Received: 17 December 2017 | Revised: 28 May 2018 | Accepted: 29 May 2018

DOI: 10.1111/mec.14771

ORIGINAL ARTICLE

WILEY MOLECULAR ECOLOGY

Genome-wide DNA methylation signatures of infection status in Trinidadian guppies (*Poecilia reticulata*)

Juntao Hu[1,2] | Felipe Pérez-Jvostov[1,2] | Léa Blondel[1,2] | Rowan D. H. Barrett[1,2]

[1]Redpath Museum, McGill University, Montreal, Quebec, Canada

[2]Department of Biology, McGill University, Montreal, Quebec, Canada

Correspondence
Juntao Hu, Redpath Museum, McGill University, 859 Sherbrooke Street West, Montreal, Quebec, Canada H3A 0C4.
Email: juntao.hu@mail.mcgill.ca

Funding information
China Scholarship Council Fellowship, Grant/Award Number: 201406350023; NSERC CREATE, Grant/Award Number: 2015-466283; NSERC Discovery Grant and Canada Research Chair

Abstract

Epigenetic modification, especially DNA methylation, can play an important role in mediating gene regulatory response to environmental stressors and may be a key process affecting phenotypic plasticity and adaptation. Parasites are potent stressors with profound physiological and ecological effects on their hosts, yet it remains unclear how parasites influence host methylation patterns. Here, we used a well-studied host–parasite system, the guppy *Poecilia reticulata* and its ectoparasitic monogenean *Gyrodactylus turnbulli* to gain mechanistic insight into the dynamics of DNA methylation in host–parasite interactions. To explore this, we quantitatively measured genome-wide DNA methylation in guppy skin tissue using reduced representation bisulphite sequencing and characterized differential methylation patterns in guppies during distinct phases of infection. We identified 365, 313, and 741 differentially methylated regions (DMRs) between *infected* and *control* fish in *early infection*, *peak infection* and *recovery* phases, respectively. The magnitude of the methylation difference was moderate in DMRs, with an average of 29% (*early infection*), 27% (*peak infection*) and 30% (*recovery*) differential methylation per DMR. Approximately 50% of DMRs overlapped with CpG islands, and over half of the DMRs overlapped with gene bodies, several of which encode proteins relevant to immune response. These findings provide the first evidence of an epigenetic signature of infection by ectoparasites and demonstrate the changing relationship between epigenetic variation and immune response in distinct phases of infection.

KEYWORDS
DNA methylation, epigenetics, *Gyrodactylus turnbulli*, host–parasite interactions, phenotypic plasticity, *Poecilia reticulata*, reduced representation bisulphite sequencing

1 | INTRODUCTION

Parasitism has long been recognized as a major driver of ecological and evolutionary processes in a wide range of host taxa (Hamilton, 1980; Paterson & Piertney, 2011; Sheldon & Verhulst, 1996). This relevance is classically attributed to increased mortality of infected individuals and to parasite-induced changes in host phenotype (Hatcher, Dick, & Dunn, 2006). Examples of both mechanisms are plentiful, both in the laboratory (e.g., Hari Dass & Vyas, 2014; Lazzaro, Flores, Lorigan, & Yourth, 2008) and in the wild (e.g., Gotanda et al., 2013; van Oosterhout, Harris, & Cable, 2003), and their ecological and evolutionary implications have been well documented (Penczykowski, Laine, & Koskella, 2016). Recently, the role of epigenetic modulation in host–parasite interactions has received increased attention as a potential source of rapid and reversible phenotypic variation that can be shaped by both parasites and the host (Cheeseman & Weitzman, 2015; Gómez-Díaz, Jorda, Peinado, & Rivero, 2012; Robert McMaster, Morrison, & Kobor, 2016; Silmon de Monerri & Kim, 2014). Indeed, parasites can modulate gene expression profiles in their hosts through epigenetic modifications (Paschos &

Allday, 2010; Sessions et al., 2013), and these modifications can also be associated with an adaptive immune response of the host (Boyko & Kovalchuk, 2011; Conrath, 2011; Holeski, Jander, & Agrawal, 2012; Youngblood, Davis, & Ahmed, 2010). However, to date, the most compelling evidence for epigenetic responses to parasite infection has come from studies of endoparasites, such as bacterial pathogens infecting plants (e.g., Dowen et al., 2012) and intracellular protozoans infecting vertebrates (e.g., Hari Dass & Vyas, 2014). To our knowledge, no study has investigated epigenetic responses of hosts to ectoparasites, or if these responses change during the course of an infection. Ectoparasites are distinct from endoparasites in that they cannot manipulate host cell machinery and thus cannot directly modify intracellular signalling pathways and host transcription regulation (Cheeseman & Weitzman, 2015). Thus, the effects that ectoparasites have on host epigenome are unknown, but they are likely to be different from those of intracellular parasites. Here, we explore epigenetic modifications in a host–parasite system that is ideal for testing dynamics across distinct phases of infection, and that has been extensively studied both in nature and in the laboratory: Trinidadian guppies and their monogenean ectoparasites, *Gyrodactylus*.

Trinidadian guppies have been frequently used in evolutionary studies due to their dramatic and rapid adaptation to the local environment (for reviews: Endler, 1995; Houde, 1997; Magurran, 2005). Although initial work mainly focused on interactions between guppies and their predators (Reznick & Endler, 1982; Reznick, Shaw, Rodd, & Shaw, 1997), an extensive body of work has shown that gyrodactylid ectoparasites can influence many aspects of guppy ecology, including mate choice (Kennedy, Endler, Poynton, & McMinn, 1987; López, 1999), foraging behaviour (Kolluru et al., 2006), life history traits (Pérez-Jvostov, Hendry, Fussmann, & Scott, 2012, 2017), bacterial and fungal infections (Cusack & Cone, 1986; Kotob, Menanteau-Ledouble, Kumar, Abdelzaher, & El-Matbouli, 2016), and survival (van Oosterhout et al., 2007; Pérez-Jvostov et al., 2017). *Gyrodactylus* spp. are skin grazing parasites with a hyperviviparous life cycle, where the first-born offspring develops asexually from the adult female and contains a developing embryo (Bakke, Cable, & Harris, 2007). A single adult *Gyrodactylus* worm can produce rapid exponential population growth on individual fishes, and such infection has been shown to have significant fitness consequences (Bakke et al., 2007); for example, infected fish have higher mortality (van Oosterhout et al., 2007) and lower mating rates (Kennedy et al., 1987). Thus, studying the epigenetic mechanisms underlying responses to *Gyrodactylus* in guppies will aid in understanding a wide range of ecological and evolutionary processes in this well-studied host–parasite system.

As a first defence against skin grazing parasites like *Gyrodactylus*, fish largely rely on their innate immune system in the form of localized inflammation, which typically appears as hyperplasia, and elevated mucus secretion on the skin (Kumar et al., 2017). Increased expression of cyto- and chemokines in fish skin has been observed on infected fish mounting an immune response against a *Gyrodactylus* spp. infection (Kania, Larsen, Ingerslev, & Buchmann, 2007; Lindenstrøm, Buchmann, & Secombes, 2003; Lindenstrøm, Secombes, & Buchmann, 2004; Matejusová et al., 2006). Similarly, skin mucus contains high concentrations of lectins and immunoglobulins that also play important roles in both detecting and attacking ectoparasites (Ángeles Esteban, 2012; Salinas, Zhang, & Sunyer, 2011). Nonetheless, *Gyrodactylus* can often overcome these defences and reach such infection levels (hundreds of worms on one fish) that their grazing on the skin can diminish mucus production, and quickly decrease host health (Buchmann & Bresciani, 1997; Wells & Cone, 1990). Taken together, it is evident that *Gyrodactylus* infections can change the physiology of infected fish and can also result in changes in gene expression. However, the role of epigenetic mechanisms in regulating these changes is unknown.

We address this gap by performing an extensive epigenomic survey of guppies during three distinct phases of infection with a guppy-specific *Gyrodactylus*. Recent studies have shown increased genome-wide methylation as a general response to infection by endoparasites (Hari Dass & Vyas, 2014; Marr et al., 2014; Paschos & Allday, 2010). One explanation for this pattern is that parasites "hijack" the epigenome of the host by inducing hypermethylation in promoters of immune genes, which can result in gene repression and thus allow parasites to evade host defence mechanisms (Silmon de Monerri & Kim, 2014). In contrast, we predict that ectoparasites such as *Gyrodactylus* should not be able to manipulate the epigenome of guppies in this way, and thus, we have the opposite expectation: an active immune response by guppies should be reflected by increased expression of immune genes and thus hypomethylation of their promoters. To test this hypothesis, we specifically surveyed methylation changes over distinct phases of infection. Recent work has shown that host gene expression can vary across the course of infection, thereby indicating functional changes in immune response at different points of an infection cycle (Choi, Aliota, Mayhew, Erickson, & Christensen, 2014; Westermann, Barquist, & Vogel, 2017). If methylation responses to infection reflect an active immune response of guppies to their parasites, we expect to see the greatest methylation changes occurring in fish that are able to successfully recover from infection. We investigate three specific questions: (a) Are there general methylation patterns associated with *Gyrodactylus* infection in the guppy genome? (b) How are differentially methylated regions distributed among different regions of the genome (i.e., promoters, exons, introns and intergenic regions)? (c) How do methylation patterns change during the course of infection? Answering these questions will help us to better understand the mechanisms underlying host responses to parasite infection at the molecular level.

2 | MATERIALS AND METHODS

2.1 | Guppies

We used females from an admixed, laboratory-reared, population of guppies sampled from different locations in throughout the Northern Mountain range in Trinidad that have been in the laboratory at McGill University for at least ten generations. We selected only females because they have a broader range of peak *Gyrodactylus* loads (Cable & van Oosterhout, 2007) and because methylation

patterns are known to be sex-specific (McCarthy et al., 2014). Prior to the initiation of the experiment, we selected over 60 guppies from this laboratory population and scanned them under the microscope to identify whether *Gyrodactylus* was prevalent. From these fish, only one male was infected, and it had only one worm. Although this prevalence is very low, we do not know the history of *Gyrodactylus* infections in this laboratory-reared population, and it is thus possible that some fish have previously been infected. Therefore, we randomly selected healthy females, treated them for *Gyrodactylus* using Clout (Fritz Industries Inc., Mesquite, TX, USA)—in case we missed any worms—and kept them isolated for a 3-week quarantine period. We expect that after this quarantine, any females with prior exposure to *Gyrodactylus* would have lost any acquired immune response to *Gyrodactylus*, and respond to infection as naïve fish (Richards & Chubb, 1996; Scott, 1985). Fish were monitored daily to ensure that they were in good health, and were fed daily with brine shrimp. At the end of the 3-week quarantine period, all fish were confirmed to be free of *Gyrodactylus* infections.

2.2 | Experimental infections

Prior to the infection trials, all quarantined females were transferred to individual 1.8-L tanks in a flow-through system (Aquaneering Inc., San Diego, CA, USA) that standardizes water quality and temperature (26°C) and prevents potential movement of parasites between tanks with ultraviolet sterilizers. Females were divided into two experimental groups: *control* and *infected* (see Experimental design below; Figure 1). To initiate the infections in females in the *infected* group, each female was anaesthetized using MS-222 (buffered to a neutral pH with $NaHCO_3$) and manually infected by transferring two to three *Gyrodactylus* worms from one donor infected guppy (day 0). Females in the *control* group underwent the same procedure, except they were sham infected. For all trials, we used an isogenic strain of a guppy-specific *Gyrodactylus*, *G. turnbulli*, that was isolated from one worm from a pet store guppy in Montreal in 2008 (Dargent, Scott, Hendry, & Fussmann, 2013; Tadiri, Dargent, & Scott, 2013) and has been kept at high densities in the laboratory using pet store guppies as hosts (Tadiri, Scott, & Fussmann, 2016). Given that neither this isogenic strain of *Gyrodactylus* nor the guppies used in this experiment have had prior exposure to each other, our female guppies are naïve to this specific strain of *G. turnbulli* (hereafter referred to simply as *Gyrodactylus*).

After the experimental infection, all *control* and *infected* females were scanned for *Gyrodactylus* under a dissecting microscope every 2 days to track the development of the infection, or to confirm the absence of it in the control. This is a standard procedure to count *Gyrodactylus* worms and does not affect guppy health (Dargent et al., 2013; Pérez-Jvostov et al., 2012; Scott, 1982, 1985).

2.3 | Experimental design

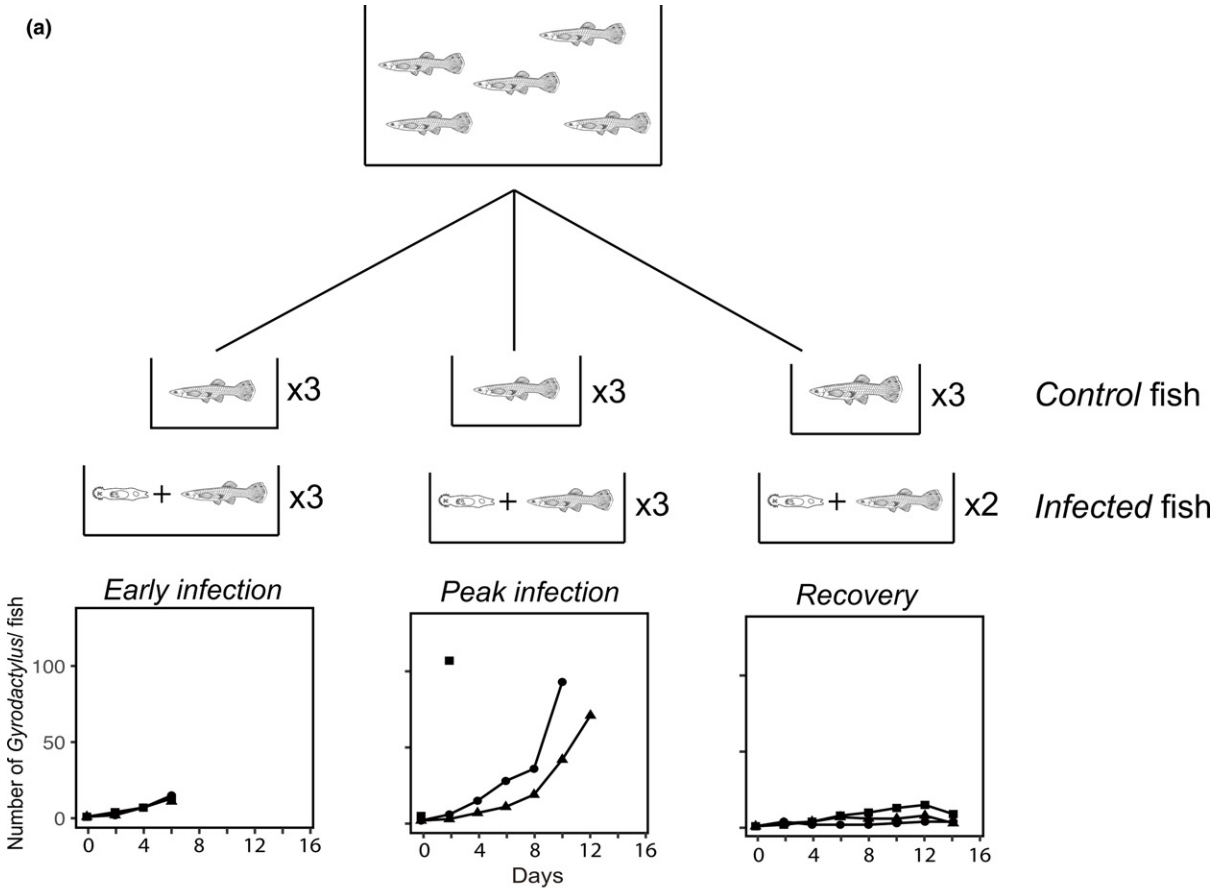
We designed our experiment with two main factors: experimental group (*infected* and *control*), and phase (*early infection*, *peak infection* and *recovery*; Figure 1). Given the high variability in parasite numbers at any specific time postinfection, we controlled for infection intensity rather than days post infection. Following Gheorghiu, Marcogliese, and Scott (2012), we characterized an *early infection* phase in which guppies had parasite loads <20 worms, and a *peak infection* phase in which guppies had ~100 parasites and/or showed erratic swimming or decreased health. We also characterized a *recovery* phase in which, after initial *Gyrodactylus* population growth, the number of worms on the fish started to decrease for two consecutive days or to half the number of the previous scan, suggesting guppy immune response was decreasing *Gyrodactylus*' population growth. We recorded the day postinfection when the number of parasites in *infected* fish reached each of the three infection phases, and found similar infection dynamics of *infected* fish within each infection phase, with the exception of one fish from *peak infection* phase, suggesting that *infected* fish had similar temporal responses to infection (Figure 1). We collected the complementary *control* fish on the same day when we collected *infected* fish to control for their age. In total, we collected three *infected* fish from each of the *early* and *peak infection* phases, as well as three complementary *control* fish. For the *recovery* phase, we were able to only sample three *infected* and two *control* fish.

2.4 | DNA extraction

We extracted DNA from epidermis of skin using phenol:chloroform: isoamyl alcohol (25:24:1) and assessed the quality and quantity using Tecan Infinite® 200 NanoQuant and Quant-iT PicoGreen® dsDNA assay kit (ThermoFisher Scientific). We used skin tissue because it is in intimate contact with the parasite, and involved in immune response to ectoparasites in fish (Ángeles Esteban, 2012). All procedures were approved by McGill University (Animal Use Protocol 2000-4570).

2.5 | Reduced representation bisulphite sequencing

To measure genome-wide DNA methylation levels, we used a high-throughput sequencing approach known as reduced representation bisulphite sequencing (RRBS) (Gu et al., 2011; Meissner et al., 2008), following the protocol described in Boyle et al. (2012) with minor modifications. For each individual, we created a library from 200 ng of genomic DNA and ligated the fragments in each library with unique Illumina TruSeq adapters. We targeted fragments of 160–340 bp (including ~120 bp of adapter sequence) using NaCl-PEG diluted SpeedBeads (Rohland & Reich, 2012). We randomly multiplexed eight and nine libraries into two pools and treated the pools with sodium bisulphite (EpiTect, Qiagen), following the protocol for formalin-fixed paraffin-embedded samples. After two rounds of bisulphite treatment to ensure complete conversion, each pool was amplified with Illumina primers and loaded in two lanes (100-bp single-end reads) of Hiseq 2500 at the McGill University and Genome Quebec Innovation Centre. Each sample was sequenced to a mean depth (±*SD*) of 19.86 ± 4.210 million reads (Supporting information



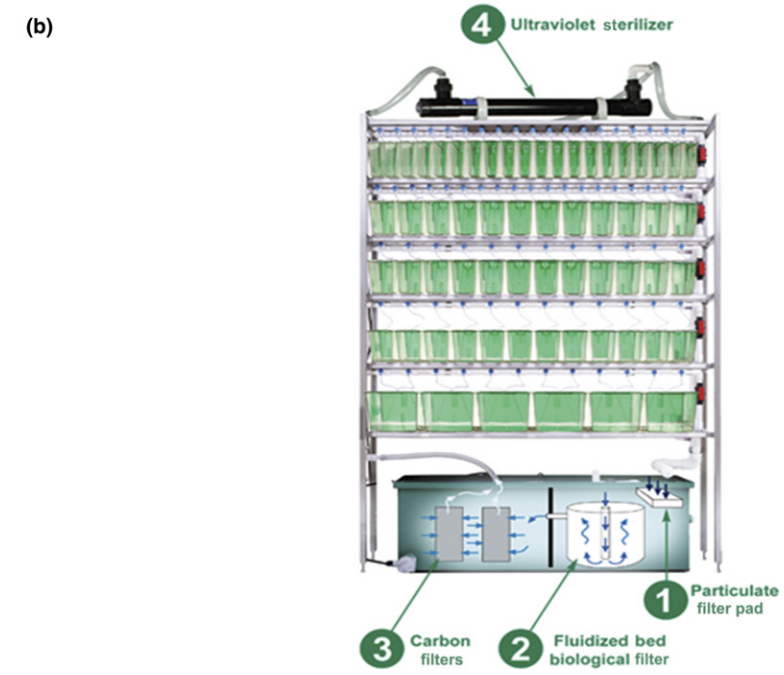


FIGURE 1 Overview of the experimental design used to set up the infection trial. (a) Guppies from a genetically homogeneous population were randomly assigned to *control* or *infected* groups in three infection phases: *early infection*, *peak infection* and *recovery* based on the number of parasites on each fish. Each *control* or *infected* group contains three replicate fish (*control* group in *recovery* contains two fish), and individual fish were kept in separated tanks until the end of the experiment. (b) The flow-through system (Aquaneering Inc., San Diego, CA, USA. Image source: http://www.aquaneering.com/zebrafish_stand_alone_systems.php). Water is filtered by particulate, biological and carbon filters, and flows into individual tanks. The in-line UV sterilizer lamp provides UV light preventing potential movement of parasites between tanks [Colour figure can be viewed at wileyonlinelibrary.com]

Table S1). We quantified methylation at non-CpG motifs and found less than 1% non-CpG cytosines were methylated, suggesting a highly efficient bisulphite conversion.

2.6 | Read filtering and mapping

To remove adapter contamination, low-quality bases and bases artificially introduced during library construction, we trimmed reads using TRIM GALORE! version 0.4.4 (https://www.bioinformatics.babraham.ac.uk/projects/trim_galore/), with the "rrbs" option. We then used the program BOWTIE2 version 2.2.9 (Langmead & Salzberg, 2012), implemented in BISMARK version 0.17.0 (Krueger & Andrews, 2011) to align trimmed reads for each sample to the guppy genome (GenBank assembly accession GCA_00063615.2) with default settings, except for tolerating one nonbisulphite mismatch per read. We only included reads that mapped uniquely to the reference genome in downstream differentially methylated cytosines (DMCs) and differentially methylated regions (DMRs). The average mapping efficiency (±*SD*) was 58.69 ± 2.03%. Only CpG context cytosine methylation was analysed because CpG methylation is the most common functional methylation in vertebrates (Suzuki & Bird, 2008).

2.7 | DMC and DMR calling

We analysed DMCs and DMRs at two steps: First, we pooled all *infected* or *control* fish as two groups and identified DMCs and DMRs between groups to determine general patterns of methylation response to parasites in the guppy genome. Second, we performed similar DMC and DMR analyses within each infection phase to examine specific epigenetic responses in each phase of infection. When pooling all *infected* or *control* fish, a total of 878,645 CpG sites met the minimum coverage requirement, consisting of an average of 1.5% of all CpG sites in the genome after alignment (Supporting information Table S1). For each phase, 978,671 (*early infection*), 1,018,862 (*peak infection*) and 1,027,149 (*recovery*) CpG sites met the minimum coverage requirement, consisting of ~2% of all CpG sites after alignment (Supporting information Table S1).

We identified individual DMCs using the R package METHYLKIT version 1.4.1 (Akalin et al., 2012). Read coverage was normalized between samples. A minimum of five reads in all samples were required at a CpG site for that site to be analysed (Walker et al., 2015; Wan et al., 2016). Sites that were in the 99.9th percentile of coverage were also removed from the analysis to account for potential PCR bias. Hierarchical cluster analysis was conducted using Ward's method based on the filtered CpG sites, and most individuals clustered primarily by experimental group (*infected* vs. *control*; Supporting information Figure S1). We used default parameters (false discovery rate correction *Q*-value <0.01), with a correction for overdispersion, and a minimum required methylation difference of 25% between *infected* and *control* fish to identify DMCs (Akalin et al., 2012; Baerwald et al., 2015). We then determined DMRs using the R package EDMR version 0.6.4.1 (Li et al., 2013) with default parameters. To be considered significant, a DMR needed to contain at least three CpG sites within an algorithm-specified genomic distance, with at least one classified as a DMC (*Q*-value <0.01), and an absolute mean methylation difference greater than 20% when comparing *infected* and *control* fish (see Li et al., 2013 for details). We analysed the shared DMRs between different infection phases by extracting and comparing the chromosomal names, and the start and end positions of each DMR. We visualized differential methylation patterns across individuals and obtained clustering of samples and DMRs in heatmaps with the "complete" clustering method on Euclidian distances, using the R package PHEATMAP version 1.0.8 (https://cran.r-project.org/web/packages/pheatmap/index.html). We clustered hyper- and hypomethylated DMRs between *infected* and *control* fish using the relative per cent DNA methylation, which is the normalized per cent DNA methylation scaled for each DMR's per cent DNA methylation (median per cent methylation as 0) of *infected* and *control* fish in heatmaps. We also clustered individual fish based on overall methylation patterns across DMRs.

2.8 | Genomic context, gene annotation and gene ontology analysis

To build null distributions of genomic features (promoters/exons/introns/intergenic regions) of DMCs, we used the sets of CpG sites that passed the filtering steps described above. We first identified the positions of CpG sites within genomic features. We gave precedence to promoters > exons > introns > intergenic regions when features overlapped, and defined promoter regions as upstream 1,000 bp and downstream 1,000 bp from the transcription starting site (TSS) (Akalin, Franke, Vlahoviček, Mason, & Schübeler, 2015). We then identified the positions of DMCs within genomic features and compared the distributions of DMCs to null distributions using *G* tests.

To perform the functional analysis of DMRs, we identified the nearest TSS and its associated transcript ID, and the position of methylated regions within genomic features for each DMR. We also identified the proximity of DMRs to CpG islands, which are CpG-rich regions that are usually unmethylated and serve as sites for transcription initiation (Jones, 2012), using python scripts (https://github.com/lucasnell/TaJoCGI) that apply an algorithm based on the methods described in Takai and Jones (2002). DMRs were considered overlapping or proximal to CpG islands when they were within or less than 4 kb away from an island and considered locating within open sea when they were outside the 4 kb window (Baerwald et al., 2015). As the guppy genome has not been fully annotated yet, we used BLASTx against the NCBI nonredundant database to identify genes that DMRs were mapped to, followed by functional category assignment, GO term mapping and node score distribution analysis implemented in BLAST2GO version 4.1 (Conesa et al., 2005; Götz et al., 2008). To specifically investigate the immune relevance of identified genes, we acquired a list of 1,843 GO terms including "immune system process" (GO:0002376) and its child terms using the R package GO.DB version 3.4.0 (Carlson 2017), and compared the blast results of DMR-associated transcripts to the list. We then created a list of

DMRs overlapping with genes or regions up to 5 kb upstream or downstream of these gene locations (Le Luyer et al., 2017). In addition, we also checked whether any of the DMRs overlapped with the promoters of immune genes, using the annotatePeakInBatch function implemented in the R package CHIPPEAKANNO version 3.14.0 (Zhu, 2013; Zhu et al., 2010).

2.9 | Pathway analysis

To identify functional associations among the genes that DMRs were mapped to, we conducted pathway analysis using the Kyoto Encyclopaedia of Genes and Genomes (KEGG) enrichment analysis implemented in BLAST2GO version 4.1 (Conesa et al., 2005; Götz et al., 2008).

3 | RESULTS

3.1 | General patterns of differential methylation in infected vs. control guppies

After pooling across phases (nine *infected* fish vs. eight *control* fish), we identified 1,540 DMCs between *infected* and *control* fish after false discovery rate correction. We found significantly more DMCs within exons (*G* test; $p = 0.028$) than expected by chance, and fewer within promoters (*G* test; $p < 0.001$) when compared to the null distribution built on all CpG sites that passed the filtering steps; however, DMCs were not significantly enriched in introns (*G* test; $p = 0.49$) or intergenic regions (*G* test; $p = 0.36$; Figure 2a). Based on the DMCs, we found 30 DMRs between *infected* and *control* fish after false discovery rate correction. Given our lower limit differential methylation cut-off (20%), methylation differences between *infected* and *control* group ranged from 20% to 45% per DMR (Supporting information Table S2). Based on Euclidean distances calculated from the 30 DMRs (Figure 2b), most individuals clustered primarily by experimental group (*infected* vs. *control*). Both DMCs and DMRs displayed more hyper- than hypomethylation in *infected* fish relative to *control* fish (872 hypermethylated and 668 hypomethylated DMCs, 18 hypermethylated and 12 hypomethylated DMRs when comparing *infected* fish to *control* fish), suggesting that an increase in genomic DNA methylation levels is a general response to parasite infection in guppies.

We mapped 27 of the 30 DMRs to 15 chromosomes, with 25 of the DMRs localized within or proximal to known genes. Chromosome LG10 (NC_024340.1) contained the most DMRs (four). Four other chromosomes, LG6, LG13, LG14 and LG16 (NC_024336.1, NC_024343.1, NC_024344.1 and NC_024346.1) contained two to three DMRs. Ten chromosomes each contained a single DMR, and three DMRs could not be mapped to a chromosome due to the incomplete nature of the guppy genome. Over half of the DMRs were found overlapping with gene bodies, and 30% of the DMRs (10/33) were found within or proximal to CpG islands. The DMRs mapped to genes annotated with a variety of gene ontology (GO) categories in Biological Process, and the gene ontology categories with the highest node scores found were ATP binding (GO:0005524) and zinc ion binding (GO:0008270) in Molecular Function, and integral component of membrane (GO:0016021) in Cellular Component (Supporting information Figure S2). One DMR mapped to a gene (*ifngr1*) annotated with immune response (Table 1).

3.2 | Patterns of differential methylation between the three distinct infection phases

After correcting for false discovery rates, we identified 11,355, 9,310 and 19,058 DMCs in *early infection*, *peak infection* and *recovery*, respectively. DMCs were distributed broadly across the genome, with no apparent clustering on specific chromosomes or chromosomal regions (Figure 3a–c). More DMCs were hypermethylated than were hypomethylated in all phases (Figure 3d), suggesting that the increase in genomic DNA methylation levels is a consistent response throughout all phases of infection. Although we found significantly fewer DMCs within promoters than expected by chance in all phases (*G* test; $p < 0.001$), DMCs were not significantly enriched in exons, introns or intergenic regions in all phases (Figure 4a–c). Based on DMCs, we identified 365 (*early infection*), 313 (*peak infection*) and 741 (*recovery*) DMRs, with most DMRs (~90%) located within or proximal to annotated genes in all phases (Figure 4d–f). Over half of the DMRs (56% in *early infection* phase; 52% in *peak infection* phase; 57% in *recovery* phase) were found overlapping with gene bodies (Figure 5a), and ~50% of the DMRs (47% in *early infection* phase; 46% in *peak infection* phase; 47% in *recovery* phase) were found within or proximal to CpG islands (Figure 5b). Methylation differences per DMR between *infected* and *control* groups ranged from 20% to 56% (*early infection*), 20% to 55% (*peak infection*) and 20% to 70% (*recovery*). Among all identified DMRs, 26, 56 and 38 DMRs were shared between *early* and *peak infection* phases, *peak infection* and *recovery phases*, and *early infection* and *recovery phases*, respectively. Seven DMRs, which were annotated with known genes (*ADCY8*, *ANXA5*, *ARFGEF3*, *LRFN5*, *PPKG1*, *PPM1B* and *PSMC3*), were shared by all three phases (Figure 6a).

About 8% of all DMRs in our study were not located within or proximal to known genes. This may be an indication of *trans*-acting regulatory elements, for example, enhancers (Taudt, Colome-Tatche, & Johannes, 2016), but could also be due to the incomplete annotation of the guppy genome, precluding identification of genes and their proximal regulatory regions. Gene ontology categories with the highest node scores were similar in all phases. These included single-organism cellular process (GO:0008150) and signal transduction (GO:0007165) in Biological Process, hydrolase activity (GO:0016787), nucleic acid binding (GO:0003676) and transferase activity (GO:0016740) in Molecular Function, and integral component of membrane (GO:0016021) in Cellular Component (Supporting information Figure S3–S5). We found seven, five and seven DMRs annotated with immune response in *early infection*, *peak infection* and *recovery* phases, respectively (Table 1). None of the DMRs overlapped with the promoters of immune genes, and none of the immune-related GO terms were shared by all individual phases (Figure 6b).

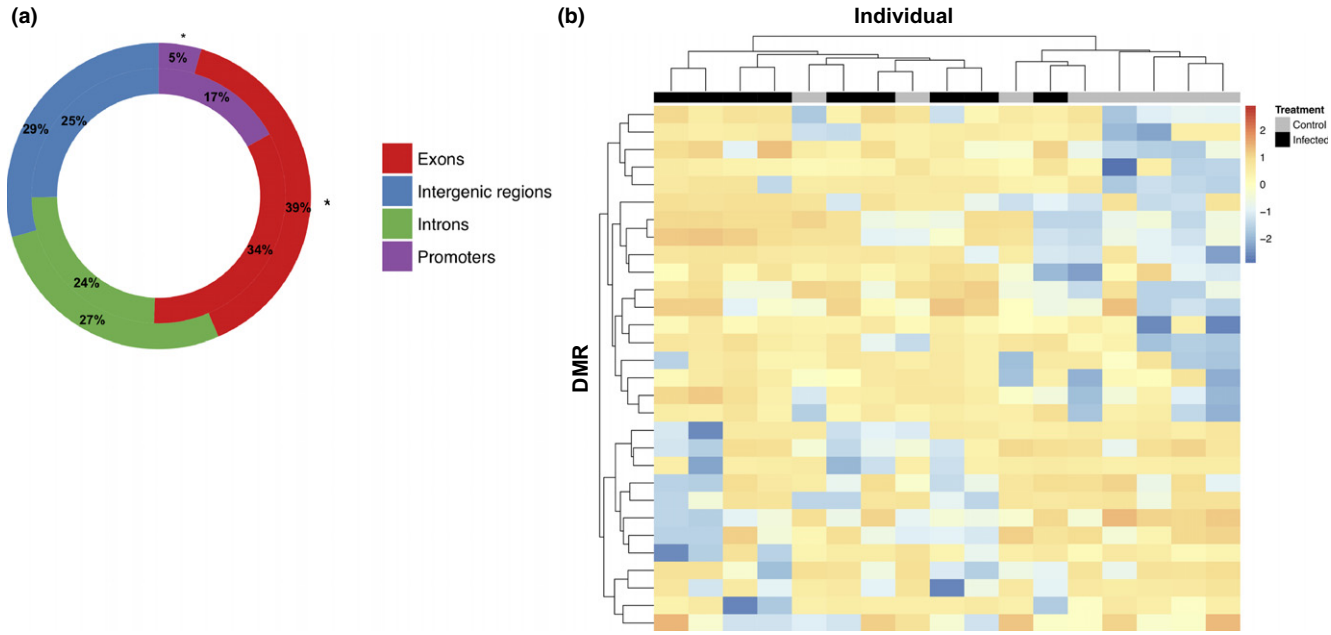


FIGURE 2 (a) The proportion of genomic features (promoters, exons, introns or intergenic regions) in DMCs compared with the genomic features of all filtered CpG sites. The outer ring describes the locations of DMCs; the inner ring describes the features of filtered CpG sites. Asterisks denote significant differences between the features of DMCs vs. the features of filtered CpG sites using a *G* test at $p < 0.05$. (b) Heatmap of methylation levels of the 30 DMRs when comparing *infected* vs. *control* fish pooling across all phases. Each column represents a colour-coded individual: black for *infected* fish and grey for *control* fish. Each row represents one of the DMRs, which are clustered based on the similarities of the methylation patterns between individuals. Darker red indicates greater methylation in an individual for that DMR. Darker blue indicates lesser methylation in an individual for that DMR. Individual dendrogram positions are based on overall methylation patterns across the 30 DMRs [Colour figure can be viewed at wileyonlinelibrary.com]

3.3 | Pathway analysis

We identified several molecular pathways associated with *Gyrodactylus* infection for each infection phase via KEGG analysis (Supporting information Table S3–S5). The top canonical pathway was purine metabolism for all three infection phases. Other top canonical pathways included aminobenzoate degradation, thiamine metabolism, Th1 and Th2 cell differentiation and the T-cell receptor signalling pathway.

4 | DISCUSSION

The modulation of host–parasite interactions through epigenetic mechanisms has received increased attention in recent years (Gómez-Díaz et al., 2012; Poulin & Thomas, 2008; Wenzel & Piertney, 2014). However, little is known about the role of epigenetics in host responses to ectoparasites, or if and how these responses change throughout the duration of an infection. We used a quantitative, single-base resolution technique (RRBS) to measure DNA methylation in female guppies during distinct phases of infection by the guppy-specific *G. turnbulli* and detected both a general epigenetic response across infection phases, and unique epigenetic responses in each phase. We found increased genomic DNA methylation levels in *infected* guppies, which is consistent with recent reports of genome-wide DNA methylation variation in infected mammal cells, in which increased DNA methylation was induced by parasite infection (Hari Dass & Vyas, 2014; Paschos & Allday, 2010). We also observed mean methylation differences of ~30% per DMR in skin tissue of *infected* vs. *control* groups, a level that is on par with methylation responses previously observed for important phenotypic changes such as migration-related phenotypes (migratory vs. nonmigratory) in rainbow trout (Baerwald et al., 2015), and distinct caste phenotypes in honeybees (Herb et al., 2012). In addition to the moderate overall magnitude of methylation differences, we identified a number of DMRs that mapped to gene regulatory regions (CpG islands), and genomic regions close to immune genes. Hierarchical clustering based on methylation patterns alone was sufficient to differentiate between *infected* and *control* guppies, although there were individual exceptions (see below). Overall, our study provides the first investigation of epigenetic changes across distinct phases of infection by an ectoparasite, and identified DMRs that may be relevant to guppy immune response.

4.1 | Linking differential DNA methylation to genomic architecture

We found approximately 50% of DMRs within or proximal to CpG islands, strongly suggesting that epigenetic modifications on the skin of *infected* guppies are influencing the transcriptional activity of associated genes. CpG islands at promoters remain predominantly unmethylated in somatic cells and play a role in regulating transcription initiation in vertebrates, where approximately 70% of all annotated promoters are associated with CpG islands (Saxonov, Berg, & Brutlag, 2006). Hypermethylation

TABLE 1 Gene ontology (GO) terms in immune-related genes

Transcript ID[a]	Transcript name	GO ID	GO term	Meth Diff[b]
Pooling infected vs. controls				
XM_008419655.2	PREDICTED: *Poecilia reticulata interferon gamma receptor 1* (*ifngr1*), mRNA	GO:0030097	Hemopoiesis	−24.5
Early infection				
XM_008405926.2	PREDICTED: *Poecilia reticulata interferon regulatory factor 8* (*irf8*), mRNA	GO:0014005	Microglia development	−28.4
		GO:0045649	Regulation of macrophage differentiation	
		GO:0045658	Regulation of neutrophil differentiation	
XM_008416346.2	PREDICTED: *Poecilia reticulata mast/stem cell growth factor receptor kita-like* (LOC103468941), transcript variant X1, mRNA	GO:0038093	Fc receptor signalling pathway	−27.6
XM_008426764.1	PREDICTED: *Poecilia reticulata ribosomal protein L35a* (*rpl35a*), mRNA	GO:0030218	Erythrocyte differentiation	29.2
XM_008431017.1	PREDICTED: *Poecilia reticulata protein C-ets-1-like* (*ets1*), transcript variant X1, mRNA	GO:0030223	Neutrophil differentiation	23.1
		GO:0060217	Hemangioblast cell differentiation	
XM_008432066.1	PREDICTED: *Poecilia reticulata cholecystokinin-like* (LOC103478316), mRNA	GO:0006955	Immune response	−20.1
XM_008432236.2	PREDICTED: *Poecilia reticulata N-myc downstream regulated 1* (*ndrg1*), mRNA	GO:0045576	Mast cell activation	23.1
XM_017310387.1	PREDICTED: *Poecilia reticulata L-amino-acid oxidase-like* (LOC103480925), transcript variant X2, mRNA	GO:0045087	Innate immune response	−23.0
Peak infection				
XM_008398390.1	PREDICTED: *Poecilia reticulata ribosomal protein S29* (*rps29*), mRNA	GO:0048821	Erythrocyte development	−25.7
		GO:0060218	Hematopoietic stem cell differentiation	
XM_008419655.2	PREDICTED: *Poecilia reticulata interferon gamma receptor 1* (*ifngr1*), mRNA	GO:0030097	Hemopoiesis	−23.6
XM_008432236.2	PREDICTED: *Poecilia reticulata N-myc downstream regulated 1* (*ndrg1*), mRNA	GO:0045576	Mast cell activation	24.4
XM_008435495.2	PREDICTED: *Poecilia reticulata tumour necrosis factor superfamily member 12* (*tnfsf12*), mRNA	GO:0006955	Immune response	−20.8
XM_008437577.2	PREDICTED: *Poecilia reticulata tumour necrosis factor superfamily member 11* (*tnfsf11*), mRNA	GO:0006955	Immune response	−31.6
Recovery				
XM_008398848.1	PREDICTED: *Poecilia reticulata ubiquitin conjugating enzyme E2 D3* (*ube2d3*), transcript variant X1, mRNA	GO:0002223	Stimulatory C-type lectin receptor signalling pathway	21.5
		GO:0035666	TRIF-dependent toll-like receptor signalling pathway	
		GO:0038095	Fc-epsilon receptor signalling pathway	
		GO:0050852	T cell receptor signalling pathway	
XM_008409600.2	PREDICTED: *Poecilia reticulata GATA-binding factor 2-like* (LOC103465060), *transcript variant X3*, mRNA	GO:0048821	Erythrocyte development	32.3
		GO:0060215	Primitive hemopoiesis	
XM_008419655.2	PREDICTED: *Poecilia reticulata interferon gamma receptor 1* (*ifngr1*), mRNA	GO:0030097	Hemopoiesis	−41.8
XM_008421008.2	PREDICTED: *Poecilia reticulata macrophage erythroblast attacher* (maea), mRNA	GO:0043249	Erythrocyte maturation	−21.2

(Continues)

TABLE 1 (Continued)

Transcript ID[a]	Transcript name	GO ID	GO term	Meth Diff[b]
XM_008426764.1	PREDICTED: *Poecilia reticulata ribosomal protein L35a* (*rpl35a*), mRNA	GO:0030218	Erythrocyte differentiation	27.2
XM_008427554.2	*Poecilia reticulata junctional adhesion molecule 2* (*jam2*), transcript variant X2, mRNA	GO:0048534	Hematopoietic or lymphoid organ development	30.5
XM_017309240.1	PREDICTED: *Poecilia reticulata protein tyrosine phosphatase, nonreceptor type 6* (*ptpn6*), transcript variant X3, mRNA	GO:0045087	Innate immune response	−37.5

[a]DMRs overlapping with genes, or regions that are 5 kb up- or downstream of gene locations are labelled in bold. [b]Per cent methylation differences averaged from all CpG sites within the defined region. The comparison is between *infected* and *control* fish with positive values representing increased methylation for *infected* fish, and negative values representing decreased methylation for *infected* fish.

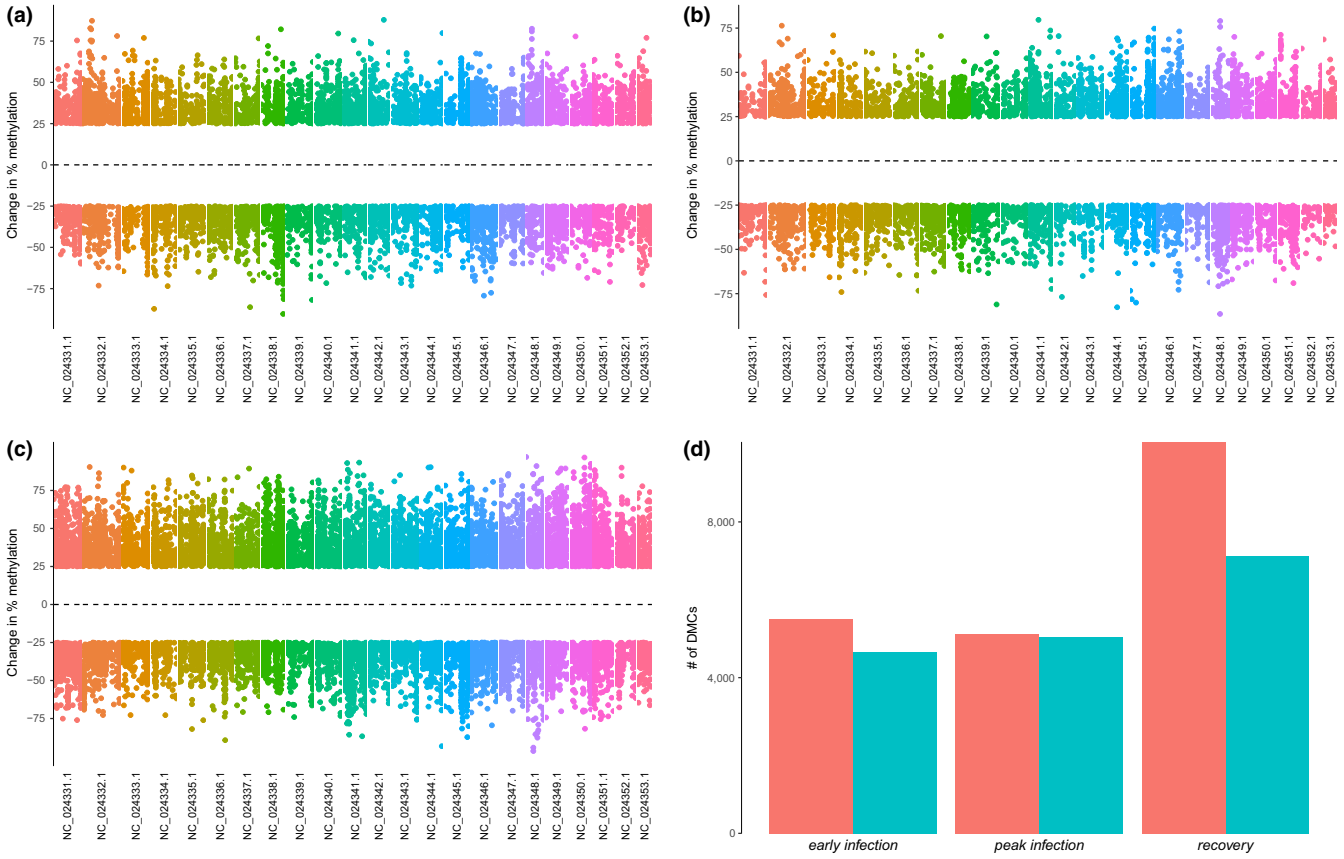


FIGURE 3 (a–c) Manhattan plots of the chromosomal positions of methylated CpG loci that differed significantly between *infected* and *control* fish in (a) *early infection*, (b) *peak infection* or (c) *recovery*. Each point represents a single DMC. The *y*-axis presents the difference in percentage methylation for that DMC in *infected* fish relative to the *control* fish. Only DMCs with more than 25% change in methylation are shown. Points above and below the horizontal dashed line are hypermethylated and hypomethylated loci, respectively. (d) Number of hypermethylated (red) and hypomethylated (blue) DMCs across the guppy genome in *early infection*, *peak infection* or *recovery* phase [Colour figure can be viewed at wileyonlinelibrary.com]

of these sites is typically associated with gene repression (Jones, 2012). Thus, the general hypermethylation response observed is somewhat unexpected; we predicted increased gene expression would occur as the host mounts an immune response to infection, or during host angiogenesis and repair in response to parasite foraging on skin. This pattern highlights the importance of understanding the functional roles of loci overlapping with DMRs. For instance, in contrast to the predominant hypermethylation occurring in *infected* guppies relative to controls, it is notable that we observed a 40% decrease in DNA methylation in the *tenascin XB* promoter of infected fish during the *recovery* phase compared to their controls. The *tenascin XB* locus is involved in wound healing and maintaining skin tissue structure (Gbadegesin et al., 2013), and hypomethylation of its promoter suggests that this gene is more stably expressed in the skin tissue of *infected* fish, and is thus likely to be involved in healing and skin repair after *Gyrodactylus* infection.

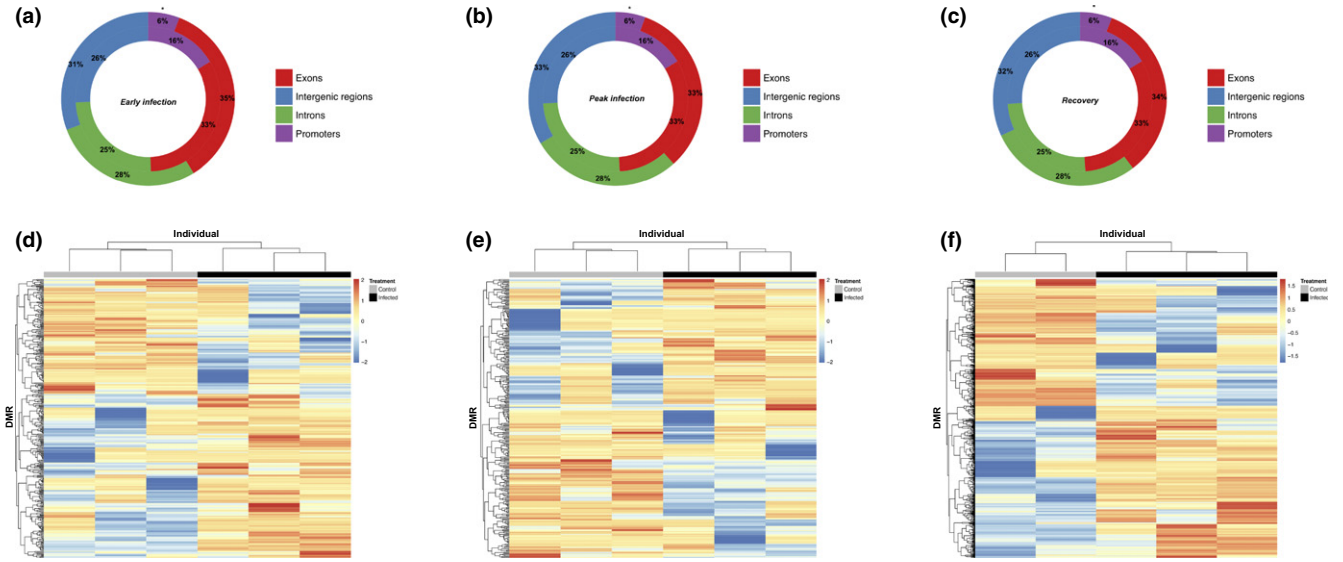


FIGURE 4 (a–c) The proportion of genomic features (promoters, exons, introns or intergenic regions) in DMCs compared with genomic features of filtered CpG sites in (a) *early infection*, (b) *peak infection* and (c) *recovery*. Outer rings describe the locations of DMCs; inner rings describe the features of filtered CpG sites. Asterisks denote significant differences between the features of DMCs vs. the features of all filtered CpG sites using a *G* test at $p < 0.01$. (d–f) Heatmap of methylation levels of DMRs when comparing *infected* vs. *control* fish in (d) *early infection*, (e) *peak infection* and (f) *recovery*. Each column represents a colour-coded individual: black for *infected* fish and grey for *control* fish. Each row represents one of the DMRs identified within a phase. DMRs are clustered based on the similarities of the methylation patterns between individuals. The darker the red, the more methylated that individual is for that DMR. The darker the blue, the less methylated that individual is for that DMR. Individual dendrogram positions are based on their overall methylation patterns across DMRs identified within a phase [Colour figure can be viewed at wileyonlinelibrary.com]

Although DMCs were not significantly enriched in gene bodies, over half of all DMRs in our study were found to be overlapping with gene bodies, and all DMRs overlapping with immune gene locations were located within gene bodies. This is unexpected because, in contrast to promoters, gene bodies are typically CpG-poor, extensively methylated and contain repetitive and transposable elements (Jones, 2012). Although the functions of methylation in gene bodies remain unknown, there is some evidence suggesting that variation in DNA methylation in gene bodies can result in alternative splicing (Jones, 2012), facilitate mutation by providing genetic variation during somatic hypermutation in immune genes (Racanelli & Rehermann, 2006) and even regulate the activation of transposable elements to facilitate systemic responses to parasite infection (Wenzel & Piertney, 2014). Indeed, when compared with controls, guppies in the *infected* group—regardless of infection phase—showed 43% higher DNA methylation levels in the gene body of the *protein phosphatase 2 regulatory subunit B'delta* (*PPP2R5D*). This gene controls substrate specificity and cellular localization, and plays a role as a regulator of tumorigenesis, drug resistance and immune surveillance (Ruvolo, 2016). Thus, methylation variation in this gene may result in a different transcript that facilitates guppies' resistance to infection, or over the long term, may produce new mutations for parasite-mediated selection to act on.

4.2 | Immunological relevance of DMRs

Our gene ontology analysis identified multiple genes relevant to immune response within DMRs (Table 1). DMRs mapped to genes involved in antiviral response (Robertsen, 2006), immune complexes (Schraml, Baker, & Reilly, 2006), antimicrobial peptide production (Fernandes & Smith, 2004) and T-regulatory cell activation, central and peripheral tolerance establishment (Dougall et al., 1999; González-Suárez & Sanz-Moreno, 2016; Theill, Boyle, & Penninger, 2002). DMRs also mapped to genes that are mainly associated with the development and differentiation of leucocyte cells in the epithelium (e.g., mast cell, macrophage, neutrophil granulocyte), which is important for immune response because altering leucocyte cells can make consuming mucus energetically unfavourable for parasites (Buchmann & Lindenstrøm, 2002; Dalgaard, Nielsen, & Buchmann, 2003; Jones, 2001). The involvement of this collection of genes suggests that *Gyrodactylus* can induce diverse immune responses in guppies. Interestingly, only a few of the immune genes and GO terms were shared between individual phases of infection, which suggests that interactions between guppies and *Gyrodactylus* change throughout the development of infection. This observation is consistent with previous studies showing temporal shifts in gene expression in animals infected with intracellular parasites (Choi et al., 2014; Westermann, Gorski, & Vogel, 2012). However, none of the immune-related DMRs overlapped with the promoters of immune genes, which does not provide evidence in support of our hypothesis that an active response of the host to infection would be reflected by hypomethylation in the promoters of immune genes.

Previous studies have identified several genes (e.g., MHC) that are under selection during *Gyrodactylus* infection (Fraser & Neff, 2009; Fraser, Ramnarine, & Neff, 2010; Kjaerner-Semb et al., 2016; van Oosterhout et al., 2006; Tonteri, VasemÄGi, Lumme, & Primmer,

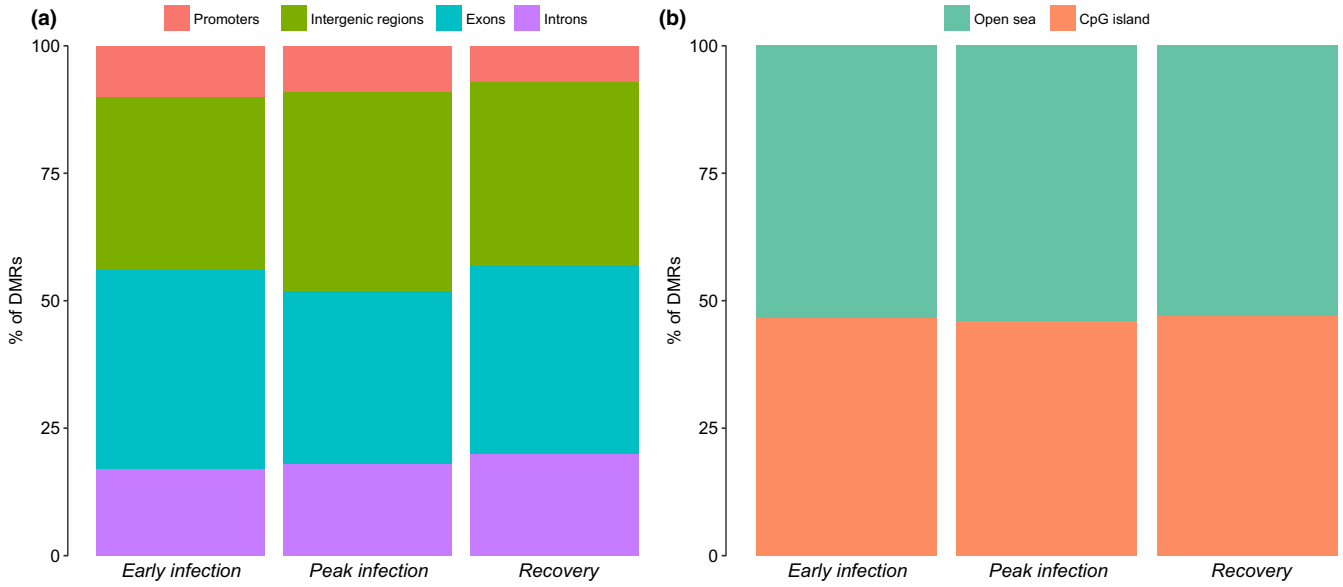


FIGURE 5 Genomic architecture of DMRs. (a) Proportion of DMRs overlapping with genomic features (promoters, exons, introns or intergenic regions) in *early infection*, *peak infection* and *recovery* phases. Overlapping genomic features were given the precedence promoters > exons > introns > intergenic regions. (b) Proportion of DMRs that are within or proximal to CpG islands or open sea in *early infection*, *peak infection* and *recovery* phases [Colour figure can be viewed at wileyonlinelibrary.com]

2010) and that are differentially expressed in fish with different parasite loads (Lindenstrøm et al., 2004; Tadiso et al., 2011). Surprisingly, however, we found no overlap between these previously identified candidate genes for infection response and those mapping to DMRs in our study. This may suggest that the observed epigenetic differences are due to *trans*-acting genetic variants as opposed to more local *cis*-acting variants. However, the lack of overlap could also suggest that genetic variation and epigenetic variation represent independent mechanisms for facilitating adaptation or acclimation to infection (Klironomos, Berg, & Collins, 2013). This possibility highlights the importance of studies such as ours that can uncover complementary sources of candidate loci relevant to immune response to parasite infections.

4.3 | Functional associations between DMRs and immune response

Our KEGG analysis on annotated DMRs revealed important functional associations in canonical pathways and common gene networks. In particular, we identified several molecular pathways associated with disease and xenobiotic metabolism, including the following top three pathways that were shared by all infection phases: purine metabolism, aminobenzoate degradation and thiamine metabolism (Supporting information Table S3–S5). Purine metabolism can affect immunity, stress tolerance and resistance to infectious diseases in fish (Dawood, Koshio, & Esteban, 2017; Gil, 2002; Li & Gatlin, 2006) and aminobenzoate degradation is associated with disease severity and stress response (Gevers et al., 2014). The thiamine metabolism pathway also helps to regulate the immune system through the activation of immune cells (Manzetti, Zhang, & van der Spoel, 2014). However, in general, the physiological processes revealed by our ontology and pathway analyses were diverse, and genes annotated with immune response were only a small subset of all genes that were differentially methylated. This is consistent with the view that parasite infection impacts physiological condition through a wide range of cellular processes in addition to strict immune responses (Hill, 2011; Wenzel & Piertney, 2014).

4.4 | Unique epigenetic responses in distinct infection phases

Among the different infection phases, we found that the number of DMRs was highest in the *recovery* phase. This might be expected, as higher gene regulation during this phase could represent a fully mounted and active response to *Gyrodactylus*, and would also explain the reduction in infection levels observed in these fish. We also found that the majority of DMRs were unique to only a single infection phase. This may reflect changes in methylation driven by the dynamics of the infection cycle (e.g., Tadiso et al., 2011). However, it is also possible that some aspects of infection dynamics are produced by changes in methylation. Indeed, we found some DMRs that mapped to genes that are themselves involved in regulating epigenetic modifications (e.g., N-lysine methyltransferase), and active methylation changes at these epiloci may regulate methylation levels elsewhere in the genome. There is mounting evidence suggesting that infection-associated changes in methylation patterns are not primarily driven by the host, but are rather adaptive parasite-induced manipulations (Schmid Hempel & Schmid-Hempel, 2011). Nonetheless, our finding that differential methylation was greatest in fish that were able to limit and reduce infection could

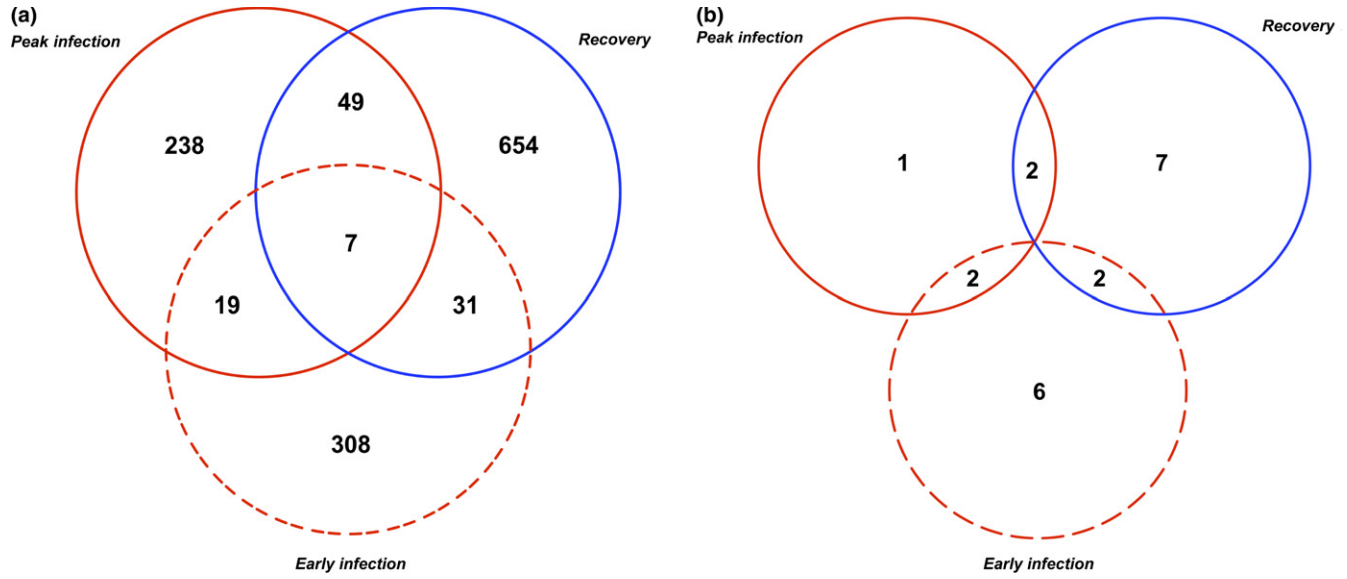


FIGURE 6 Venn diagrams showing characteristics of DMRs. (a) The number of DMRs that are unique in each phase and shared between different phases. (b) The number of GO terms annotated by immune-related DMRs that are unique in each phase and shared between different phases [Colour figure can be viewed at wileyonlinelibrary.com]

suggest that hosts are also able to induce their own adaptive methylation responses.

4.5 | Potential caveats

Our experiment has some limitations that should be noted. The most common technique used to measure DNA methylation is methylation-sensitive AFLP (Schrey et al., 2013), which identifies global methylation changes, but does not provide the single-base resolution needed to extract functional genomic information. The RRBS approach used in our study has several advantages compared to the MS-AFLP, with the most important being its single nucleotide resolution and greater genomic coverage, which allows for a more complete analysis of genomic sequences underlying differentially methylated regions. However, the RRBS approach does require a well-assembled reference genome with good annotations for alignment and functional analysis purposes. Because the guppy genome was annotated using the "The NCBI Eukaryotic Genome Annotation Pipeline" with predicted functions but no gene ontology terms (Künstner et al., 2016), we could not always convert gene IDs from the guppy genome to ENSEMBL IDs in corresponding model species, resulting in an incomplete analysis of DMR-associated gene functions.

Second, although we measured parasite load, we did not measure the infection-induced phenotypic change of each fish, for example, mate choice, and our sampling procedure is terminal, so the direct associations between epigenetic and phenotypic variation and fitness remain unclear. The relationships between the hyper/hypomethylation of DMRs and gene expression of the loci that they map to are complex, and although we have speculated about the potential effects of DMRs on phenotype (and possibly fitness) based on genomic architecture and gene ontology, these inferences are necessarily speculative pending future work to directly investigate the phenotypic and fitness consequences of differential methylation.

Third, although our analyses only included reads that uniquely aligned to one location, we cannot distinguish all gene family members due to the extensive repetitive sequences (approximately 20%) in the guppy genome (Künstner et al., 2016). Several of the DMR-associated genes we observed are members of well-known gene families (e.g., *WNT2*, *ANXA5*, *CDCA8*). Thus, it is possible that in some cases we have misidentified the specific gene family member showing differential methylation. In the absence of experiments (e.g., bisulphite cloning) to distinguish gene members, results should be interpreted with caution.

Fourth, we only used skin tissue from adult individuals, whereas DNA methylation is known to be specific to tissue and development stage, and infection can induce both local and systematic immune response. Thus, using different tissues at the same infection phase, or sampling fish from other developmental stages, might have allowed us a more complete understanding of immune responses.

In summary, although we provided evidence for broad epigenetic changes that were induced by parasite infection, and differed across infection phases, the evolutionary consequences of these changes remain unexplored. The relevance of epigenetic variation for evolution rests on whether epigenetically induced responses are under genetic control, and whether these responses can improve fitness (Hu & Barrett, 2017; Richards, 2006). It has been suggested that certain epigenetically induced responses in animals can be inherited over several generations in the laboratory (Daxinger & Whitelaw, 2012; Heard & Martienssen, 2014; Lim & Brunet, 2013); however, the stability of these responses over longer evolutionary timescales is unclear. If the methylation patterns observed here are heritable across generations, they could potentially increase fitness by providing offspring with innate resistance to parasites.

5 | CONCLUSIONS

Consistent with previous studies of endoparasites, here we present the first evidence that ectoparasites can have important effects on genomic DNA methylation of their host. Our genome-wide methylation data show significant epigenetic changes in guppies infected with an isogenic strain of the guppy-specific *G. turnbulli* and indicate that these changes vary across different phases of infection. We found an underrepresentation of methylation variation in promoters, and over half of the DMRs overlapping with gene bodies, suggesting an important role of gene body methylation in host–parasite interactions. Although discriminating between the causes and consequences of methylation variation is challenging, the high number of DMRs in fish showing successful recovery from infection suggests that these modifications could potentially be driven by an active response of the host as opposed to being regulated by the parasite. Our study adds to the large body of literature on guppy–*Gyrodactylus* interactions by characterizing the epigenetic modifications associated with infection dynamics and demonstrates that epigenetic modifications in guppies play an important role in the immune response to *Gyrodactylus*. Further investigation of DNA methylation patterns across natural host and parasite populations may be key to explaining the variation in resistance to infection observed in nature, as well as the evolution of complex phenotypic traits in the context of host–parasite interactions.

ACKNOWLEDGEMENTS

The authors would like to thank members of DryBar, Amanda Lea, Daniel Schoen, and the three anonymous reviewers for useful feedback on the manuscript. This work was supported by China Scholarship Council Fellowship 201406350023 to JH, a Postdoctoral Fellowship to FPJ and a Doctoral Award to LB by the NSERC CREATE 2015-466283, and a NSERC Discovery Grant and Canada Research Chair to RDHB.

CONFLICT OF INTEREST

The authors have no conflict of interests.

DATA ACCESSIBILITY

Raw Illumina sequencing reads for the 17 analysed individuals can be downloaded from the NCBI Short Read Archive (SRA Accession no.: SRP145142).

AUTHOR CONTRIBUTIONS

J.H. and R.D.H.B. conceived the study. J.H., F.P.J. and L.B. ran the experiment and collected the data, J.H. analysed the data, and J.H. wrote the manuscript with input from F.P.J., L.B. and R.D.H.B.

ORCID

Juntao Hu http://orcid.org/0000-0003-1857-8700
Léa Blondel http://orcid.org/0000-0002-4255-2140

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

How to cite this article: Hu J, Pérez-Jvostov F, Blondel L, Barrett RDH. Genome-wide DNA methylation signatures of infection status in Trinidadian guppies (*Poecilia reticulata*). *Mol Ecol*. 2018;27:3087–3102. https://doi.org/10.1111/mec.14771