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Gene expression remodelling and immune response during adaptive divergence in an African cichlid fish

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Abstract

Variation in gene expression contributes to ecological speciation by facilitating population persistence in novel environments. Likewise, immune responses can be of relevance in speciation driven by adaptation to different environments. Previous studies examining gene expression differences between recently diverged ecotypes have often relied on only one pair of populations, targeted the expression of only a subset of genes or used wild-caught individuals. Here, we investigated the contribution of habitat-specific parasites and symbionts and the underlying immunological abilities of ecotype hosts to adaptive divergence in lake–river population pairs of the cichlid fish *Astatotilapia burtoni*. To shed light on the role of phenotypic plasticity in adaptive divergence, we compared parasite and microbiota communities, immune response, and gene expression patterns of fish from natural habitats and a lake-like pond set-up. In all investigated population pairs, lake fish were more heavily parasitized than river fish, in terms of both parasite taxon composition and infection abundance. The innate immune response in the wild was higher in lake than in river populations and was elevated in a river population exposed to lake parasites in the pond set-up. Environmental differences between lake and river habitat and their distinct parasite communities have shaped differential gene expression, involving genes functioning in osmoregulation and immune response. Most changes in gene expression between lake and river samples in the wild and in the pond set-up were based on a plastic response. Finally, gene expression and bacterial communities of wild-caught individuals and individuals acclimatized to lake-like pond conditions showed shifts underlying adaptive phenotypic plasticity.

KEYWORDS
cichlid, habitat-specific gene expression, lake–river, microbiota, parasites, plasticity

1 | INTRODUCTION

Colonization of novel environments is a key step in ecological divergence between populations. Adaptation to new environments can occur through the fixation of pre-existing genotypic differences, through novel mutations, via phenotypic plasticity or a combination of these (Pfennig et al., 2010; Price et al., 2003; Schneider & Meyer, 2017; West-Eberhard, 2003).

Adaptive phenotypic plasticity—that is, the ability to generate a phenotype from the same genotype that is better suited for a novel environment (Ghalambor et al., 2007)—can promote the expansion of populations into new niches (Richards et al., 2006; Thibert-Plante & Hendry, 2011; Yeh & Price, 2004). Adaptive phenotypic plasticity can temporarily shield genetic diversity from the direct impact of natural selection. This can generate a time-lapse between the emergence of beneficial mutations and their spread within a population, which may eventually result in genetic differentiation (Schlichting, 2004). While theoretical models often assume that the capacity to exhibit a plastic response is genetically based and that variation in the responsible locus/loci exists in natural populations, there is currently limited empirical evidence supporting this assumption (Nussey et al., 2005; Oostra et al., 2018). The process by which originally plastic traits can become genetically fixed in a homogeneous environment has been termed "genetic assimilation" (Waddington, 1942, 1953). However, we are far from understanding how this process operates at the molecular level (Ehrenreich & Pfennig, 2016).

Variation in gene expression has been suggested to play an important role in ecological speciation (Pavey et al., 2010) by (a) facilitating population persistence in a new environment and (b) contributing to adaptive genetic divergence, which can lead to reproductive isolation and ultimately to speciation. The comparative analysis of gene expression patterns can uncover ecologically important phenotypes that are not immediately evident from the inspection of overall morphology, such as physiological differences (Pavey et al., 2010). Evidence of parallel trajectories in gene expression (i.e., repeated regulation of gene expression in the same direction in one environment compared to another) allows for a more complete evaluation of the degree of parallelism in adaptive divergence than focusing only on parallel genomic divergence (Derome et al., 2006; Hanson et al., 2017). Furthermore, studying gene expression plasticity leads to a more inclusive understanding of the role of phenotypic plasticity in adaptive evolution, moving away from purely theoretical predictions (Ghalambor et al., 2007; Price et al., 2003; West-Eberhard, 2005), in particular when applied to the level of entire transcriptomes of wild populations (Aubin-Horth & Renn, 2009; Todd et al., 2016). Previous studies examining gene expression differences between recently diverged ecotypes led to valuable insights but often relied on comparisons involving only one pair of populations (Ghalambor et al., 2015; Jeukens et al., 2010; Lenz et al., 2013), targeted the expression of only a subset of genes (Di Poi et al., 2016; McCairns & Bernatchez, 2010; Morris et al., 2014) or used wild caught-individuals (Huang et al., 2016).

Transplant and common garden experiments are often used to characterize the relative contribution of heritability versus plasticity in ecologically relevant traits (Schlichting & Pigliucci, 1998; West-Eberhard, 2003). However, most of the available studies focused on obvious phenotypic traits such as morphology and size, neglecting traits such as immunity and physiology, which are more difficult to measure but may be equally important for performance and fitness (Lohman et al., 2017).

In addition, some drivers of divergent selection via adaptation to different environments, such as predation and resource competition, have received considerable scientific attention (Bolnick, 2004; Ingley & Johnson, 2016; Marchinko, 2009; Nosil, 2004; Nosil & Crespi, 2006; Schluter, 2003; Svanback & Bolnick, 2007; Vamosi, 2003; Vamosi & Schluter, 2002), while local adaptation related to parasite pressure has often been neglected (Rundle & Nosil, 2005). Differences in the abiotic and biotic conditions in varying habitats can result in the development and maintenance of contrasting parasite communities (Eizaguirre et al., 2011; Halmetoja et al., 2000). Selection on immune system diversification can be particularly strong when hosts encounter novel parasites such as after colonization of a new habitat (Diepeveen et al., 2013; Jones et al., 2012; Matthews et al., 2010; Scharsack et al., 2007). In aquatic environments, parasite composition and abundance often vary between lake and river environments (Scharsack et al., 2007). The presence of parasites can be a persistent selective force in freshwater fish populations and has been suggested to drive adaptive divergence between fish species (Blais et al., 2007; Eizaguirre et al., 2012a, 2012b; Eizaguirre et al., 2009; MacColl, 2009; MacColl & Chapman, 2010). The co-evolutionary dynamics of hosts and their parasites are expected to lead to local immunogenetic adaptation in the hosts as well as to local adaptation of parasite infectivity and virulence (Eizaguirre & Lenz, 2010; Kaltz & Shykoff, 1998; Kawecki & Ebert, 2004). The immune system of vertebrates consists of two components: (a) the innate immune response is the first line of defence and consists of nonspecific mechanisms to protect hosts from infection (Medzhitov & Janeway, 2002), and (b) the adaptive immune response is characterized by a specific antigen recognition that drives a secondary pathogen-specific reaction (Castro & Tafalla, 2015).

The vertebrate immune system is also affected by host-associated microorganisms, whose importance and ubiquity (for a review see McFall-Ngai et al., 2013) have stimulated work on the role of the microbiome in animal speciation (Brucker & Bordenstein, 2012, 2013; Sharon et al., 2010; Shropshire & Bordenstein, 2016). Plasticity of the gut microbiota has been proposed as an essential factor determining the phenomic plasticity of vertebrates, but this hypothesis remains largely unexplored as most studies to date have focused on gut microbiota composition at one point in time rather than its plasticity (Alberdi et al., 2016).

The haplochromine cichlid fish *Astatotilapia burtoni* inhabits Lake Tanganyika and affluent rivers (Figure 1, Table S1)—environments that differ in water chemistry, habitat structure and prey availability (Theis et al., 2014). *A. burtoni* lake and river ecotypes provide an excellent model to study population pairs along a "speciation continuum," from
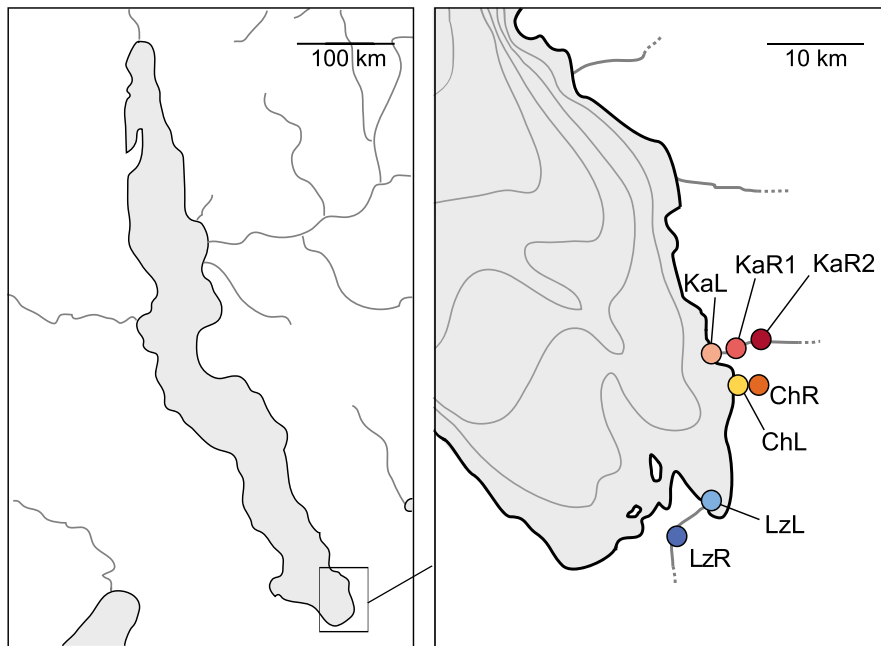
FIGURE 1 *Astatotilpia burtoni* lake and river populations from the south-east of Lake Tanganyika used in this study. KaL, Kalambo lake; KaR1, Kalambo river; KaR2, Kalambo river upstream; ChL, Chitili lake; ChR, Chitili river; LzL, Lunzua lake; LzR, Lunzua river

early divergence to a considerable degree of reproductive isolation (Egger et al., 2017; Rajkov et al., 2018a, 2018b; Theis et al., 2014, 2017). The ecotypes show habitat-specific adaptations in body shape and trophic structures that correspond to different diets (Theis et al., 2014). Common garden experiments have demonstrated that differences in body shape and gill raker length have both a plastic and a genetic component (Theis et al., 2014). A transplant experiment using F_1 lake–river crosses raised in a common garden lake-like set-up identified a substantial contribution of adaptive phenotypic plasticity to increased immigrant fitness (Rajkov et al., 2018a). River and lake ecotype also differ in number and relative area of so-called egg-spots (ovoid markings on the anal fins of males), both of which correlate with adaptive immune response (Theis et al., 2017). Furthermore, genome scans identified candidate genes with immune-related functions that show differences in expression between lake and river populations (Egger et al., 2017).

Here, we use *A. burtoni* lake–river population pairs to investigate the potential role of habitat-specific parasites and symbionts and the underlying immunological capabilities of ecotype hosts in lake–river divergence. To better understand the role of phenotypic plasticity in adaptive divergence (Rajkov et al., 2018a), we compare gene expression patterns, parasite and microbiota communities of fish from natural habitats and a lake-like pond set-up. We employ RNA-sequencing (RNA-seq) of replicate populations from two different environments, including their natural habitat range and a pond lake-like set-up, to evaluate plasticity of expression patterns between divergent ecotypes.

2 | MATERIALS AND METHODS

2.1 | Study populations and pond experiment

For parasite screening and transcriptome sequencing (RNA-seq), we sampled between 16 and 22 adult *Astatilapia burtoni* specimens

per locality at an ~1:1 sex ratio from lake and river populations at the Kalambo River (Kalambo lake—KaL, Kalambo river—KaR1 and Kalambo river upstream—KaR2) and the Lunzua River (Lunzua lake—LzL, Lunzua river—LzR) (Figure 1) in August 2017 (see Table 1 for sampling locations and sample sizes). Additionally, 10 adults per locality at an [~]1:1 sex ratio were sampled for parasite screening from the Chitili River (Chitili lake—ChL, Chitili river—ChR) in September 2018. For microbiota genotyping, 20 individuals per population were sampled from the same locations at Kalambo and Lunzua River in August 2017. At each location, conductivity and pH of the water were measured with a pH meter (HI 73127; Hanna Instruments). Conductivity is a measure of water's ability to pass electrical flow. This ability is directly related to the concentration of ions in the water. Water salinity is usually not measured directly, but is instead derived from the conductivity measurement (Baker, 2007; Pickard & Emery, 1990). Fish were caught by hook and line fishing and transported to the field station at Kalambo Lodge in buckets filled with water from sampling locations. Specimens were either processed within a few hours of capture or kept alive for one night in concrete ponds filled with water from the respective sampling location. To evaluate plasticity in parasite and microbiota communities as well as in gene expression, we included 26 specimens from a previous experiment involving wild-caught adults (November 2015) from Kalambo lake (KaL) and river (KaR2) populations. These fish were kept in two separate concrete ponds (dimensions: 2 × 1 × 1 m; length × width × depth) supplied with lake water mimicking lake-like conditions with respect to water temperature, chemistry and flow, at high density (~100 individuals per pond) from July 2016 until August 2017 and fed daily with commercial flake food. Fish were killed by pithing and immediately photographed, measured (±0.5 mm), weighed (±5 mg), sexed by visual inspection of external coloration and the genital papilla, and fin-clipped. Sampling and parasite screening were performed under study permits number 003376 and 004264 issued by

TABLE 1 Geographical coordinates of sampling locations, and sample size details for parasite screening and splenosomatic index (SI), RNA-seq and microbiota analysis (16S seq); for Chitili lake and stream populations only parasite data were collected

Site	System	Habitat	Year	GPS coordinates Latitude	Longitude	Sample size parasite screening and SI Males	Females	Juveniles	Total	Sample size RNA-seq Males	Females	Total	Sample size 16S seq Males	Females	Total
LzL	Lunzua	Wild	2017	8°44'57.13"S	31°10'21.86"E	11	8	1	20	4	4	8	4	4	8
LzR	Lunzua	Wild	2017	8°47'23.51"S	31°8'14.33"E	11	9	0	20	4	4	8	4	4	8
KaL	Kalambo	Wild	2017	8°36'6.27"S	31°11'13.24"E	12	10	0	22	4	4	8	4	4	8
KaR1	Kalambo	Wild	2017	8°35'35.23"S	31°11'6.18"E	11	5	0	16	4	4	8	5	3	8
KaR2	Kalambo	Wild	2017	8°35'6.24"S	31°12'29.32"E	13	6	0	19	4	4	8	4	4	8
KaL pond	Kalambo	Pond	2017	8°37'25.5"S	31°12'03.7"E	6	6	0	12	4	4	8	5	3	8
KaR2 pond	Kalambo	Pond	2017	8°37'25.5"S	31°12'03.7"E	8	6	0	14	4	4	8	4	4	8
ChL	Chitili	Wild	2018	8°38'18.42"S	31°11'55.34"E	6	5	0	11	NA	NA	NA	NA	NA	NA
ChR	Chitili	Wild	2018	8°38'16.91"S	31°124.02"E	6	6	0	12	NA	NA	NA	NA	NA	NA

Lake Tanganyika Research Unit, Department of Fisheries, and the Immigration Department, Republic of Zambia.

2.2 | Parasitological survey

Immediately after measuring each fish (see above), we dissected gills, skin, fins, eyes, intestinal tract, heart and liver, and exhaustively screened these organs for metazoan ecto- and endoparasites using a field stereomicroscope (Nikon, SMZ445). We first screened the outer surface of each *A. burtoni* specimen for monogeneans and crustaceans. Next, fins and gills were dissected and screened separately for ectoparasites. The gastrointestinal tract was excised from oesophagus to anus and placed in saline solution (0.9% NaCl). Livers and hearts were dissected and inspected in a Petri dish filled with lake water. Finally, the intestinal content and teased internal organs were pressed between two glasses of a Petri dish for examination. Endoparasite specimens were separated according to higher helminth taxa (Acanthocephala, Cestoda, Digenea and Nematoda) following Paperna (1996).

2.3 | RNA-seq library preparation and sequencing

We focused on immune- and homeostasis-related organs: spleen and gills, as those are most likely to be involved in adaptation to different osmotic habitats and parasite pressure. Immediately after parasite screening of the gills, one gill from each side as well as the spleen were dissected and preserved in RNAlater (Sigma-Aldrich) and later transferred to Individual TRIzol tubes which were weighed (±0.05 mg) before and after the spleen was added to calculate spleen weight. For RNA extraction in the laboratory, we used Zymo Directzol-96 RNA plates (Zymo Research), after bead-beating for homogenization in TRIzol and chloroform phase separation. Quality control was done using Nanodrop (Thermo Scientific) and TapeStation 4200 (Agilent) devices. Eight individuals per sampling site were selected for RNA-seq based on RNA quality while ensuring an equal sex distribution for each population. Sample selection was performed ignoring parasite screening results. Individual libraries from 112 samples (56 specimens, two tissues) were constructed using the TruSeq Stranded Total RNA Library Prep Kit Ribo-Zero Gold (Illumina). Single-end sequencing to 125-bp reads in five Illumina HiSeq2500 lanes was performed at the Genomics Facility Basel jointly operated by the Department of Biosystem Science and Engineering (D-BSSE), ETH Zürich, and the University of Basel.

2.4 | Microbiota

Whole intestines and swabs of the buccal mucosa were placed in 99% ethanol for subsequent DNA extraction. The stomach was removed from the intestines before the extraction to remove undigested content. DNA from ethanol-preserved cotton swabs was

extracted following a modified DNeasy Blood & Tissue Kit (Qiagen) protocol (Keller et al., 2017). The modifications included evaporating EtOH at 60°C under vacuum (Concentrator plus, Eppendorf), overnight incubation at 56°C after the addition of ATL buffer and proteinase K into the swab tube, incubation at 56°C for 30 min after addition of AL buffer into the swab tube to increase the recovery of Gram-positive bacteria, and final elution in 2×60 µl. DNA from ethanol-preserved intestines was extracted using a modified version of repeated bead-beating plus column (RBB + C) protocol following Baldo et al. (2015). For Illumina sequencing of amplicons, DNA was amplified with 16S rRNA gene primers that target the V3–V4 hypervariable region. The library preparation protocol followed Baldo et al. (2017), with minor modifications (see Table S1 for primer sequences). A subset of eight individuals per population with [~]1:1 sex ratio was selected for library preparation after an initial PCR (polymerase chain reaction) step to test for positive amplification. We used 25 cycles for the first and 15 cycles for the second PCR. Amplicons were cleaned individually with the UltraClean 96 PCR Cleanup Kit (Qiagen). All samples were then quantified with a Qubit High Sensitivity Kit (Thermo Scientific) and pooled at equimolar concentrations to create the final library. This library was sequenced on an Illumina MiSeq version 3 (600 cycle cartridge, 300-bp paired-end) with 15% PhiX, at the Genomics Facility Basel.

2.5 | Data analysis

2.5.1 | Parasites and immune response

Infection presence, prevalence (percentage of infected fish per population), abundance (sum of parasite individuals on/in infected fish divided by the number of dissected fish) and median infection intensity (median number of parasite individuals on infected fish) were calculated following Rózsa et al. (2000) for each group of parasites and each population. We calculated three summary variables as estimates of overall parasite infestation rate: (a) number of parasite taxa (the number of parasite taxa infecting one fish), (b) total parasite load (the sum of all parasites infecting one fish) and (c) gill parasite load (the sum of all gill parasites infecting one fish).

Differences in (a) the number of parasite taxa, and (b) total and (c) gill parasite load between different populations were analysed using zero-inflated generalized linear models (GLMs) with a negative binomial (number of parasite taxa) or Poisson (parasite load) probability distribution, and with population, standard length and sex as predictors using the zeroinfl function in the PSCL package (version 1.5.2) (Zeilies et al., 2008). Significance of the model terms was determined based on likelihood ratio tests using lrtest in the LMTEST package (version 0.9.35) (Achim & Hothorn, 2002).

We also calculated the splenosomatic index (SI—spleen mass to body mass ratio) to estimate the innate immune response to parasite infection (Hadidi et al., 2008; Kaufmann et al., 2017; Lefebvre et al., 2004). Immune response was analysed in a linear model with the splenosomatic index as a response variable and population, total number of parasites per individual, size and sex as fixed effects.

Total (gill and gut) parasite load, gill parasite load and splenosomatic index were analysed in all wild populations (model 1). Gill parasite load and splenosomatic index were analysed separately in the Kalambo lake–river pair (KaL–KaR2) (model 2) to compare these populations in the wild and in the experimental set-up. Tukey–Kramer post-hoc tests were applied to test for the significance of pairwise comparisons between populations using the LSMEANS package (version 2.27.61) (Lenth, 2016). Statistical analyses were performed in R version 3.3.2 (R Core Team, 2016).

2.5.2 | RNA-seq

Read filtering and mapping
Illumina strand-specific single-end sequences of each library were filtered using TRIMMOMATIC version 0.33 (Bolger et al., 2014) with a 4-bp window size, required window quality of 15 and a read minimum length of 80 bp (two-thirds of the initial read length). Adapters were removed using TRIMMOMATIC. Cleaned reads were mapped against the Nile tilapia (*Oreochromis niloticus*) genome assembly (RefSeq assembly version GCF_001858045.1_ASM185804v2, Brawand et al., 2014) with STAR version 2.5.2a (Dobin et al., 2013) with the following settings: --outFilterMultimapNmax 1 --outFilterMatchNminOverLread 0.4 --outFilterScoreMinOverLread 0.4. Unique alignments were reported in sorted BAM format and assigned to genes using the HTSEQ-count script from the HTSEQ framework (version 0.6.1p1) (Anders et al., 2015).

Global expression patterns
Prior to all analyses, we excluded genes with very low expression levels, which we considered as noise (present in fewer than four samples with less than three counts per sample). Expression values were then normalized with the DESEQ2 (version 1.24.0.) R (version 3.5.0) package (Love et al., 2014). The tissue type (gills vs. spleen), habitat type (lake vs. river), population (Kalambo vs. Lunzua) and experimental condition (wild vs. pond) were included in the DESEQ2 design. Variance stabilizing transformation (VST) was applied to the normalized expression data to minimize differences between samples when plotting the data. We used principal components analysis (PCA) to illustrate the global patterns of gene expression differences as implemented in DESEQ2. To summarize the data, replicates of each population within each organ were grouped by calculating their median expression. The summarized expression values were then transformed into transcripts per kilobase million (TPM) values. These values were used for all downstream analyses apart from differential expression analyses.

Rate of gene expression evolution
Following the method of Brawand et al. (2011), "expression trees" were constructed using the neighbour-joining approach on the

pairwise Euclidean distance between populations for all protein-coding genes (28,938 genes) and separately for each organ. All neighbour-joining trees were assembled using the R package APE (version 5.3) (Paradis et al., 2004). Branch lengths were represented as the sum of the branch lengths for river samples and the sum of the branch lengths for lake samples.

Differential gene expression analyses

To identify differentially expressed (DE) genes between the two habitats, we compared lake and river samples for each organ (gills and spleen) with DESEQ2 by grouping KaR1, KaR2 and LzR samples into a river group and KaL and LzL into a lake group. To examine the effect of the KaR2 nonparasitized population on gene expression, we also performed a gene expression analysis excluding the KaR2 samples from the river populations and another analysis excluding the KaR1 samples from the river populations. We added the lake–river system information (Kalambo and Lunzua) as an interaction factor in our model, following recommendations for DESEQ2 (Love et al., 2014), as we identified phylogenetic signal in the expression data. Genes with an adjusted *p*-value (false discover rate [FDR]) below .05 were considered as DE. We then intersected all pairwise gene expression comparisons and reported all intersecting genes.

Modules of co-expressed genes and module–trait associations

To identify co-expressed genes associated with habitat type, we constructed signed weighted gene co-expression networks with the R package WGCNA (version 1.66) (Langfelder & Horvath, 2008). We followed the filtering steps recommended by Langfelder and Horvath (2008), which resulted in a matrix of 23,770 genes retaining 39 specimens for the spleen samples and 25,209 genes retaining 38 samples for the gill samples after outlier exclusions. Spleen and gills were analysed separately and only natural populations (LzR, LzL, KaR1, KaR2 and KaL) were used for the analysis. The infection status for each specimen per parasite taxon was used as a Boolean value (infected true or false). Module–trait association analyses were calculated using a weighted Pearson correlation as recommended by Langfelder and Horvath (2008).

Habitat-specific variation

We pooled the sequencing reads of the two organs per individual and quality-filtered and trimmed them with TRIMMOMATIC 0.33 (Bolger et al., 2014) with a 4 bp window size, a required window quality of 15 and a minimum read length of 30 bp. We then performed reference-free de novo variant calling per population (Kalambo and Lunzua) with KISSPLICE pipeline 2.4.0, which filters out single nucleotide polymorphisms (SNPs) with globally low counts of both alleles. The method used here has been developed to address this issue in the best way and shows a high precision rate such as that for condition-specific SNPs, the difference of expressed allele frequencies corresponds very well to the true difference of allele frequencies (Lopez-Maestre et al., 2016). Only KaR2 samples were used as river samples for the Kalambo system (KaR1 was excluded prior to the analysis as this population is geographically and genetically very

close to the lake population; Egger et al., 2017). KISSPLICE was run with -s 1 -t 4 -u and -experimental. The SNPs thus identified were placed on the Nile tilapia genome assembly with STAR 2.5.2a (Dobin et al., 2013) (--outFilterMultimapxNmax 1 --outFilterMatchNminOverLread 0.4 --outFilterScoreMinOverLread 0.4). The genome index used for this mapping step was generated with the corresponding STAR parameters: --runMode genomeGenerate, --sjdbOverhang 124, --sjdbGTFfeatureExon exon and the genome annotation file (RefSeq GCF_001858045.1_ASM185804v2). KISSPLICE2REFGENOME version 2.0.0 (default parameters) was used to classify KISSPLICE events aligned to the Nile tilapia reference genome and KISSDE 1.4.0 (default parameters) was used to determine variants that differ between the two habitats (river and lake). The KISSPLICE events were filtered according to the following attributes in R 3.5.1: only SNPs were kept; SNPs placed on mitochondrial DNA or on unplaced scaffolds of the reference genome were removed; only SNPs with significant *p*-values for an allele difference between habitats were kept ($p \leq .05$ after adjustment for multiple testing following the Benjamini & Hochberg method). We then defined candidate genes as genes with SNPs with significant *p*-values in both populations (73 genes, Table S10).

Plasticity and genetic assimilation

By performing differential gene expression analysis between lake and river samples in the wild and in pond conditions, we were able to approximate which proportion of gene expression variation is plastic and which is genetically fixed between populations. As the plastic response, we defined all genes that were DE in the wild river–lake contrast but not in the pond river–lake contrast. On the other hand, we assigned genes that were DE in both river–lake contrasts (pond and wild) to genetically fixed gene expression variation. We performed this analysis for the two organs separately and reported how many and which genes were assigned to each category (genetic assimilation or plastic response).

Enrichment analyses

All GO enrichment graphs and all GO enrichment tables were produced within BLAST2GO (version 5.1) (Conesa et al., 2005). Enrichment analyses were run in BLAST2GO using a two-tailed Fisher's exact test with the complete Nile tilapia transcriptome as the background set. This set was functionally annotated with BLAST2GO based on the blast output against the nonredundant database (September 2017) with default settings. Gene ontology (GO) enrichment analyses were done for all genes (longest isoform as representative of the gene) within modules that have the highest correlation with the habitat in the gills and in the spleen. GO terms of enrichment test outcomes were reduced to the most specific GO terms.

2.5.3 | Microbiota

PCR-negative control samples resulted in very low read coverage (≤ 505), suggesting that contamination was negligible and were hence excluded from the final data set. Sequence raw reads were

quality-filtered, assembled into contigs and classified using MO-THUR (version 1.39.5) (Schloss et al., 2009) according to the MOTHUR Illumina MiSeq SOP. Taxonomic classification was performed against the RDP database (Cole et al., 2014) using the classify.seqs function with a bootstrap cut-off of 80%. Chloroplasts, mitochondria and nonbacterial sequences were removed. To obtain operational taxonomic units (OTUs), sequences were clustered at a 0.03 distance level. The OTU table output of MOTHUR was imported into R (version 3.3.2) for further processing using the R package PHYLOSEQ (version 1.19.1) (McMurdie & Holmes, 2013).

We retained only OTUs shared across two or more samples, and the OTU abundance table was subsampled to an even sequencing depth using the rarefy_even_depth function in PHYLOSEQ. Alpha (inverse Simpson index) and beta (Bray–Curtis distances) diversity measures were calculated in PHYLOSEQ. Pairwise Bray–Curtis distances among samples were visualized using nonmetric multidimensional scaling (NMDS). The permutational analysis of variance (PERMANOVA) (10,000 permutations) was performed with the VEGAN package (version 2.4-5) in R separately for gut and mouth microbiota to test the habitat effects, the population effects and their interaction.

3 | RESULTS

3.1 | Higher parasite diversity and abundance in lake than in river habitats

The parasite community differed substantially between lake and river fish within lake–river systems (Table 2). Gut parasites (nematodes and Acantocephala), as well as parasitic copepods on the gills were only present in the lake populations (except for one individual from the Chitili River that could be a recent migrant). River populations KaR1 and LzR showed only monogenean infections and no parasites were detected at site KaR2. However, KaR2 fish kept in the lake-like pond set-up acquired copepod and monogenean parasites.

The number of parasite taxa depended on population ($\chi^2_{df=6} = 80.68$, $p < .001$; Table S2; Figure 2a). LzL fish had higher taxon richness compared to river fish from both the Lunzua and the Kalambo River (post-hoc test, all $p < .05$). Fish from the upstream Kalambo population (KaR2) had lower taxon richness than any lake population (post-hoc test, all $p < .05$). There was no significant difference in the number of parasite taxa between geographically closer lake and river populations from the same system with very low genetic divergence (median $F_{ST} = 0$, Egger et al., 2017) (post-hoc test: KaL-KaR1, $p = .131$; ChL-ChR, $p = .266$).

The total parasite load depended on population ($\chi^2_{df=6} = 147.03$, $p < .001$) and sex of the individual ($\chi^2_{df=2} = 6.55$, $p = .038$) (Table S3; Figure 2b). LzL fish had a higher parasite load than any river fish (post-hoc test, all $p < .05$), and Kalambo lake fish had a higher parasite load than Kalambo river fish (post-hoc test: KaL–KaR1, $p = .049$; KaL–KaR2, $p = .03$). The difference in total parasite load between Chitili lake and river fish was not significant (post-hoc test: ChL–ChR, $p = .075$). Males had higher parasite load than females (post-hoc test: f-m, $p = .034$).
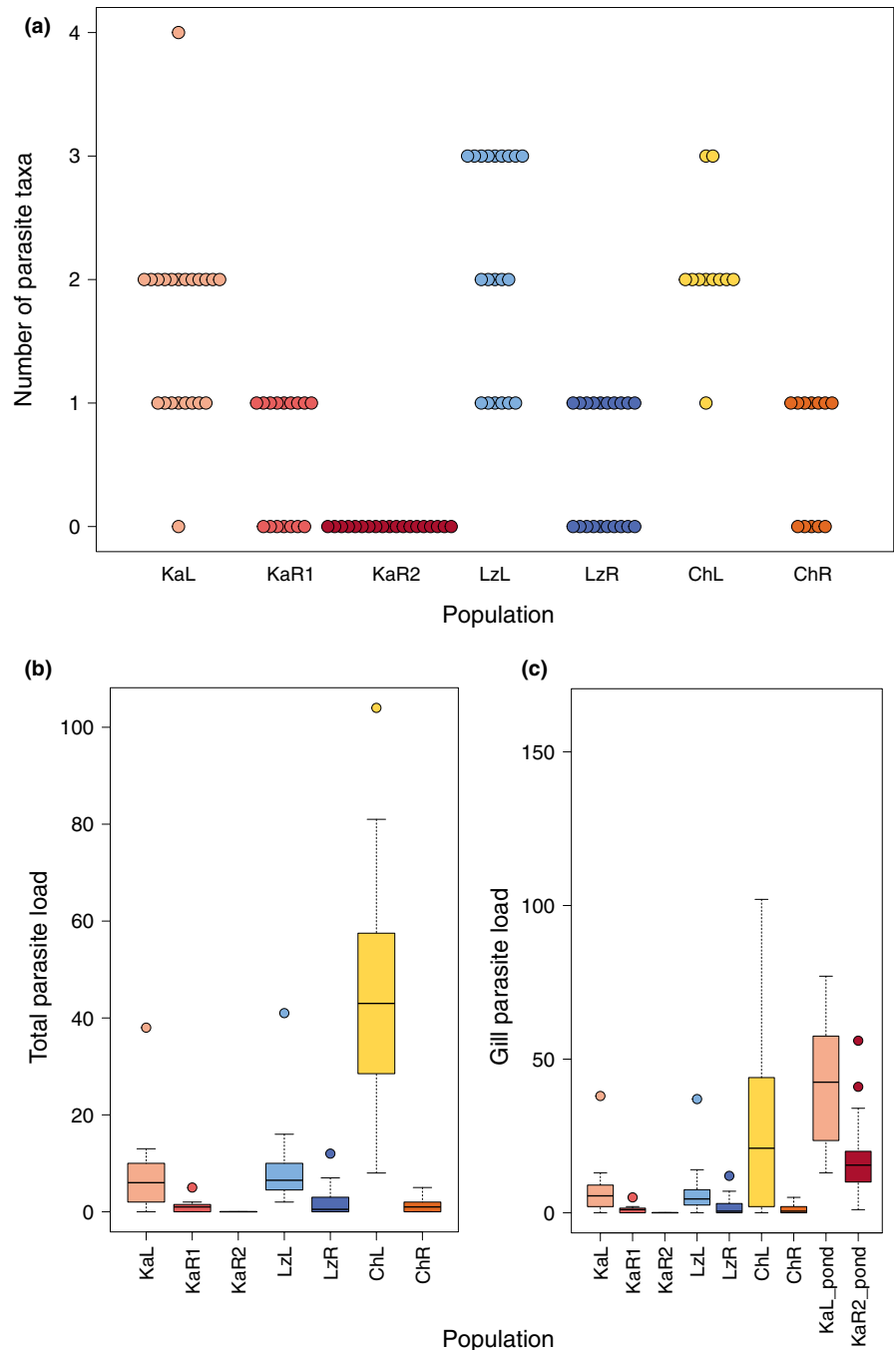
Gill parasite load in the wild (model 1) depended on population ($\chi^2_{df=6} = 1,065.50$, $p < .001$), fish size ($\chi^2_{df=1} = 15.09$, $p < .001$) and sex ($\chi^2_{df=2} = 9.95$, $p = .007$) (model 1; Table S4; Figure 2c). Lake fish had a significantly higher gill parasite load than river fish (post-hoc test: all $p < .001$), except for LzL–LzR ($p = .266$) and KaL–LzR ($p = .088$). Males had a higher gill parasite load than females (post-hoc test: f–m, $p = .004$), and larger individuals had a higher gill parasite load in the lake habitat and in the ponds (Figure S1).

In the ponds versus wild comparison, gill parasite load depended on population ($\chi^2_{df=3} = 102.01$, $p < .001$) (model 2; Table S4; Figure 2c). There was a significant difference in gill parasite load between populations in all pairwise comparisons (post-hoc test: KaL–KaR2, KaLpond–KaR2_pond, KaLpond–KaL, KaLpond–KaR2, KaR2pond–KaR2; all $p < .05$) except between KaL in the wild and KaR2 in the pond set-up (post-hoc test: KaL–KaR2pond, $p = .115$).

TABLE 2 Sampling site, lake–river system, habitat type, sample size and prevalence (%)/mean abundance/median intensity of different groups of parasites for five populations of *Astatotilapia burtoni* from the wild and two from a pond set-up

Site	System		Habitat	N	Gill ectoparasites		Gut endoparasites	
					Monogenea	Copepoda	Acantocephala	Nematoda
LzL	Lunzua	Wild	Lake	20	55/2.1/2	90/4.45/3	5/0.05/1	65/2.3/4
LzR	Lunzua	Wild	River	20	50/2.2/3	0/0/—	0/0/—	0/0/—
KaL	Kalambo	Wild	Lake	22	59.09/3.32/4	81.82/3.77/4	13.64/0.09/1	9.09/0.14/1
KaR1	Kalambo	Wild	River	16	56.25/1/1	0/0/—	0/0/—	0/0/—
KaR2	Kalambo	Wild	River	19	0/0/—	0/0/—	0/0/—	0/0/—
KaL pond	Kalambo	Pond	Lake	12	100/41.83/42.5	16.67/0.25/1.5	NA	NA
KaR2 pond	Kalambo	Pond	River	14	100/17.43/12.5	57.14/1.71/2.5	NA	NA
ChL	Chitili	Wild	Lake	11	100/43/33	72.73/3.18/4	36.36/0.45/1	0/0/—
ChR	Chitili	Wild	River	12	50/1.25/2	8.33/0.08/1	0/0/—	0/0/—

FIGURE 2 Parasite diversity and abundance per population. (a) Number of parasite taxa in the wild. (b) Total parasite load in the wild. (c) Gill parasite load including pond set-up. Colour code according to Figure 1. KaL, Kalambo lake; KaR1, Kalambo river; KaR2, Kalambo river upstream; ChL, Chitili lake; ChR, Chitili river; LzL, Lunzua lake; LzR, Lunzua river; KaL_pond, Kalambo lake population in ponds; KaR2_pond, Kalambo river upstream population in ponds

3.2 | Immune response reflects differences in parasite abundance

The splenosomatic index (SI) was analysed in all wild populations (model 1) and separately in the Kalambo lake–river pair (KaL–KaR2) (model 2) to compare these populations in the wild and in the lake-like experimental set-up. SI depended on population in both models (model 1: $F_{4,83} = 16.38$, $p < .001$; model 2: $F_{3,59} = 7.00$, $p < .001$; Table S5; Figure 3a). In the populations from the wild, the SI also depended on total parasite load (model 1: $F_{1,83} = 8.30$, $p = .005$; Table S5) with fish having a higher total parasite load also having higher SI (Figure 3b). In the wild (model 1), lake fish had a higher SI than river fish (post-hoc test, all pairwise $p < .05$; Table S5). When

fish from the pond set-up were compared with their respective source population (model 2), the KaR2 river population had a higher SI in the ponds than in the wild (post-hoc test, $p = .014$; Table S5; Figure 3a).

3.3 | Global patterns of expression divergence among organs and habitats

A PCA separated the two organs along the first principal component (PC1), accounting for 92% of the observed variance (Figure 4a). There was a strikingly higher variation among individuals in the gene expression in the spleen compared to the gills. A PCA of gill samples
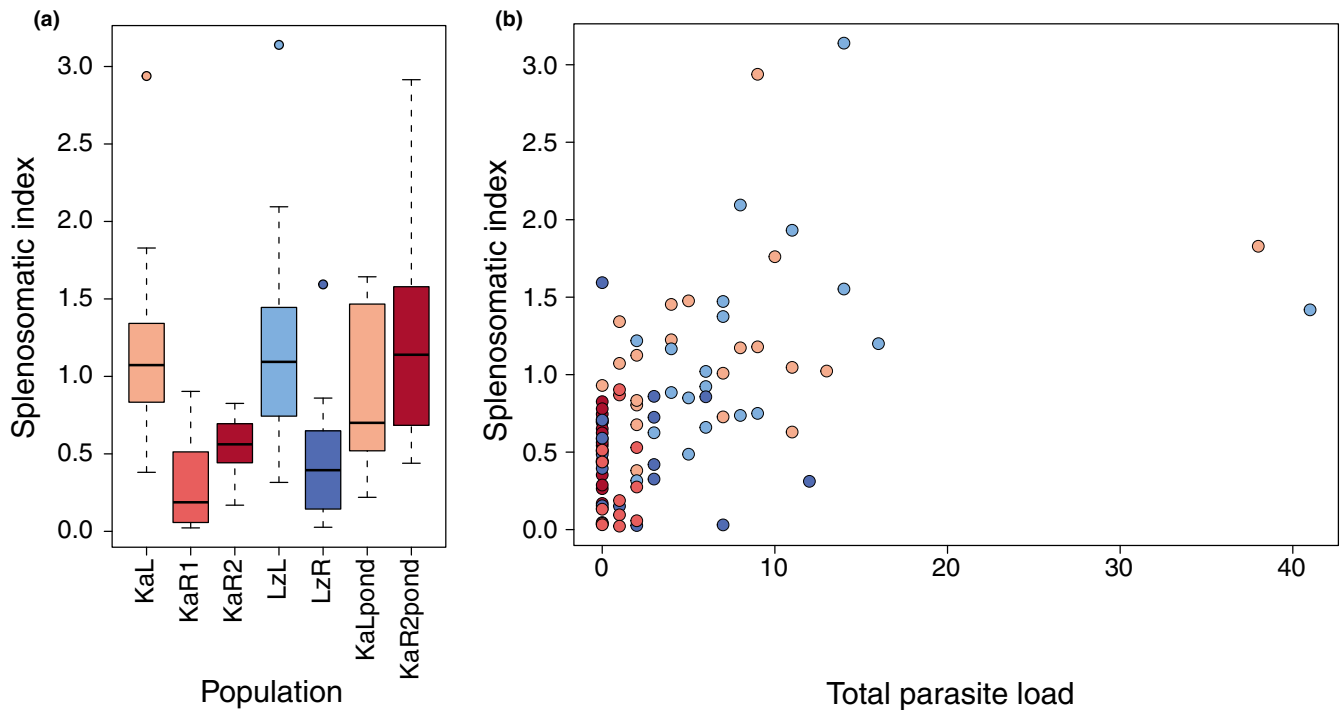
FIGURE 3 Innate immune response (splenosomatic index) depending on (a) population and (b) total parasite load in the wild populations. Colour code according to Figure 1. KaL, Kalambo lake; KaR1, Kalambo river; KaR2, Kalambo river upstream; LzL, Lunzua lake; LzR, Lunzua river
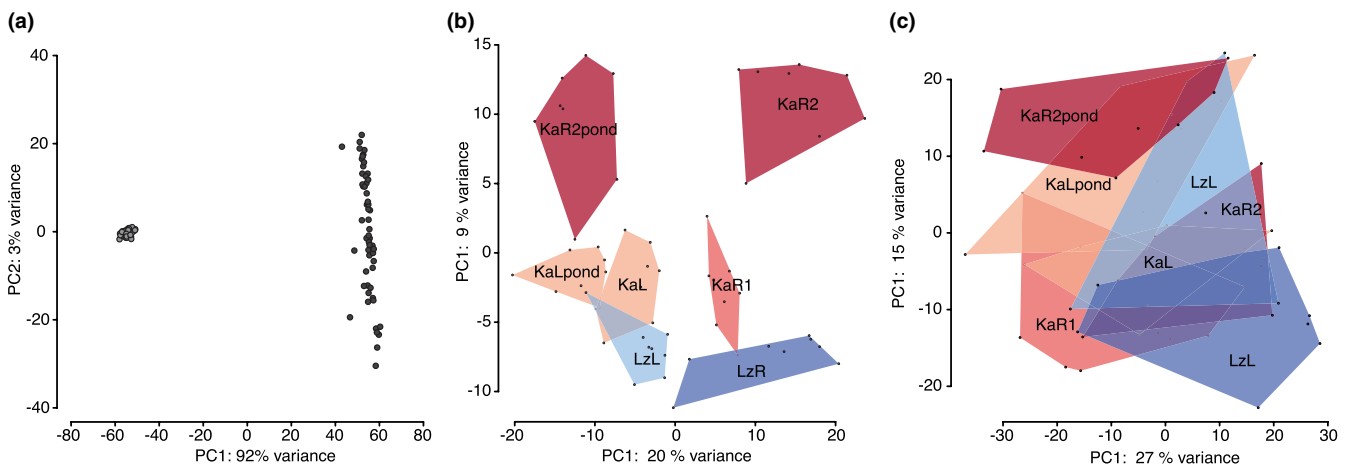

FIGURE 4 Global patterns of gene expression differences among samples. (a) PCA plot of overall gene expression levels. Samples are coloured according to organs (dark grey: gills, light grey: spleen), and proportion of the variance explained by the principal components is indicated next to the axes. (b) PCA plot of gene expression levels in gill samples. (c) PCA plot of gene expression levels in spleen samples. Replicate samples are connected with polygons, polygons are coloured according to habitats (dark grey: lake, light grey: river) and the name of the population is placed in the middle of the respective polygon. KaL, Kalambo lake; KaR1, Kalambo river; KaR2, Kalambo river upstream; LzL, Lunzua lake; LzR, Lunzua river; KaLpond, Kalambo lake population in ponds; KaR2pond, Kalambo river upstream population in ponds

only (Figure 4b) revealed a clear separation of river and lake samples along PC1 (PC1 explained 20% of the variation): The upstream river population (KaR2) clustered separately, followed by other river populations (KaR1 and LzR), the lake populations (Kal and LzL) and the pond samples (KaR2pond and KaLpond). PC2 (9% of the variation) separated the upstream Kalambo River population (both from

the wild [KaR2] and from the pond [KaR2pond]) from all the other populations, and the Kalambo system from the Lunzua system.

A PCA of spleen samples did not show a clear clustering according to population (Figure 4c). Pond individuals clustered together with wild-caught lake individuals with high total parasite load along PC2 that explained 15% of the variance (Figure S2).

3.3.1 | Rate of expression changes across organs and natural habitats

Overall, in concordance with the PCA, spleen showed a higher rate of expression changes compared to gills in the expression trees (Figure 5a). We further noted a higher rate of expression changes in river samples compared to lake samples in both organs. The difference in branch lengths suggests that gills and spleen transcriptomes may have experienced different selection regimes throughout the colonization of new habitats (Figure 5b,c). We observed the same difference between the two organs for the two types of habitat. Yet, overall, there was a greater expression of divergence in the river samples compared to the lake samples. This suggests that the lake samples may have experienced stronger purifying selection or less positive selection on gene expression than the river samples.

3.3.2 | Differences in gene expression between lake and river samples

A total of 2,772 genes showed significant differential expression (DE; up-regulation and down-regulation) between combined lake (KaL and LzL) and river habitats (combination of KaR1, KaR2 and LzR population) (Figure 6a; Table S6) in the gills and 590 in the spleen. From these DE genes, 1,137 were exclusively down-regulated in the gills, 1,462 up-regulated in the gills, 227 down-regulated in spleen and 190 up-regulated in the spleen (Figure 6b). When the nonparasitized river population (KaR2) was excluded from the differential analyses, fewer genes were DE for both tissues: only 1,787 genes were DE in the gills (2,772 if including KaR2 population) and 271 genes were DE in the spleen (290 genes if including the KaR2 population) (Figure S3).

3.3.3 | Gene-expression in parasitized versus non parasitized fish

To evaluate the effect of parasitism on gene expression, we performed differential expression analyses between infected and noninfected fish (Figure S4). We performed analyses separately for four different parasite groups: Monogenea, Copepoda, Nematoda and Acantocephala. As reported above, we only found Monogenea-infected fish in lake populations. When comparing differential expression between Monogenea-infected fish and Monogenea-noninfected fish, we observed more DE genes in the spleen compared to gills. On the other hand, we observed that for the gills, there were more DE genes in river populations compared with lake populations, while the opposite was observed in the spleen (more genes DE in the lake compared to river) (Figure S4a, Table S7). When comparing the number of DE genes for the different parasite categories and the different tissues, we observed more DE genes in fish infected by Nematode parasites than by any other parasite taxon, and more DE genes in spleen tissue than in gills (Figure S4b, Table S8). By doing so, we identified for each parasite category potential candidate genes for the host response to parasitic infection. To further connect gene expression signatures to the innate immune response and parasite infection, we constructed signed weighted gene co-expression networks from gill and spleen expression profiles and correlated the obtained networks to the following parameters: population, SI, river–lake system (Kalambo, Lunzua), habitat (lake, river) and parasite type (Figure 7). The gill gene-expression network (Figure 7a) consisted of 28 modules comprising between 27 and 1,692 genes (mean module size = 265, gene module memberships are reported in Table S9a). Here, the highest correlations were obtained for the greenyellow (223 genes, $r = .86$) and the magenta (262 genes, $r = -.86$) modules, both with habitat type (lake and river). A GO enrichment analysis (Figure S5a,b) identified GO terms related to osmoregulation to be enriched in these modules (enrichment for GO terms in
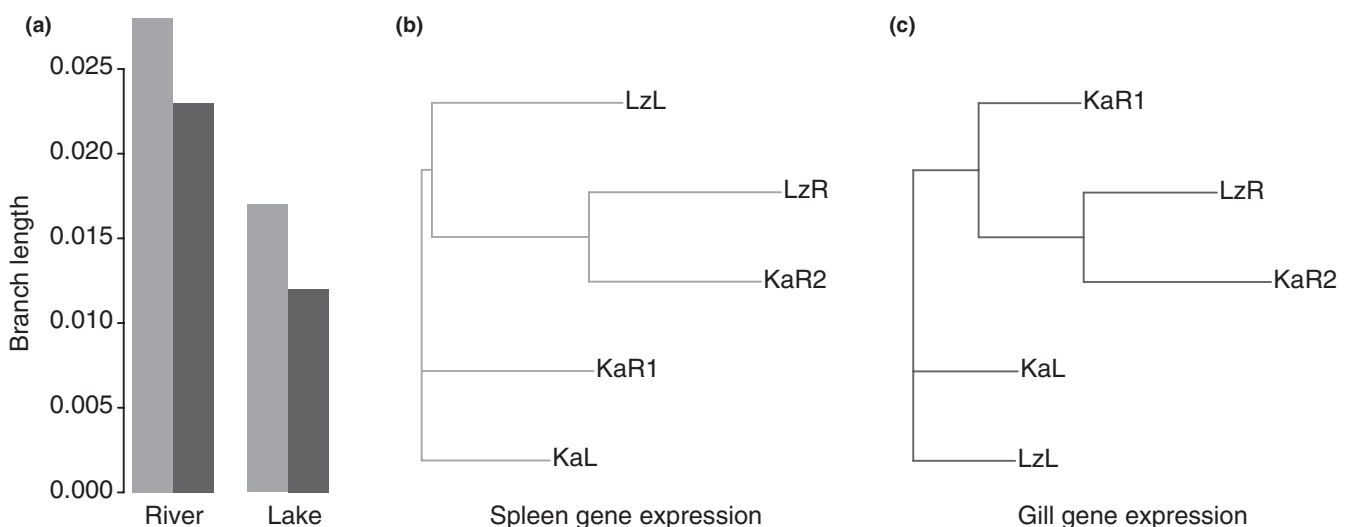

FIGURE 5 Expression divergence among organs and habitat. (a) Barplots of the branch lengths of protein-coding expression trees of river and lake samples. The barplots are coloured according to the organ (light grey: spleen, dark grey: gills). (b) Protein-coding gene expression trees in the gills. KaL, Kalambo lake; KaR1, Kalambo river; KaR2, Kalambo river upstream; LzL, Lunzua lake; LzR, Lunzua river. (c) Protein-coding gene expression trees in the spleen
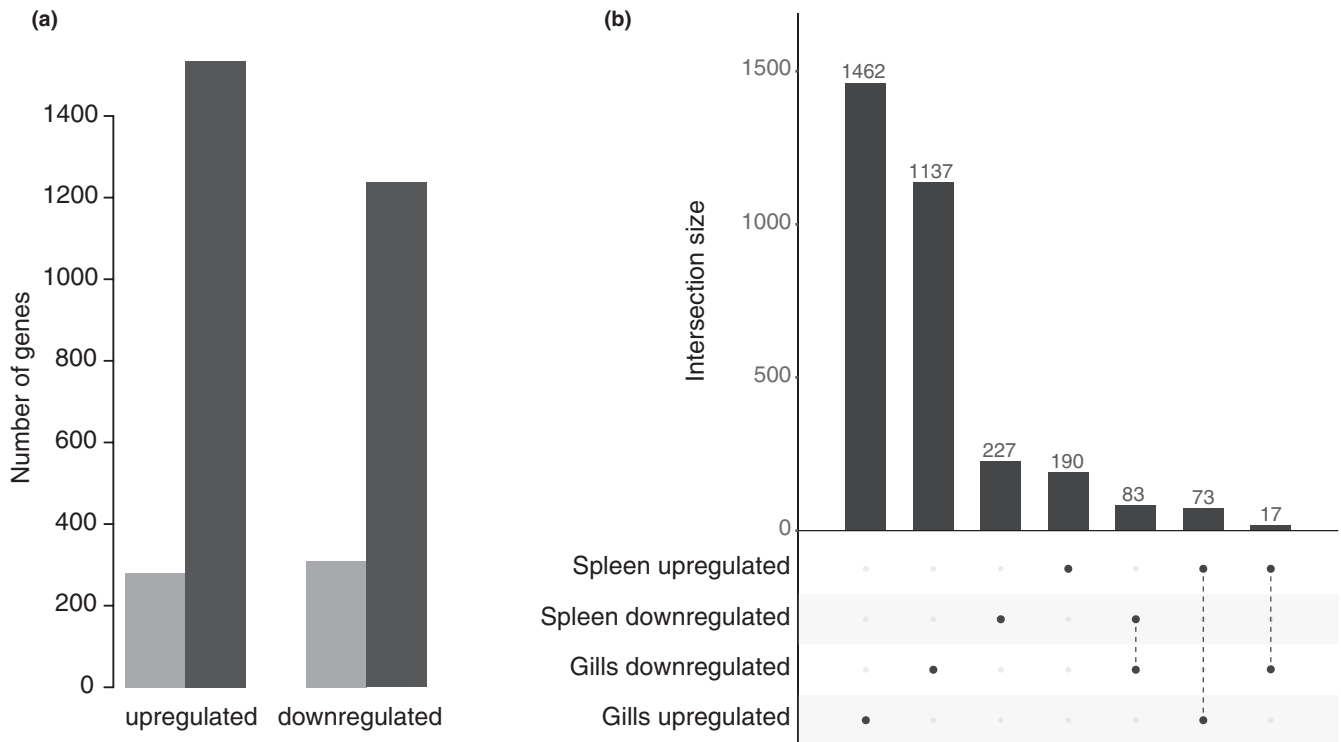
FIGURE 6 Lake–river differential expression. (a) Barplot of the number of genes up-regulated and down-regulated in the lake–river contrast (Kalmabo and Lunzua population) in the two different organs (dark grey: gills, light grey: spleen). (b) Intersection plot between the up-regulated (up) and down-regulated (down) genes in the two organs

greenyellow module [Figure S5a]: e.g., "regulation of pH," "sodium ion transmembrane transport," "sodium:proton antiporter activity," "voltage-gated chloride channel activity"; in magenta module [Figure S5b]: e.g., "intracellular signal transduction," "hydrolase activity," "nitrogen utilization"). In this tissue we also found three modules correlated with copepod infection, which included the highly habitat-correlated greenyellow module.

The spleen network (Figure 7b) consisted of 31 modules (22–3,326 genes, mean module size = 365, gene module memberships are reported in Table S9b). The strongest negative correlation was observed for the red module with SI (463 genes, $r = -.69$) and the darkturquoise module with habitat type (64 genes, $r = -.69$), which also positively correlated with SI ($r = .65$). The orange module correlated with habitat (51 genes, $r = .64$). A GO analysis (Figure S5c,d) clearly linked the red and darkturqoise module to immune response (enrichment for GO terms in red module [Figure S5c]: e.g., "autophagy", "stress-activated MAPK cascade", "extracellular exosome", all of which belong to the broader GO category "defence mechanism"; in darkturquoise module [Figure S5d]: e.g., "antigen processing and presentation of exogenous protein antigen via MHC class", "positive regulation of antigen processing and presentation of peptide antigen via MHC class").

3.3.4 | Habitat-specific genetic variation

We detected 73 significant SNPs (FDR < 0.05) between lake and river habitats in Kalambo and Lunzua system (Table S10). From these, 68 are located in protein-coding genes, two in one long non-coding RNA (LOC109196944) and three in uncharacterized genes. Among the annotated candidate genes, several genes have functions involved in ion transport and homeostasis (sodium/potassium-transporting ATPase subunit alpha-1, sodium/potassium-transporting ATPase subunit alpha-3) and immunity (basigin, ubiquitin carboxyl-terminal hydrolase CYLD, interferon regulatory factor 9, unconventional myosin-Ig, H-2 class I histocompatibility antigen Q9 alpha chain). In our samples, sodium/potassium-transporting ATPase subunit alpha-1 (*Atp1a1*) was up-regulated in all river populations in gills and in LzR also in spleen. Ubiquitin carboxyl-terminal hydrolase CYLD was up-regulated in lake populations (in comparisons KaL–KaR2 and LzL–LzR) in spleen. Basigin was up-regulated in river populations (in comparisons KaL–KaR2 and LzL–LzR) in spleen and gills. Interferon regulatory factor 9 was up-regulated in lake populations (KaL–KaR2 and LzL–LzR) in spleen. Cofilin was up-regulated in river populations (KaL–KaR2 and LzL–LzR) in spleen. Among the GO terms identified for those candidate genes was "immune system process" (Figure S6), illustrating this as an unsupervised method. In addition, in the gills, we identified two candidates (NCBI gene names and IDs: rnmt [100691696], and LOC100699477 [100699477]) included in the highly correlated "greenyellow" module and one candidate (100700200) in the highly correlated magenta module. In the spleen, we identified three candidates (NCBI gene IDs: 100694281, 100696602 and 100708925) included in the highly correlated "red" module and one candidate (100701947) in the highly correlated darkturquoise module.
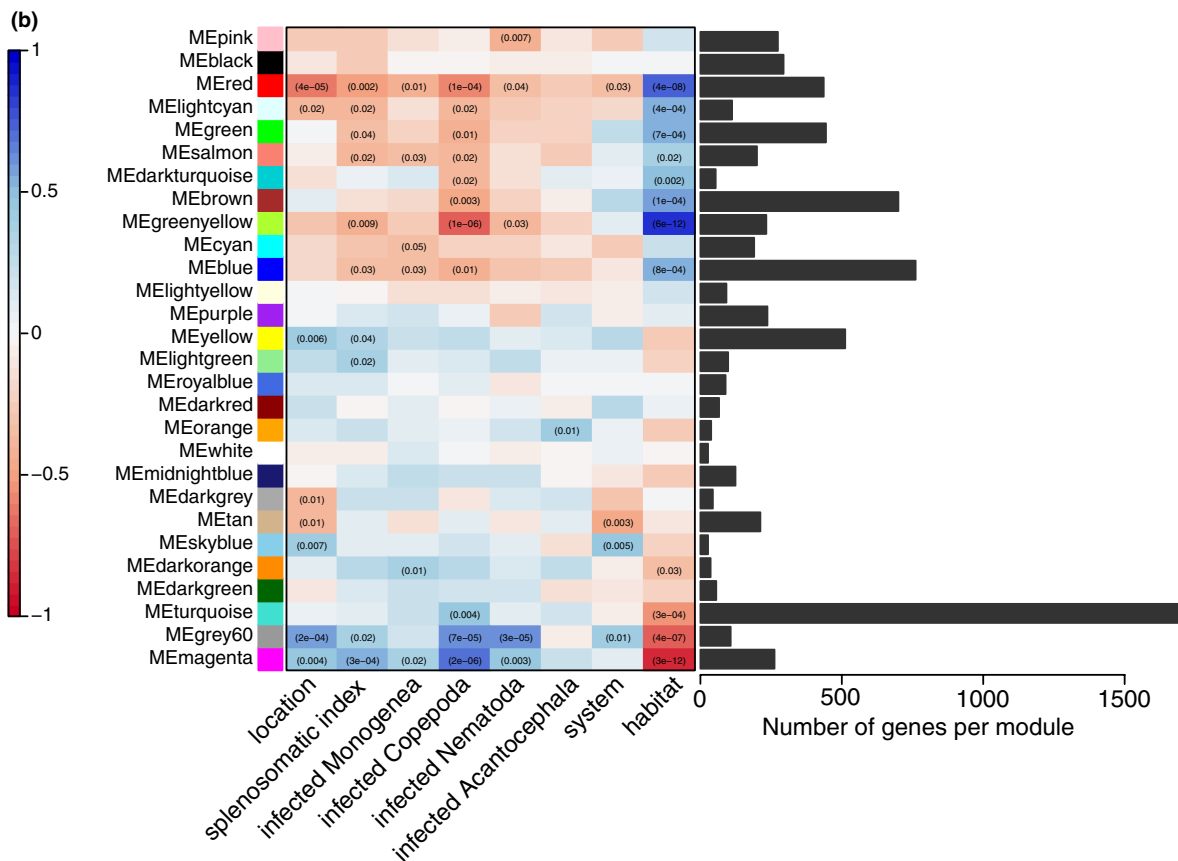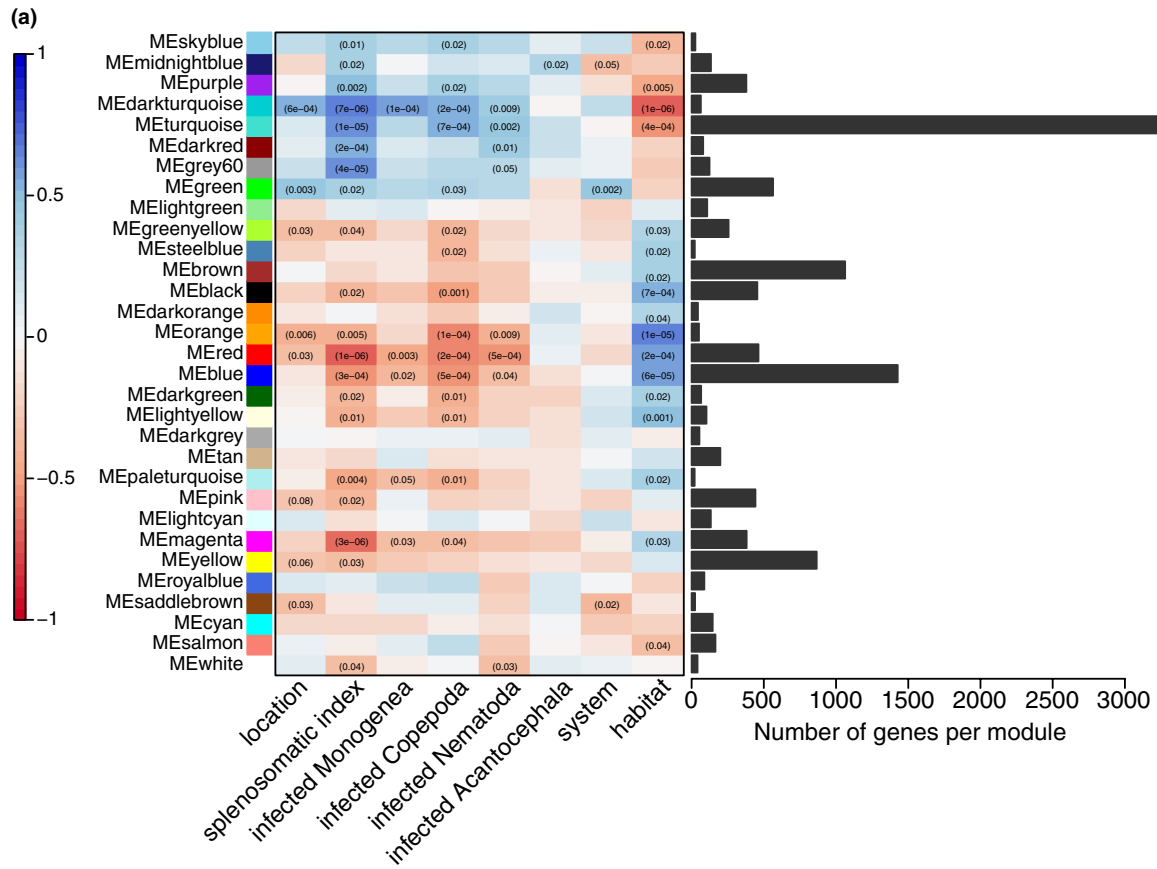
FIGURE 7 Module–trait correlation for (a) the gills and (b) the spleen. The number of genes included in each module is represented in the barplots on the right. The colour scale illustrates the correlation (Pearson coefficient) values and the numbers on the heatmap indicate the significance level (only p-values < .05 are displayed)

3.3.5 | Expression plasticity and genetic assimilation

We identified genes that were DE (lake–river contrast) in wild and pond samples, for each of the two organs. In agreement with the analysis above, we observed more DE genes in gills (in the wild and in the pond environment) than in spleen (Figure 8a). Generally, more genes were DE between the samples from the wild than those from the pond, suggesting a down-regulation of the gene expression in response to environmental changes (Figure 8a). Only a minor fraction of genes showed the same direction of expression between river and lake fish, in both the natural habitat and the lake-like experimental set-up, suggesting that these genes are prime candidates for fixed gene expression changes as a response to the source environment (potential genetic assimilation) (Figure 8b). We report all genes assigned to either genetic assimilation (Table S11) or plastic response (Table S12) as candidate genes identified by an experiment in an artificial environment that require further research.

We constructed a PCA plot of pond and wild samples for the two categories of genes, those with a potential genetically fixed expression change and those showing a plastic response in the two different organs (Figure 9). As expected, in the PCA plots of the genes that showed a plastic response (Figure 9a,c), PC1 clearly separates samples of the river environment (KaR2) from samples from the lake-like environment (KaL, KaLpond and KaR2pond) in both organs. By contrast, the PCA of genes that are probably subject to genetic assimilation (Figure 9b,d) separates samples according to source populations along PC1 (phylogenetic signal). This separation was more pronounced in spleen than in gill samples, albeit with lower numbers of DE genes (Figure 9).

3.4 | Microbiota

The microbial community of the buccal and intestinal mucosa over all samples consisted of 77,646 OTUs identified with a 97% identity threshold belonging to 1,000 genera in 35 phyla (22,489 OTUs comprising 903 genera remained after filtering out singletons). The most abundant phyla in the gut mucosa were Fusobacteria (25%), Proteobacteria (16%) and Firmicutes (8%), and in the buccal mucosa were Proteobacteria (33%), Bacteroidetes (11%) and Fusobacteria (4%) (relative abundance percentages given after filtering out low-abundance sequences [≤0.02]; Figure S7).

The microbiota composition (β-diversity) of all sampled OTUs differed between the habitats (nested PERMANOVA habitat: buccal mucosa $F_{2,53} = 13.49$, $p < .001$/intestinal mucosa $F_{2,55} = 9.84$, $p < .001$). Sex had no effect on the microbial community (nested PERMANOVA sex: buccal mucosa $F_{1,53} = 1.15$, $p = .267$/intestinal mucosa $F_{1,55} = 1.51$, $p = .135$). Microbial community richness
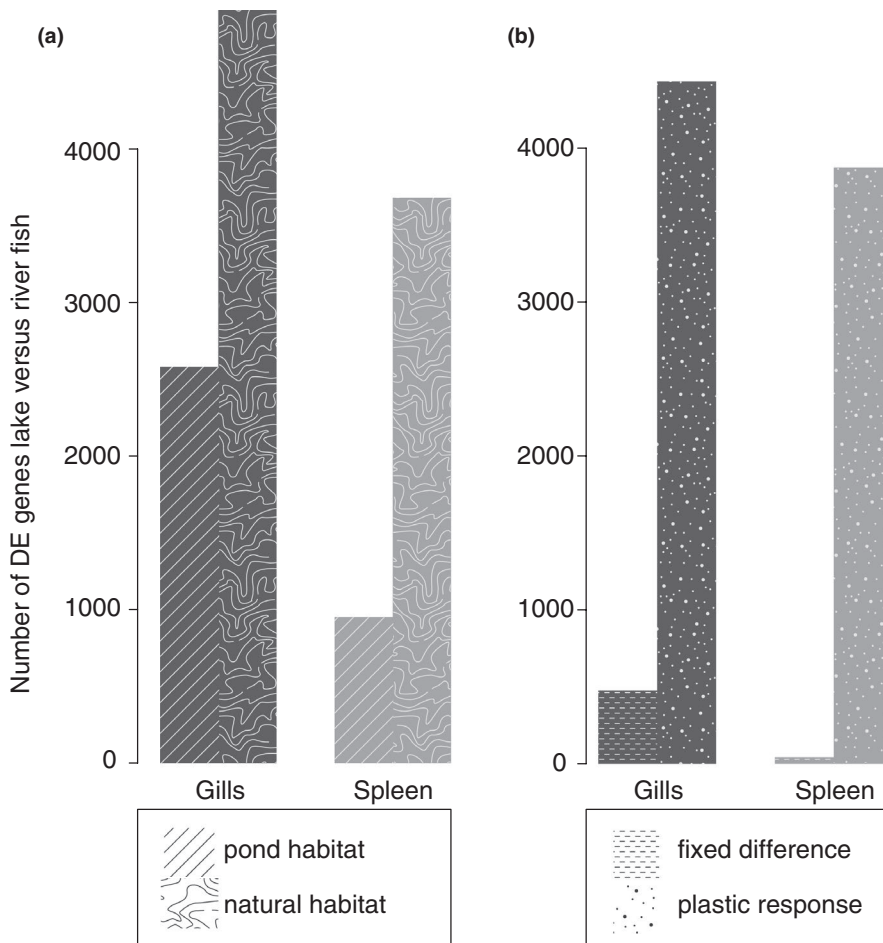

FIGURE 8 Lake–river differential expression in pond and wild populations. (a) Barplot of the number of genes differentially expressed in lake and river populations sampled in the wild (dark grey) or in the ponds (light grey). Differential expression was assessed for gills and spleen separately. (b) Barplot of the number of genes with plastic expression variation (dark grey) or genetically assimilated (fixed) expression (light grey) for gills and spleen samples
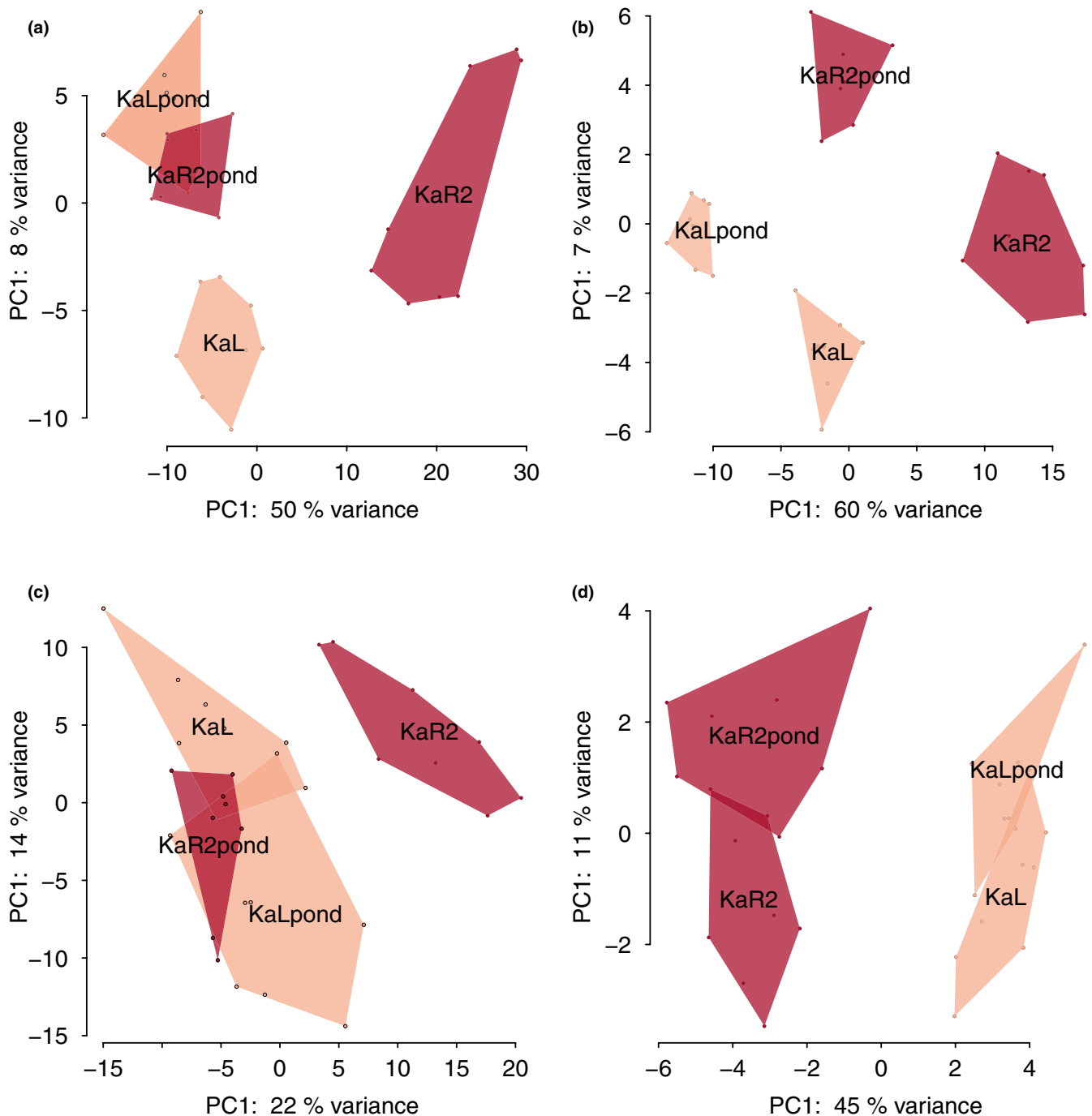
FIGURE 9 Gene expression variation for different parts of the transcriptome. PCA plots of gills (a, b) and spleen (c, d) samples. The PCA plots were produced using the candidate genes for plastic response to environment changes (a, c) and the genes for which gene expression variation was genetically fixed (b, d). Replicate samples are connected via polygons, polygons are coloured according to population (red: lake, orange: river) and the name of the population is placed in the middle of the respective polygon. KaL, Kalambo lake; KaR1, Kalambo river; KaR2, Kalambo river upstream; LzL, Lunzua lake; LzR, Lunzua river; KaLpond, Kalambo lake population in ponds; KaR2pond, Kalambo river upstream population in ponds. The proportion of the variance explained by the principal components is indicated next to the axes

(α-diversity, inverse Simpson index) was higher in the mouth than in the gut samples, with no differences between sexes. NMDS analysis of bacterial communities based on Bray–Curtis distances showed clustering by tissue and by habitat, as well as a shift in the river communities towards lake-like in the pond environment (Figure 10) paralleling the gene expression patterns.

4 | DISCUSSION

4.1 | Higher parasite diversity and abundance in the lake than in the rivers

Parasite communities of riverine *Astatotilapia burtoni* populations

were less diverse than those of their adjacent lake populations, which is consistent with findings from stickleback lake–stream population comparisons (Eizaguirre et al., 2011, 2012; Feulner et al., 2015; Stutz & Bolnick, 2017). Parasitic helminths (nematodes and acantocephalans), as well as parasitic copepods, were only found in the lake habitat. The only parasite group found in both habitats—monogeneans—was more abundant in the KaL lake population than in the river populations. There are several possible explanations for these patterns. Complex life cycles with invertebrate intermediate hosts and mainly vertebrate final hosts are common in nematodes and acantocephalans (Anderson, 1988; Benesh et al., 2014; Chubb et al., 2010). Lake Tanganyika is home to several groups that underwent adaptive radiations including cichlids, Mastacembelid eels, Synodontis catfish and crabs (Brown et al., 2010; Day et al., 2009; Marijnissen et al., 2006), and is thus expected to harbour a higher diversity and abundance of potential hosts and, consequently, also parasites than the tributary rivers. The rate of parasite infection is expected to increase with host population size (Anderson & May, 1979; McCallum et al., 2002). In agreement with this, estimated effective population sizes in *A. burtoni* are much higher for lake than for river populations (Egger et al., 2017). Furthermore, a large host population can allow for the persistence of parasite species with low reproductive rates that are unable to persist in smaller populations (Dobson & Carper, 1996; Lindstrom et al., 2004).

At four of our study sites (KaL, KaR2, ChL, ChR), gills of adult individuals caught in November 2015 (wet season) were screened for parasites as a part of a pilot study by experienced parasitologists who found the same pattern: no parasites in KaR2 and 75% prevalence in KaL (C. Rahmouni et al., unpubl. data). In the lake–river pair from the Chitili system, which shows the lowest genetic divergence (median F_{ST} ChL–ChR = 0, Egger et al., 2017), they found 50% versus 37% prevalence in lake versus river specimens that are separated by just 300 m. This shows that the pattern is consistent between different years and different seasons, providing opportunities for consistent parasite-driven divergent selection and fulfilling one more condition for parasite-driven speciation (Karvonen & Seehausen, 2012).

We further found that differences in parasite taxon composition were positively correlated with the extent of genetic differentiation corrected for geographical distance. Differentiation in infections was present even in the population pairs featuring the lowest genetic divergence (KaL–KaR1, ChL–ChR, both median $F_{ST} = 0$). This is consistent with the idea that differentiation in infections is present as soon as two populations occupy different environments, proceeding genetic differentiation.

4.2 | Immune response reflects parasite abundance in lake and river

In the wild, lake populations showed increased levels of innate immune response, as estimated by SI. When exposed to lake parasites in lake-like pond environment, river fish showed an elevated innate immune response as well, reflected by both spleen size and spleen gene expression profiles resembling those of lake fish.

Although lake fish in the pond experiment had higher gill parasite abundance than river fish, their SI was lower. This suggests that lake fish are well adapted to parasites that they normally encounter in the wild, unlike river fish, whose immune response was elevated in comparison to the wild. Models predict that parasite–host coevolution can facilitate speciation in host populations when they can adapt to the parasite community that infects them (reviewed by Greischar & Koskella, 2007; Kaltz & Shykoff, 1998; Kawecki & Ebert, 2004; Summers et al., 2003). In this case, gene flow from nonadapted host
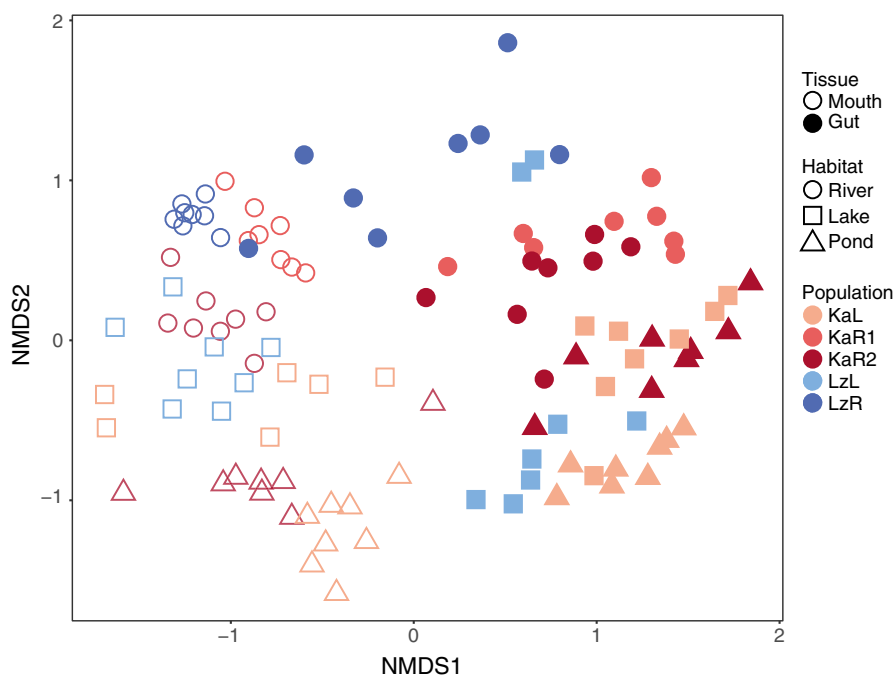

FIGURE 10 Nonmetric multidimensional scaling (NMDS) analysis of bacterial communities based on Bray–Curtis distances. Colours represent populations: circles, river habitat; squares, lake habitat; triangles, ponds; filled symbols, intestinal samples; hollow symbols, buccal samples. KaL, Kalambo lake; KaR1, Kalambo river; KaR2, Kalambo river upstream; LzL, Lunzua lake; LzR, Lunzua river. Stress = 0.171

populations could be maladaptive, and assortative mating between host populations may evolve (Karvonen & Seehausen, 2012).

Unlike the innate immune response estimated here, the proportion of cells of the adaptive immune system (i.e., the lymphocyte ratio) has previously been found to be higher in river than in lake populations of *A. burtoni* (Figure S8; Theis et al., 2017). However, as already noted by Theis et al. (2017), a shift in the lymphocyte ratio could also imply that there are more monocytes present, which represent the first line of the immune defence. This suggests that lake fish have a higher monocyte to lymphocyte ratio than river fish, in turn supporting our result that lake fish have an increased innate immune response compared to river fish. Alternatively, a higher adaptive immune response in river compared to lake fish could also reflect investments into alternative immune strategies. The benefit of using different arms of the immune system varies with the abundance of parasites in the environment (Lindstrom et al., 2004; McDade et al., 2016). The innate immune system provides the primary defence system against pathogen invasion, but it is energetically costly (Kraaijeveld & Godfrey, 1997; Lochmiller & Deerenberg, 2000; Moret & Schmid-Hempel, 2000; Sheldon & Verhulst, 1996). An induced response that is only deployed after an invader has been recognized could have evolved to avoid a costly permanent defence capability at the price of a delayed response and the potential risk that the parasite escapes host control (Schmid-Hempel & Ebert, 2003; Shudo & Iwasa, 2001). In lake–river stickleback, the cost of mounting an immune response induces associated fitness costs and might lead to selection for river fish with a relatively low level of innate immune response (Kaufmann et al., 2017), consistent with our findings. The results from the same study (Kaufmann et al., 2017) suggest that varying parasite communities can lead to population-specific immune responses that contribute to varying host–parasite co-evolutionary trajectories, further corroborating that, even though innate immune responses are thought to be rather unspecific, they can contribute to local adaptation (Tschirren et al., 2013).

4.3 | Comparative gene expression in spleen and gill tissue

Studying gene expression in wild animals permits a view on differential expression responses caused by both genetic and environmental factors (Huang et al., 2016). While the gene expression patterns in gill tissue showed clear clustering by habitat and population (Figure 4b), spleen expression patterns showed clustering by infection status and parasite load (Figure 4c). This pattern was further reflected by higher correlations of the SI with gene expression modules from the spleen network than the gill network and stronger correlations of habitat type with gill than spleen modules.

Fish gills are a multifunctional organ involved in gas exchange, ion regulation, osmoregulation, acid–base balance, ammonia excretion, hormone production, modification of circulating metabolites and immune defence (Evans, 2005; Secombes & Wang, 2012). Gills are constantly in direct contact with water and are likely to show

the greatest transcriptomic response to environmental differences such as water chemistry between different aquatic habitats (Gibbons et al., 2017; Hughes et al., 2017; Kavembe et al., 2015; Lam et al., 2014; Xu et al., 2013). Gill transcriptome remodelling in fish after exposure to elevated salinity has been observed even after 1 week (Jeffries et al., 2019). Here we find that the gene expression pattern in gills along PC1 (Figure 4b) is associated with different environmental conditions in lake and river habitats. We also found that gill gene expression modules were most strongly correlated with habitat type with the genes in these modules being involved in osmoregulation. The observed shift of the gill gene expression profiles of the KaR2 population along the PC1 axis in the pond set-up supports the important role of adaptive phenotypic plasticity in adaptation to different environments. It has previously been shown, in a transplant experiment performed in lake habitat, that KaR2 individuals raised in a common garden set-up performed equally well as lake fish, unlike wild-caught KaR2 individuals (Rajkov et al., 2018a). On the other hand, the separation of the upstream Kalambo river population (both from the wild [KaR2] and from the pond [KaR2pond]) from all the other populations along the PC2 axis, and the separation of the Kalambo from the Lunzua system, suggests a strong phylogenetic signal (Egger et al., 2017) in gene expression patterns.

As an immune response-generating and pathogen-neutralizing organ (Press & Evensen, 1999), the spleen is expected to reflect immune system reaction in its transcriptomic response (Huang et al., 2016). Indeed, we found that spleen expression patterns seemed to reflect stress related to parasite exposure: (a) pond individuals with high parasite load clustered together with lake individuals from the wild that had a high parasite load and (b) we identified gene expression modules with a function in the innate immune response as being correlated with SI. The number of DE genes in the river–lake contrast is lower in the spleen than in the gills (Figures 6a and 8). This result was expected, as the general expression pattern observed in the spleen does not show a clear separation between lake and river samples (Figure 4c). However, when looking at the genes that showed a plastic response in both organs, the separation of spleen samples is more pronounced than that of gill samples (Figure 9), which might suggest that some organs are more susceptible to expression changes than others (Tang et al., 2011). As expected, when inspecting the PCA plots of genes that showed signatures of adaptive plasticity, PC1 (Figure 9a,c) clearly separated samples from the river environment (KaR2) from samples from the lake and the lake-like artificial environment (KaL, KaLpond and KaR2pond) in both organs. Phenotypic plasticity in gene expression has been found to contribute to divergence of locally adapted fish populations (Dayan et al., 2015), where phenotypic plasticity and adaptation operate on different suites of genes for the majority of significant differences in gene expression levels.

Several interesting habitat-specific genetic variations correlating with habitat type were identified in the context of our study. Among those candidates is the sodium/potassium-transporting ATPase subunit alpha-1 (*Atp1a1*). This gene is involved in the maintenance of the ion balance and electrolyte homeostasis in different osmoregulatory

epithelia (Evans, 2005). *Atp1a1* is known as key gene in the adaptation to different osmotic environments in many fish species (e.g., lake whitefish, stickleback, killifish: Scott, 2004; bull shark: Reilly et al., 2011; brown trout: Larsen et al., 2008); is one of the main candidates for the marine to freshwater transition in different fish species (DeFaveri et al., 2011; Jones et al., 2012; McCairns & Bernatchez, 2010); and shows clear haplotype clustering by habitat in sticklebacks (Roesti et al., 2014). *Atp1a1* and *Atp1a3* were also found to be up-regulated in freshwater euryhaline Mozambique tilapia gills in comparison to seawater (Lam et al., 2014). The difference in conductivity between freshwater and marine stickleback habitat is on a similar order of magnitude (e.g., typical conductivity of seawater, 55,000 $\mu S\ cm^{-1}$; freshwater, 100–200 $\mu S\ cm^{-1}$) as the difference in conductivity between river and lake habitat of *A. burtoni* investigated here (Lake Tanganyika, 660–670 $\mu S\ cm^{-1}$; river, 18–60 $\mu S\ cm^{-1}$, table S9 in Rajkov et al., 2018b). Ubiquitin carboxyl-terminal hydrolase CYLD is one of the four genes identified as repeated F_{ST} outliers in lake whitefish (Renaut et al., 2011) and is methylated in response to salinity stress in Pacific oyster (Zhang et al., 2017). Basigin has a role in various physiological and pathological contexts (e.g., immune response in tilapia spleen following *Streptococcus* challenge) (Zhu et al., 2017) and is a receptor essential for erythrocyte invasion by *Plasmodium falciparum* parasite in humans (Crosnier et al., 2011). Interferon regulatory factor 9 has an immune function. Cofilin has previously been found to be involved in changes related to plasticity in fish (Dayan et al., 2015; Debes et al., 2012). The overlap of candidate genes across analyses in our study suggests that genetic and expression changes involve similar biological functions.

4.4 | Microbiota

The capacity of the gut microorganism community to change its composition or gene-expression pattern in response to the host's physiological changes and variations of the external environment— that is, metagenomic plasticity—is probably an essential factor in host acclimation and adaptation to environmental change (Alberdi et al., 2016). Fish are known to experience a complete turnover of their microbiomes during transitions between different aquatic environments (Lokesh & Kiron, 2016; Schmidt et al., 2015) that are expected to cause drastic challenges to their immune system.

While an increasing number of studies have investigated fish gut microbiota and their role in diversification (Baldo et al., 2017; Baldo et al., 2019; Baldo et al., 2015; Franchini et al., 2014; Härer et al., 2019; Rennison et al., 2019; Sevellec et al., 2014, 2018; Sullam et al., 2015), the present study is among the first to compare microbiota communities in two different tissues, one in direct contact with the (novel) habitat (mouth), and another that is known to be affected by food type (gut) (Sullam et al., 2012), and characterized how these communities change upon exposure to a novel habitat in a seminatural set-up.

In a previous study that compared gut microbiota of different cichlid species (Baldo et al., 2015), wild *A. burtoni* carried the most diverse microbiota of all investigated species, being significantly distinct from all other species and from samples of the same species kept in the laboratory, with laboratory individuals displaying a highly reduced microbiota diversity compared to the wild population. While another study found changes in foregut microbiota in laboratory-bred *A. burtoni* females depending on the mouthbrooding stage (Faber-Hammond et al., 2019), we found no differences in mouth or gut microbiota between the sexes.

Host diet and host genotype are the most likely causes of the parallel shifts we observed in microbiome composition across two river systems. Whereas gut microbial communities in our study converged towards lake-like in the ponds with lake water, mouth bacterial communities in the ponds were distinct from the mouth microbial communities of wild-caught fish, indicating that other factors in the pond lake-like set-up affect the mouth microbiota communities. Higher microbial community richness observed in the mouth in comparison to the gut indicates that mouth microbiota is, as expected, more strongly influenced by external environment. Overall, our results support the hypothesis that the plasticity of the gut microbiota might be an important factor in the phenomic plasticity of vertebrates.

4.5 | Conclusion

Here we describe, for the first time, parasite and microbiota communities in *A. burtoni* lake–river ecotypes. Lake populations were more heavily parasitized than river populations, in terms of both parasite taxa composition and abundance. The innate immune response in the wild was higher in lake than in river populations. When a river population was exposed to lake parasites in a pond lake-like set-up the immune response was elevated in comparison to the wild. The present study thus demonstrates the potential for parasite-mediated divergent selection between populations occupying contrasting habitats even at the incipient stages of differentiation. Our RNA-seq data provide evidence that environmental differences between lake and river habitat and their distinct parasite communities shape differential gene expression patterns in *A. burtoni*. By comparing gene expression and bacterial communities between wild-caught individuals and individuals acclimatized to lake-like pond conditions, we show that plasticity in gene expression and microbiota composition contribute to previously identified adaptive phenotypic plasticity.

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AUTHOR CONTRIBUTIONS

J.R. and A.E.T. designed the study with input from B.E. and W.S. J.R. and B.E. performed the sampling and parasite screening. J.R., A.E.T. and B.E. performed the wet laboratory work. J.R. analysed the parasite and the microbiota data. A.E.T. analysed the RNA-seq data with the help of A.B. and drafted corresponding methods and results. J.R. wrote the manuscript with feedback from all co-authors.

DATA AVAILABILITY STATEMENT

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