, future experiments using immunosuppressed hosts or attenuated parasites, for example, would be highly beneficial. The extent to which the magnitude of host responses contributes to the virulence of an infection still remains an open question. Future sequencing efforts of both malaria parasites and their hosts will be useful to further tease apart species-specific responses and evaluate more general patterns of host responses.

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Statement of Authorship

O.H. planned and designed the study. G.V. and V.P. performed the experiment. E.V. performed the bioinformatic and statistical analyses. O.H. provided advice on analyses. E.V. wrote the article with input from all authors.

Data and Code Availability

All sequence data have been deposited in the European Nucleotide Archive at EMBL-EBI (PRJEB35047).

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Plasmodium Responses to Virulence 1083

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