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INVERTEBRATE MICROBIOLOGY

Consistency of Bacterial Communities in a Parasitic Worm: Variation Throughout the Life Cycle and Across Geographic Space

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Abstract

Microbial communities within metazoans are increasingly linked with development, health and behaviour, possibly functioning as integrated evolutionary units with the animal in which they live. This would require microbial communities to show some consistency both ontogenetically (across life stages) and geographically (among populations). We characterise the bacteriome of the parasitic trematode *Philophthalmus attenuatus*, which undergoes major life cycle transitions, and test whether its bacteriome remains consistent on developmental and spatial scales. Based on sequencing the prokaryotic 16S SSU rRNA gene, we compared the parasite bacteriome (i) across three life stages (rediae in snails, cercariae exiting snails, adults in birds) in one locality and (ii) among three geographic localities for rediae only. We found that each life stage harbours a bacteriome different from that of its host (except the adult stage) and the external environment. Very few bacterial taxa were shared among life stages, suggesting substantial ontogenetic turnover in bacteriome composition. Rediae from the three different localities also had different bacteriomes, with dissimilarities increasing with geographical distance. However, rediae from different localities nevertheless shared more bacterial taxa than did different life stages from the same locality. Changes in the bacteriome along the parasite's developmental history but some degree of geographical stability within a given life stage point toward non-random, stage-specific acquisition, selection and/or propagation of bacteria.

Keywords Bacteria · Ontogenetic variation · Life cycle · *Philophthalmus attenuatus* · Spatial variation · Trematode

Introduction

Research on animal microbiomes (communities of microbes, such as bacteria, archaea and viruses, living within animals) has revolutionised ecology and evolutionary biology [1]. The microbes inhabiting a metazoan can have parental or environmental origins, that is, they can be inherited vertically from parent to offspring or acquired horizontally from the outside environment [2–4]. Far from being mere passengers, the impact of microbial symbionts extends to processes ranging from the behaviour and health of individuals [5–9] to the structure of ecosystems [10]. The traditional view of the "organism" is being swept aside and replaced by the holobiont concept [11, 12]. Although still controversial [13], the holobiont provides a holistic perspective in which animals and their microbes may form integrated entities that function as true evolutionary and/or ecological units. In this light, an animal's phenotype and interactions within the ecosystem are the combined product of its genotype and that of the myriad microbes it harbours. However, for selection to act on the animal-microbe associations as a unit and result in co-adaptations across generations and among populations, some degree of stability in the associations is a requirement.

There are at least two dimensions along which microbiome composition may vary while still being consistent with the holobiont concept. Firstly, an animal's microbiome may or may not change over the course of its life, either in its taxonomic composition or in the relative abundance of different microbial taxa [14–16]. In particular, this may be expected in animals that undergo major transitions in habitats and/or external conditions during their ontogeny. These changes not

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only expose the individual to distinct pools of microbes that may be acquired horizontally, but they also exert different selective pressures on the microbial community in the form of changing thermal conditions, nutrient types and availability, etc. Helminth parasites with complex life cycles are great examples of organisms that experience major changes in morphology and living conditions, as they pass through the external environment as well as a series of completely distinct host taxa across their ontogeny. Recently, analysis of the microbiome of the trematode parasite *Coitocaecum parvum* has revealed that it consists of a small core of bacterial taxa, distinct from those of the hosts or external environment, that persist across all life stages, as well as bacteria specific to each life stage in their different hosts [17]. The existence of an ontogenetically stable core of bacterial taxa most likely vertically transmitted hints at an integrated and coevolved unit. Whether this finding applies to other multi-host helminth parasites remains to be tested.

Secondly, microbiome composition may vary spatially, among distinct populations of the same animal species, whether the latter is free-living or parasitic [e.g. 18, 19]. For helminth parasites, the microbiome of a population might reflect the microbial communities of their local host population or of the local external environment, if it is predominantly assembled by horizontal acquisition from the local pool of available microbes [20]. This does not imply that the helminth's microbiome would comprise a random subset of locally available microbes; on the contrary, a selectivity filter may limit which microbes can successfully colonise the helminth's body, with local adaptation possibly playing a role in determining which microbes become established and/or their relative abundance [21]. In this case, we would expect differences in microbiome composition among geographically distinct helminth populations, with the similarity among them decreasing with increasing distance, as seen in ecological communities in general [22, 23]. Alternatively, or in combination with the above mechanism, the microbiome may comprise many ancient microbe-helminth associations, maintained through vertical transmission across generations and representing mutually beneficial interactions. We may then expect much less pronounced geographic variation in microbiome composition, at least on moderate spatial scales. In such cases, differences in microbial composition between geographically distinct helminth populations would be clearer at lower levels of taxonomic resolution; analyses at higher taxonomic levels might reveal a sharing of related microbes that may be functionally equivalent.

Here, we test for both ontogenetic and geographic variation in the composition of bacterial communities (hereafter, bacteriome) in the parasitic trematode *Philophthalmus attenuatus*. Adult worms infect the eyes of New Zealand gulls (Laridae), attaching beneath the eyelids and nictitating membranes [24]. Eggs are released in bird tears; ciliated larvae hatched from those eggs seek and infect the parasite's intermediate host, the mud snail *Zeacumantus subcarinatus* (Batillariidae). Within the snail host, the parasite multiplies asexually to form a colony of clonal stages known as rediae. After further growth, rediae produce and release infective stages known as cercariae; these cercariae leave the snail and after a brief free-swimming period, attach to and encyst on the outside surfaces of gastropod shells or crab carapaces [25]. The encysted juveniles, known as metacercariae, await ingestion by a definitive gull host to grow into adults and complete their life cycle.

From the ontogenetic perspective, there are at least two bottlenecks in the parasite's life cycle that present challenges for the maintenance of a core bacteriome across all life stages: the egg and the production of cercariae. During these stages of the life cycle, vertical transmission is unlikely to be achieved every time. The eggs of *P. attenuatus* are < 80 µm in length [24], and each tiny egg may inherit only a fraction of the adult worm's bacteriome. In the case of cercarial production, studies on a different trematode species have demonstrated that although they are all clones issued from the same redial colony within the same snail, only a portion of cercariae acquire particular bacteria from the previous life stage [26, 27]. Adding to these obstacles to the maintenance of a core bacteriome across the life cycle, two life stages of *P. attenuatus* actively feed on host tissues, i.e. the adult and rediae feeding on bird and snail tissue, respectively. This provides more opportunities for host microbes to colonise the parasite bacteriome and dilute the relative abundance of any parasite-specific microbe, than in a previously studied trematode species where only the adult stage feeds on host tissue and where a small core parasite bacteriome was observed [17].

From the geographic perspective, one factor may contribute to the partial homogenisation of the parasite's bacteriome among distinct populations: host mobility and its influence on parasite migration rates among localities. Gulls should be capable of dispersing parasites across coastal locations on scales of 10–100 km. Indeed, an analysis of genetic structure (based on the cytochrome oxidase subunit I gene) among the same three populations of the trematode *P. attenuatus* studied here revealed only weak genetic structure and no evidence of isolation by distance, a pattern consistent with dispersal by bird hosts [28]. Thus, for host-mediated exchanges of parasites among populations to result in similarity in bacteriome composition, the horizontal acquisition of distinctive microbes from the locally available microbial pool would need to be overshadowed by predominantly vertical transmission and strong inter-generational fidelity in microbe-parasite associations.

Knowledge of helminth microbiomes is still limited at present [29–32]. However, microbes carried by parasites can influence parasite virulence [33] and modulate host-parasite

interactions [34]; therefore, elucidating their origins and the spatial and temporal (within and across generations) variation in microbiome composition is of central importance for our understanding of disease dynamics and evolution. In the present study, we quantify ontogenetic and geographic variation in the composition of the *P. attenuatus* bacteriome, to test whether the parasite and at least some of its microbes form spatially and temporally consistent associations or whether they instead represent opportunistic associations based on local availability. Specifically, we (i) compare the bacteriome of three life stages of *P. attenuatus* from one locality, (ii) investigate the sources of this bacteriome and (iii) contrast the bacteriome of *P. attenuatus* at one life stage across three localities. In addressing these objectives, we test whether the parasite's bacteriome is more consistent on an ontogenetic versus a geographic scale.

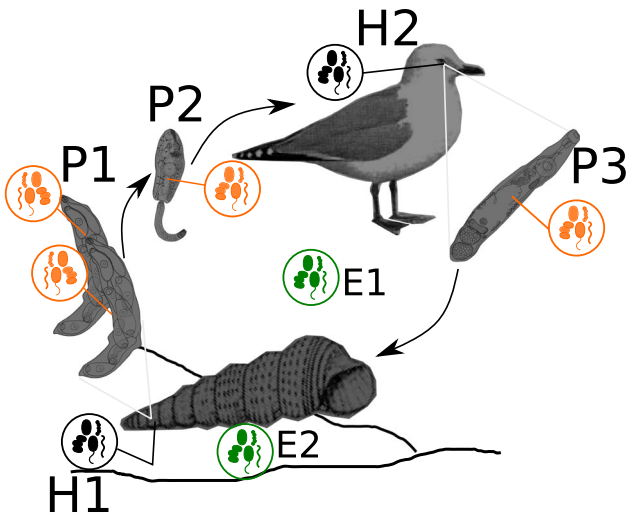

Fig. 1 Study design for sampling the bacteriome of *Philophthalmus attenuatus* across its life cycle, its respective hosts and environment. The parasite bacteriome (orange) was sampled from rediae (P1), cercariae (P2) and adult (P3) life stages. Host bacteriome (black) was sampled from snail (H1) and bird (H2) tissue sample which was in contact with the respective parasites. Two environmental samples (green) were collected, water (E1) and substrate (E2), from where snail hosts were collected

Material and Methods

Sample Collection and Processing

Ontogenetic variation in the *P. attenuatus* bacteriome was studied with samples from Portobello Bay near Dunedin, South Island, New Zealand (Lat. − 45.83°, Long. 170.67°), whereas geographic variation in bacteriome composition was investigated by sampling three localities spanning over 500 km along the South Island's east coastline: Portobello Bay, McCormacks Bay near Christchurch (Lat. − 43.56°, Long. 172.73°) and Green Point Domain in Bluff (Lat. − 46.57°, Long. 168.31°). Three life stages (rediae, cercariae and adults; Fig. 1) were considered for the ontogenetic study; however, because access to birds (and therefore adult worms) is restricted, only rediae were included in the geographic study.

Snail first intermediate hosts, *Z. subcarinatus*, were collected at low tide in the intertidal zone of Portobello Bay during the 2020 austral summer and in McCormacks Bay and Green Point Domain in both the 2019 and 2020 austral summers. Prior to snail collection at each locality and year, two types of environmental samples (water, N = 2; substrate, N = 2) were collected with sterile cotton swabs (Fig. 1). Two controls for the swabs themselves were also taken by opening the swab and exposing it to ambient air. Environmental and control samples were saved in a PowerBead Pro Tube (QIAGEN), frozen in dry ice and stored in a − 80 °C freezer immediately upon arrival at the laboratory. Water from the three localities was also collected into sterile containers for maintenance of snails in the laboratory (in multiple sterile 4 L containers, at room temperature) until processing. In the laboratory, within 1 day after collection, snails were placed in individual sterile wells with sea water from the collection site and incubated for 2 days at 25 °C under a 12 h dark:12 h light photoperiod to identify *P. attenuatus*-infected individuals through cercarial shedding.

Opportunistic sampling of the parasite's definitive host, the black-backed gull *Larus dominicanus*, was possible thanks to birds dead of natural causes donated by the Dunedin Wildlife Hospital (Dunedin, New Zealand). The dead individuals originate from the Dunedin area, i.e. within 10–20 km of Portobello Bay. Adult parasite samples collected from these birds were included in the ontogenetic study. Five birds which had their eyes closed were defrosted, and their eyes were removed using a sterile scalpel and placed in individual sterile wells with sterile PBS.

Infected snails from each locality and year and the birds were all dissected separately in a sterile laminar flow cabinet. During sample isolation, sample tools were cleaned with bleach and sterilised with 70% ethanol and burning flame between each sample. Infected snails were brushed with sterile interdental brush in 70% ethanol and rinsed thoroughly in PBS prior to dissections. Reproductive rediae (two per snail) and free cercariae (one per snail, Portobello only) were isolated from several infected snails (Christchurch 2019, N = 3; Bluff 2019, N = 3; Portobello 2020, N = 15; Christchurch 2020, N = 5; Bluff 2020, N = 10), together with snail host tissue (Christchurch 2019, N = 1; Bluff 2019, N = 2; Portobello 2020, N = 15; Christchurch 2020, N = 3; Bluff 2020, N = 3). Because of their small size, entire parasite bodies were used for bacteriome analyses, whereas for the snails, we took a small piece of tissue (about 3 mm across) immediately

adjacent to the parasite mass, consisting of gonads, which was thoroughly washed in PBS to remove any parasite tissue. Prior to dissection, bird eyes were rinsed thoroughly in PBS. Adult trematodes (up to 2 per eye, total N = 9 from 4 birds) and eye tissue (N = 6, from 4 birds) were also isolated. Again, we took entire parasite bodies for analysis, and a small piece (about 3 mm long) of the nictitating membrane (where the parasites occur) from the birds' eyes.

All tissue samples, from both parasite and host, were cleaned from surface microbiota by repeatedly pipetting up and down in PBS in sterile wells. Samples of the surface microbiota for each sample type were collected by pipetting 75 µl of the resulting "washing" (two samples per host type and parasite life stage). A sample of the PBS solution was taken at the end of each isolation procedure to account for any possible contamination of the solution. Samples were snap frozen and kept in a − 80 °C freezer until DNA isolation. Metadata for ontogenetic and geographic datasets including sample type, life stage, locality and host ID are given in Table S1 and Table S2, respectively, in Appendix 1.

Bacterial 16S rRNA Amplicon Sequencing

Five different library pools (Portobello 2019, Christchurch and Bluff 2019, adult parasites 2019, Portobello 2020, Christchurch and Bluff 2020) were prepared for sequencing targeting the V4 hypervariable region of the prokaryotic bacterial 16S SSU rRNA gene. DNA extraction and library preparations were performed as described in [17], including the use of the two types of ZymoBIOMICS microbial community standard samples (MCS and MCS DNA). Barcoded libraries were sequenced on an Illumina MiSeq platform using the V2 reagent cartridge (250 bp, paired-end) through the Otago Genomics & Bioinformatics Facility (New Zealand).

Sequence Processing

Data from each of the five sequencing runs, received separately as demultiplexed paired-end raw sequences, were individually processed and initially analysed using the Quantitative Insights Into Microbial Ecology (QIIME) 2 software package (qiime2-2020.2-py36-linux) [35]. Adapters and primers were removed from raw sequences using the plugin *cutadapt* (with 0 error rate and minimum length of 240 bp) [36] and quality filtered using the *dada2* plugin [37]. Quality profile plots were inspected for each run separately, with the same values to truncate both forward and reverse used for all runs. The resulting amplicon sequence variants (ASVs) tables were filtered to exclude non-bacterial, mitochondrial, chloroplast, ASVs without a phylum assignment, contaminants (i.e. ASVs found in blanks and exclusive to lab environment), ASVs found in the respective cleaning PBS for each life stage and samples with low sequencing depth (i.e. frequency lower than 1000 and/or with less than 8 ASVs) using the *feature-table* plugin. Two main datasets were created: the ontogenetic dataset included libraries from Portobello 2020 and adult worms 2019 and the geographic variation dataset included libraries from Christchurch 2019 and 2020, Portobello 2020 and Bluff 2019 and 2020. Each final dataset was assembled after merging the respective feature tables and representative sequences. Different taxonomic levels were assigned to the ASVs using the plugin *feature-classifier* [38] against the Greengenes 16S rRNA reference database (13_8 release) pre-trained on the 515F/806R region [39].

Prior to analyses, ASVs were aligned using the mafft program [40] and used to construct a phylogenetic tree using the fasttree2 program [41] with the *phylogeny* plugin. Diversity analyses were performed primarily using the R packages vegan [42] and phyloseq [43]. For analysis in R v. 3.6.3 [44], the filtered ASVs, taxonomy tables and rooted tree were imported into RStudio [45] with the QIIME 2R package v. 0.99.34 [46] and together with the metadata combined into a phyloseq object.

Possible methodological bias was evaluated based on the observed composition and sequence quality of the two ZymoBIOMICS microbial community standards (MCS and MCS DNA), against the expected data of these mock communities, using the *quality-control* plugin (see Supplementary Information in [17] for details).

Bacteriome Ontogenetic Development Analysis

We started by analysing variation in bacteriomes across the three life stages sampled. Firstly, abundances were rarefied to even depth (N = 1139) using *rarefy_even_depth()*. Then, differences in diversity among parasite life stages (alpha diversity) were estimated using Faith's phylogenetic diversity, Shannon evenness and Shannon diversity metrics. Comparisons between alpha diversity estimates for ASV, genus, family, class and phylum level among life stages were calculated with Kruskal–Wallis tests. Phylogenetic-based indices, unweighted unifrac [47] and quantitative weighted unifrac [48] distance metrics were calculated to determine if there were differences among the bacterial communities of the three life stages. Distance matrices were calculated with *distance()*, for the ASV, genus, family, class and phylum level. Statistically significant differences among life stages were determined with permutational ANOVA performed with *adonis* and with multilevel pairwise comparisons with *pairwise.adonis()* using Benjamini and Hochberg's [49] ("BH-FDR") correction for multiple testing with 9999 permutations. Homogeneity of dispersion within sample types was evaluated with *betadisper()*. Principal coordinates plots (PCoA) were created with *plot_ordination()* adding hulls

as defined with *find_hull()* from the R package erictools (https://rdrr.io/github/elittmann/erictools/man/find_hull.html). Group divergence was estimated as the average dissimilarity of each sample from the respective life stage group mean with the function *divergence()* from the R package microbiome [50]. A non-rarefied phyloseq object was used to explore differential abundances of bacterial phylotypes between life stages with DESeq2 [51] using geoMeans to estimate size factors. *DESeq()* was called for ASV and genus levels with default parameters, and results were contrasted by life stage with an adjusted p cut-off of 0.05.

After exploring possible sources of dissimilarity among the life stages, we then investigated bacterial taxa being shared among them. Venn diagrams for ASV, genus and family levels were plotted with the function *ps_venn()* from the R package MicEco [52]. A core bacteriome was quantified for the pooled life stages, and for each life stage at ASV, genus and family level with the *core()* function of the *microbiome* package; we defined the core bacteriome as the set of all taxa with a prevalence higher than 0.50. We then explored the sharing of ASV and their respective proportion within the bacteriomes for each pair of samples representing two parasites sharing the same host individual, i.e. redia vs redia (N = 20 pairs), cercaria vs redia (N = 15 pairs) co-infecting the same snail individual, and between adults co-infecting the same bird eye (N = 8 pairs); for this, we used a modified version of the R script from [53].

Source of Parasite Bacteriome

We then compared the observed parasite bacteriome composition (N = 42) with that of snail hosts (N = 9 and snail PBS washing N = 2) and bird hosts (N = 4), and with the environmental samples (environment N = 4, lab environment N = 4). Bacterial diversity was compared among samples using Faith's phylogenetic diversity, Shannon evenness and Shannon diversity metrics. Differences in bacterial composition among sample types were estimated based on unweighted and weighted unifrac distances. PCoA plots and Venn diagrams were obtained to visualise and identify similarities and dissimilarities among communities. Sharing of ASVs between individual hosts and their infecting parasites was also assessed. To infer possible sources of parasite bacteriomes, ASVs composing each parasite sample were classified as "unique" to a particular parasite sample if that same ASV was not found in either the co-infecting parasite or the host, "shared with both" if it was found in both the co-infecting parasite and the host, "shared with host" if it was found in the host but not in the co-infecting parasite and as "shared with parasite" if it was found in the co-infecting parasite but not in the host. For this, only parasite samples for which both co-infecting parasites and respective hosts samples were available were considered for analyses (rediae N = 12, cercariae N = 6, adults N = 8). To explore a possible environmental source of the "unique" ASVs for each life stage, we investigated whether those ASVs were found among the sequenced environmental bacterial community. And finally, to explore whether sharing of ASVs with hosts could be related to the abundance of those ASVs in the host, we compared the relative abundances of those ASVs between parasite and host samples.

Geographic Variation Data Analysis

To determine how the parasite's bacteriome compares across localities, we focused on bacteriome diversity and composition of the redial life stage, their snail hosts and environment from Christchurch, Portobello and Bluff. Alpha and beta diversity metrics were estimated, and Venn diagrams were created for each locality. For the redial samples, a core bacteriome was also estimated at different taxonomic levels. The distance-decay relationship was then explored based on Bray–Curtis dissimilarity distances as computed with the *plot_distance()* of the R package phylogeo [54]. A Spearman correlation was then calculated between geographical distance and community differences with *cor.test()*.

Finally, we investigated the nature of ASVs unique to the parasite at a particular locality (ASVs observed in Venn diagrams for the pooled parasite samples and not shared with other localities), to test whether they were truly unique to rediae of that locality because those ASVs were not found in environmental and/or host samples from other localities or whether they were in fact present environmentally and/or in hosts across localities but not shared by the parasites. Each ASV found to be unique to the parasite from a particular locality was classified as either "parasite unique" if it was not found in either environment or host samples from the same locality or from any of the other two localities, "locality unique" if found only in the parasite and in environment and/or host samples from the same locality and "shared" if it was found in any environment and/or host sample from either of the other two localities.

Results

How Does the Bacteriome Evolve Along the Parasite's Ontogenetic Development?

The parasite dataset was composed of 42 samples (rediae, N = 23; cercariae, N = 10 and adults, N = 9), with 1039 ASVs with a total frequency of 182,823 reads after quality data processing. At broad taxonomic level, the bacterial communities across the three parasite life stages sampled were mainly characterised by two taxa from a total of 25 represented phyla, Proteobacteria and Bacteroidetes

(51.47 ± 3.33% and 19.39 ± 13.41%, respectively, of the total reads obtained for each life stage). At lower levels, the taxonomic composition of the 1039 ASVs of the parasite bacteriome included taxa belonging to 195 families and 324 genera (including unclassified taxa at those levels). Most ASVs were found to be unique to one particular sample.

The three life stages presented significant differences in diversity estimates for all three measures of community richness (Table S3 in Appendix 1). Such differences in alpha diversity were also observed at a higher taxonomic level. Pairwise comparisons showed that bacteriomes of rediae have significantly higher diversity based on both Faith's and Shannon's metrics than those of cercariae and adults but did not differ in terms of evenness (Fig. 2a, Table S3).

PERMANOVA analyses and PCoA plots (Fig. 2b) indicate that the three life stages also differed in terms of bacterial community composition (unweighted unifrac: $F_{2,41} = 3.929$, $R2 = 0.168$, $p = 1e^{-04}$; weighted unifrac: $F_{2,41} = 5.727$, $R2 = 0.227$, $p = 1e^{-04}$). While the three life stages differed among each other in terms of composition (unweighted unifrac: all 3 pairwise comparisons p.adj < 0.001), when considering abundances of bacterial taxa, adults harboured a different community from both rediae and cercariae (weighted unifrac: p.adj < 0.001), but rediae and cercariae did not significantly differ in their bacterial communities (weighted unifrac: p.adj = 0.054). Both results are mostly consistent at higher taxonomic levels, the exception being for weighted unifrac estimates where rediae and cercariae do significantly differ in their bacterial community composition at genus level (p.adj = 0.0351, Table S4 in Appendix 1). While the three life stages did not have different dispersion scores (betadisper for both unweighted and weighted unifrac, p > 0.05), the bacteriomes of adults had a smaller intra-individual dispersion than those of rediae, but not those of cercariae (see Table S5 in Appendix 1 for adjusted p-values, for the pairwise Wilcoxon rank sum test). To further explore which ASVs contributed to the differences among the bacterial communities, we used

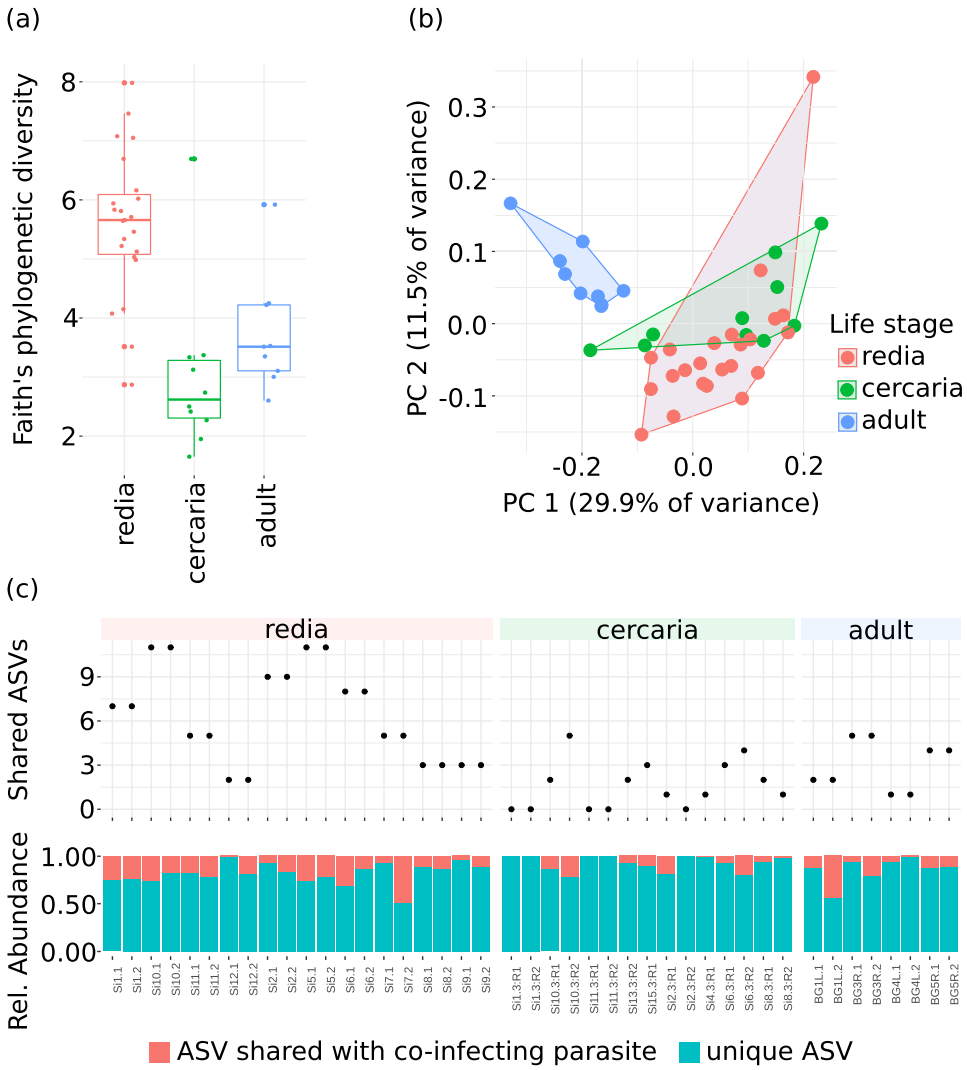

Fig. 2 Characterisation of the bacterial communities of the trematode *Philophthalmus attenuatus*. **a** Alpha diversity in the three different life stages, as measured using Faith's phylogenetic diversity index at ASV-level. **b** Principal coordinates analyses ordination based on weighted unifrac distances of bacterial communities from the three life stages, with hulls delimiting groups of samples for each life stage. **c** Number of ASVs shared between co-infecting parasites (i.e. parasites occurring in the same individual host) and relative abundance of the shared and non-shared portion of the bacteriome for each sample (for rediae, comparisons were made against co-infecting rediae; for cercariae, comparisons were made with the two co-infecting rediae; for adults, comparisons were made with co-infecting adults found in the same eye)

the DESeq2 to identify differential abundances (Table S6 in Appendix 1). Pairwise contrasts between consecutive life stages showed that while rediae and cercariae shared 74 ASVs, only one was found to be relatively less abundant in rediae; in contrast, cercariae and adults only shared one ASV, and it did not have differential abundance between the two life stages. Although rediae and adults are not consecutive life stages (the egg stage separates them), they did share six ASVs. However, overall community composition differs significantly between these two life stages: the DESeq2 analysis, which also considers ASVs with zero abundance in a particular life stage, identified seven ASVs found at higher abundances in rediae, nineteen at higher abundances in adults and several ASVs being in fact unique to each life stage. To determine if any bacterial taxa were potentially integral components of the community, we estimated how many had a prevalence above 50% for the pooled life stages and for each life stage. For the pooled life stages, no ASV met this criterion, with the highest prevalence being 33% for one Proteobacteria, *Bradyrhizobium*, which was the only ASV shared by all life stages. However, comparisons with environment and host samples revealed that this ASV was not unique to the parasite and was also present in those two types of samples (Table S7 in Appendix 1). A core bacteriome was only identified at a higher taxonomic level, with three genera [Cyanobacteria: Xenococcaceae gen. sp. (not present in adults), Bacteroidetes: Saprospiraceae gen. sp. (not present in adults) and Proteobacteria: Phyllobacteriaceae gen. sp. (shared by all)] and seven families [Cyanobacteria: Xenococcaceae (not present in adults); Bacteroidetes: Flavobacteriaceae (shared by all) and Saprospiraceae (not present in adults); Proteobacteria: Comamonadaceae (shared by all), Rhodobacteraceae (shared by all, and in fact with prevalence above 0.90), Bradyrhizobiaceae (shared by all) and Phyllobacteriaceae (shared by all)] having a prevalence above 50% among all parasite samples at those levels. When considering each life stage separately, only adults had two ASVs representing a potential core [Actinobacteria: Nocardioidaceae sp. (unique to adults) and Proteobacteria: Comamonadaceae sp. (ASV unique to adults and the bird host)]. At higher taxonomic levels, we observed some consistency between the core bacteriomes of the parasite life stages. Rediae and cercariae shared one taxon at genus level (Saprospiraceae gen. sp.) and three at family level, one of which was the only family shared by all three life-stage cores (Proteobacteria: Rhodobacteraceae).

Although each life stage seems to harbour a distinct bacterial community, with only a small proportion of taxa shared among life stages, we checked whether at the inter-individual level we could observe greater bacteriome similarity between individuals of the same or subsequent life stages (i.e. rediae and cercariae) collected from the same host tissue. Only a small proportion of taxa was shared between co-infecting parasites (Fig. 2c). The relative abundance of shared taxa was higher in rediae and adults than in cercariae (rediae: 0.182 ± 0.108, cercariae: 0.071 ± 0.079, adults: 0.142 ± 0.134, $p = 0.005$, Kruskal–Wallis rank sum test). Indeed, cercariae shared significantly fewer taxa with co-infecting rediae than co-infecting rediae shared with each other (Dunn test, p.adj = 0.004).

How Does the Parasite Bacteriome Compare to That of the Hosts and Environment?

To further explore potential sources of the parasite bacteriome and its transmission, we started by assessing the relationship between the parasite bacterial community composition and that of their direct environment, i.e. their respective hosts (Table S8 in Appendix 1). Redial bacteriomes presented higher diversity estimates than those of snails (Faith's PD, p.adj = 0.005, Shannon p.adj = 0.006) but did not differ in terms of evenness (p.adj = 0.084). Cercaria and adult life stages had similar estimated diversity as their respective hosts (both cases, p.adj > 0.05). When comparing community composition, both redial and cercarial bacteriomes differed from those of their snail hosts (Fig. 3a), a finding consistent at all taxonomic levels (Table S9 in Appendix 1). On the contrary, the bacterial community of adults did not differ from that of their host bird eyes. When comparing parasite microbiomes with the bacterial communities found in the external environment, all life stages significantly differed from the environment based on unweighted unifrac distances, but rediae and cercariae did not differ anymore when bacterial relative abundances were considered (Table S9). These results are consistent with the fact that both rediae and cercariae shared a much higher percentage of ASVs with the environment than adults did (rediae: 25.04%; cercariae: 20.38% and adults: 3.74%). However, environmental samples were collected at the place of collection of the snails; we have no data on the specific habitat used by the bird hosts.

To infer how parasite bacteriomes may be assembled, we identified how many and which percentage of the bacterial communities of each individual parasite were shared with either co-infecting parasites and/or hosts (Fig. 3b). More than 50% of the community composition of each individual parasite was unique (rediae: $78.87 \pm 12.15\%$, cercariae: $90.25 \pm 10.02\%$, adults: $69.56 \pm 21.43\%$). Adults shared on average a higher percentage of their bacterial taxa only with the host (rediae: $1.75 \pm 2.95\%$, cercariae: $4.77 \pm 5.23\%$, adults: $16.13 \pm 23.46\%$), not accounting for taxa shared with both co-infecting parasites and host. Only for cercariae were we able to assess bacterial sharing between consecutive life stages in comparison with transmission from the host. However, cercariae were in fact the life stage that shared the least with either co-infecting parasites and/or host. We then further investigated if those unique (not shared with

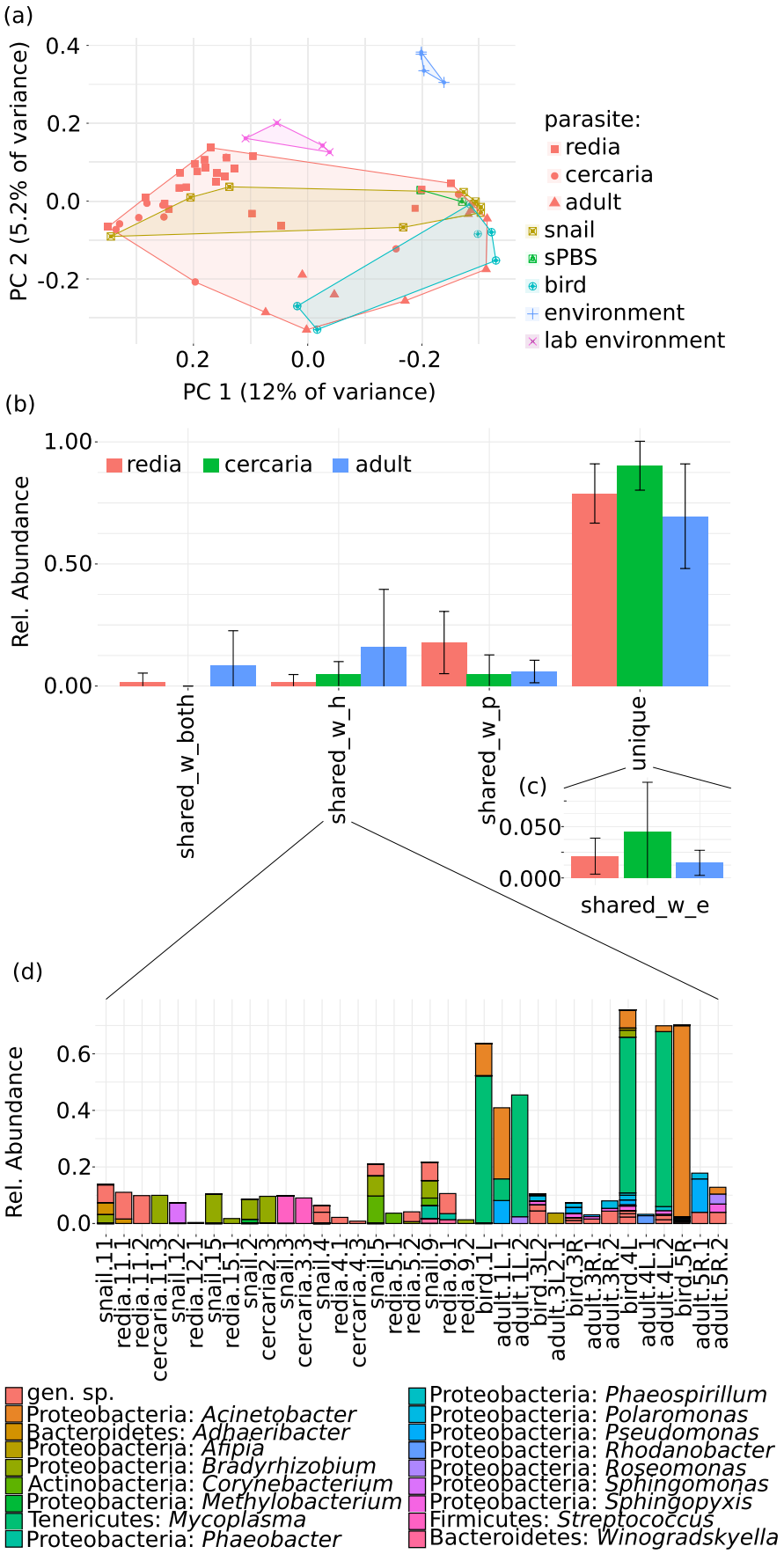

Fig. 3 Comparison of the bacterial communities of the trematode *Philophthalmus attenuatus* with bacteria from the host and environment. **a** Principal coordinates analyses ordination based on unweighted unifrac distances of bacterial communities, with hulls delimiting each group of samples. **b** Average relative abundance of bacteria in each parasite life stage that are either shared with co-infecting parasites (shared_w_p), shared with the host (shared_w_h), shared with both host and co-infecting parasites (shared_w_both) or unique to the parasite sample (unique). **c** Average relative abundance of the fraction of the "unique" parasite bacteriome that is shared with the environment. **d** Relative abundance of ASVs, with their taxonomic classification at the genus-level, that were shared with host, shown for both parasite samples and the host they came from (matching codes)

the host) components of parasite microbiomes were found in the natural environment (Fig. 3c). However, only a very small percentage of those taxa unique to each parasite were in fact found within the environmental community estimated from water and substrate samples (rediae: 2.12 ± 1.76%, cercariae: 4.55 ± 4.78%, adults: 1.47 ± 1.22%). Comparisons of the relative abundance of shared ASVs between parasite and host revealed very few instances, in adults and bird eyes, where the shared ASVs were only jointly abundant (Fig. 3d). Taking a closer look at the highly prevalent family Rhodobacteraceae, we found that while it occurred in all types of samples (Table S7 in Appendix 1), sharing between an individual parasite and its given host was only observed in one instance, between a redia and its snail host (relative abundance in redia = 0.021 and in snail = 0.045).

How Does the Parasite Bacteriome Vary With Increasing Geographic Distance?

To further understand how parasite bacteriomes are established, we assessed the geographic variation of redial bacteriomes among three localities separated by up to 500 km. While there was no difference in community diversity among localities (for all three metrics estimates and for all taxonomic levels, $p > 0.05$, Table S10 in Appendix 1, Fig. 4a), rediae from the three localities had different bacterial community compositions (unweighted unifrac: $F_{2,51} = 1.654$, $R2 = 0.063$, $p = 1e^{-04}$; weighted unifrac: $F_{2,51} = 1.601$, $R2 = 0.061$, $p = 0.033$, Fig. 4b). However, in pairwise comparisons, only Portobello had a significantly different community composition from the other two localities (for both unweighted and weighted unifrac, $p.adj < 0.05$). The distinctness of the bacterial communities in rediae from Portobello is also manifested at higher taxonomic levels (from ASV to genus level for unweighted, and from ASV to class level for weighted unifrac distances, Table S11 in Appendix 1). When assessing variation in bacteriome composition as a function of geographic distance, we observed that the two closest localities (Portobello and Bluff) shared a higher number of ASVs, and we found a significant positive relationship between geographic distance and community dissimilarity based on Bray–Curtis dissimilarity estimates ($rho = 0.333$, $p < 0.05$, Fig. 4c). There was also a higher number of ASVs found to have significantly differential abundances between Portobello and Christchurch (geographic distance ~ 300 km), than between Portobello and Bluff (geographic distance ~ 200 km; Table S12 in Appendix 1). However, for the two most distant localities (Bluff and Christchurch, geographic distance ~ 480 km), no ASV was found to be differently more or less abundant, which was also supported by the fact that these two communities were in fact not significantly different according to PERMANOVA estimates based on phylogenetic information.

To test whether unique bacteria found in each redial community were unique because they only occurred in those specific localities, we matched each of those ASVs with the ones sequenced for each of the localities' environments and hosts. While there was in fact a fraction of the bacteriome of each redia composed of taxa only found in their locality of origin (unique also to the local environmental and snail host samples) (Fig. 4d), it did not account for more than 10% of the community composition. Some taxa found to be unique to one specific redial population were in fact also found in the other localities, in either environmental or snail host samples. However, overall, most of the unique fraction of the bacterial communities in rediae was unique to the parasites of that particular locality.

We then performed similar analyses among localities for both environment and snail samples to further investigate the variation of community composition across space. Similar to what was observed for the parasite bacteriomes, there were no significant differences among localities in terms of alpha diversity for both environment and snail samples. Patterns of similarity among the microbial communities were different, however. The environments of the three localities presented significantly different community assemblages (unweighted unifrac: $F_{2,19} = 1.915$, $R2 = 0.184$, $p = 1e^{-04}$; weighted unifrac: $F_{2,19} = 2.816$, $R2 = 0.249$, $p = 1e^{-04}$), but this was not the case for bacterial communities in snail tissue samples (unweighted unifrac: $F_{2,14} = 1.106$, $R2 = 0.156$, $p = 0.229$; weighted unifrac: $F_{2,14} = 0.761$, $R2 = 0.113$, $p = 0.693$). These results were consistent at all investigated taxonomic levels for both sample types. Estimates of distance-decay relationships were congruent for all sample types, with a strong significant positive relationship between geographic distance and community dissimilarity based on Bray–Curtis distances (environment: $rho = 0.393$, $p = 2.005e^{-08}$; snail: $rho = 0.379$, $p = 6.568e^{-05}$).

How Stable Are Bacteriomes Across Parasite Life Stages Versus Geographic Distance?

Bacterial community composition always differed significantly when comparing among the different parasite life stages from the same locality, and among the same life stage from different localities. In both cases, unique components of the bacteriome constituted the majority of the bacterial communities. However, there was greater sharing of bacterial taxa within the same life stage sampled from different localities, than among different life stages from the same locality (shared among localities: ASV = 34, genus = 94, family = 79; shared among life stages: ASV = 1, genus = 15, family = 26; Fig. 5). Estimates of core bacteriome composition also support the idea of greater consistency within the same life stage whatever the locality of origin. Although only at the family level, we found evidence of sharing of

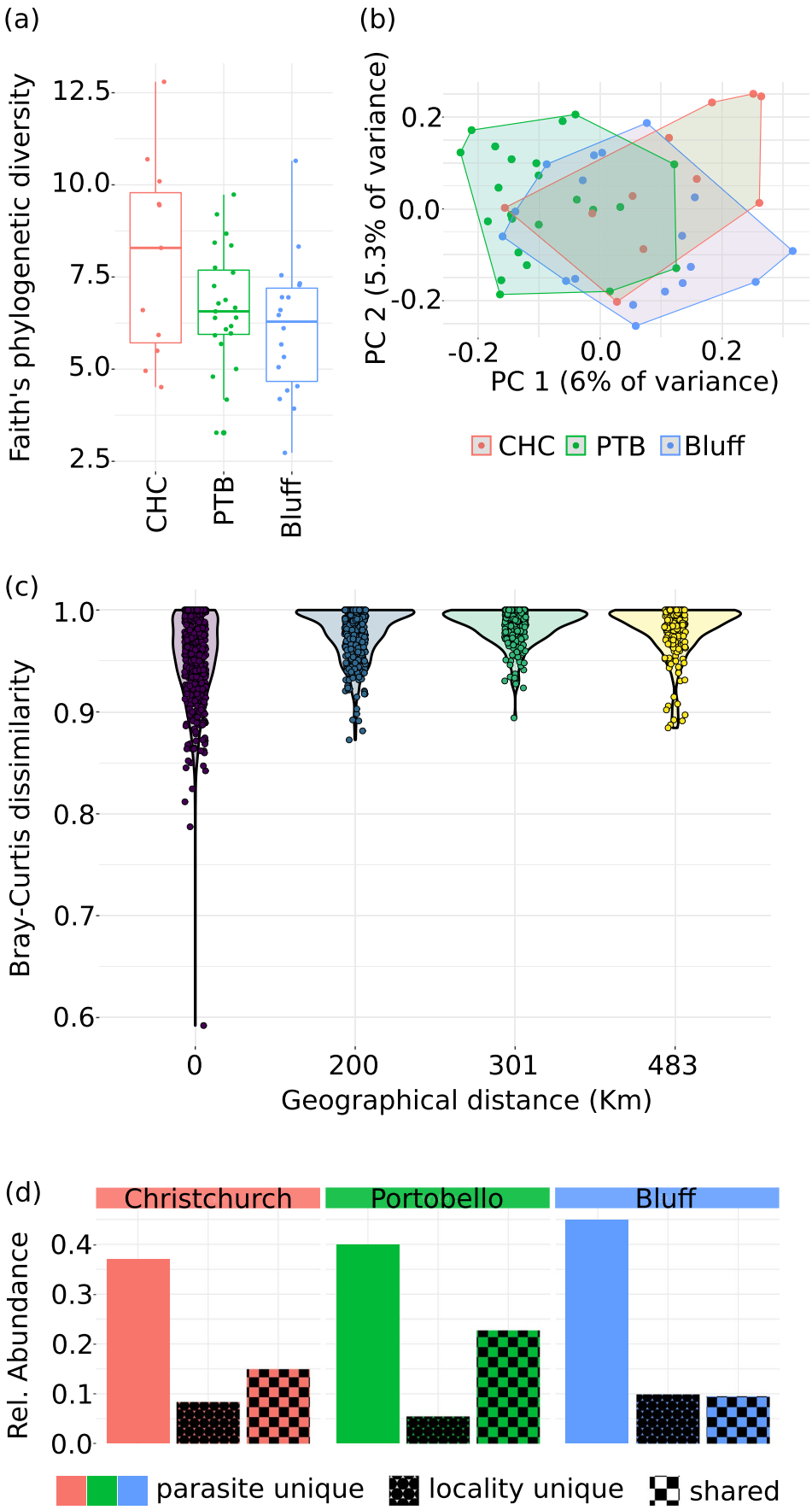

Fig. 4 Geographic variation of bacterial communities in rediae of the trematode *Philophthalmus attenuatus*. **a** Alpha diversity in the three different localities, as measured using Faith's phylogenetic diversity index at ASV-level (CHC, Christchurch; PTB, Portobello). **b** Principal coordinates analyses ordination based on unweighted unifrac distances of bacterial communities, with hulls delimiting samples from the same locality (CHC, Christchurch; PTB, Portobello). **c** Relationship between geographic distance between localities and bacterial community composition based on Bray–Curtis dissimilarity distance (distance 0 = comparisons among samples from the same locality). **d** Proportion of the relative abundance of rediae unique bacteriome (i.e. not shared with rediae from other localities) that is "parasite unique" if not found in either environment or host samples from the same locality, "locality unique" when found only in the parasite, environment and/or host samples from the same locality and "shared" when found in the environment and/or host samples from any of the other two localities

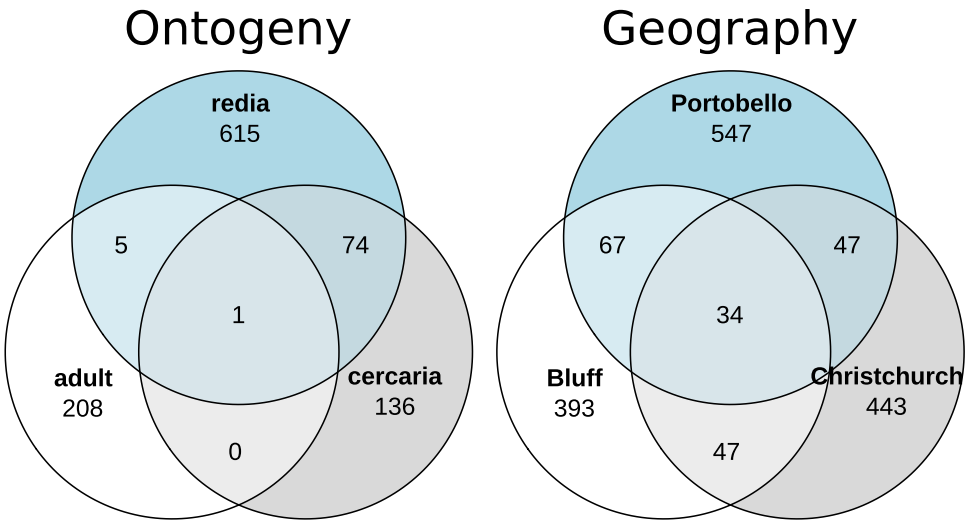

Fig. 5 Venn diagrams showing the number of shared ASVs among bacterial communities of the trematode *Philophthalmus attenuatus* across its ontogeny and geographic locations

core taxa in both cases, with the different life stages in Portobello sharing only one family at a prevalence above 50% of the samples (Proteobacteria: Rhodobacteraceae), while six families (Bacteroidetes: Flavobacteriaceae; Verrucomicrobia: Verrucomicrobiaceae; Cyanobacteria: Xenococcaceae; Planctomycetes: Pirellulaceae; Proteobacteria: Alteromonadaceae and Rhodobacteraceae) were shared at the same prevalence threshold among the core bacteriomes of rediae from all three localities.

Discussion

The phenotype and performance of individual animals is influenced by their microbial symbionts [5–9]. For animals and their microbes to function as integrated holobionts on which natural selection can act [11, 12], we would expect the animal-microbe associations to show species-level, population-level or at least life stage-level consistency and repeatability. Here, we found that the bacteriome of the parasitic trematode *Philophthalmus attenuatus* shows little consistency across life stages, with various bacteria rising and falling in abundance, even disappearing, as the parasite goes through major ontogenetic transitions and host changes. In contrast, the redial life stage of the parasite within its snail intermediate host, while showing variation across geographical space, nevertheless had greater similarity in bacteriome composition across localities separated by up to 500 km, with several core bacterial families shared among localities.

The bacteriome of *Philophthalmus attenuatus* showed substantial variation across life stages. Similar patterns have been observed in non-parasitic organisms with complex developmental cycles, for example jellyfish [55] and amphibians [56]. In those cases, as in *P. attenuatus*, there were life stage-specific bacterial community assemblages. The only available information on another parasitic helminth with a complex life cycle and passage through distinct host species allowing a comparison with our findings comes from a study on another trematode, *Coitocaecum parvum* [17]. In contrast to what we observed in *P. attenuatus*, the bacterial community composition of *C. parvum* showed much greater stability throughout its life cycle. Nevertheless, at bacterial family level, we found twice as many bacterial taxa shared among life stages in *P. attenuatus* than in *C. parvum*, suggesting some degree of consistency. Of note, the family Rhodobacteraceae was found at high prevalence across all life stages of *P. attenuatus*. Bacteria from this family are common in marine and terrestrial environments and are often found as symbionts of eukaryotes [57]. It is possible that they provide beneficial metabolic pathways for the trematode, although their presence may have no functional implication.

The two trematode species *C. parvum* and *P. attenuatus* belong to very distantly related families within the subclass Digenea [58] and use different hosts (fish definitive host for *C. parvum*, bird for *P. attenuatus*), which may account for the different stability of their bacteriomes across the life cycle. Trematode life stages can provide very different habitats to their symbionts; for example, from the point of view of thermal conditions, rediae of *P. attenuatus* live within poikilothermic snails while adults live in homeothermic birds. Different life stages also face different challenges as they pass through different hosts, from feeding on different resources to combatting vastly different immune responses. These changing opportunities for acquisition of bacteria and changing requirements of the parasite might select for partnership with different microbial symbionts. In humans, for instance, the intestinal microbiome changes with age under the influence of a range of factors including diet and environmental conditions, resulting in clear differences between children and adults [59, 60]. Whether the changing bacteriome of *P. attenuatus* is linked to the fact that each life stage

represents a distinct habitat, or because each life stage has its own bacterial requirements, remains to be determined. Nevertheless, the contrasting findings from just two trematode species hint at variability across trematode species in their ontogenetic associations with bacteria.

There are some similarities between the two trematode species, however. Interestingly, the bacterial family Comamonadaceae (Proteobacteria), which was part of the adult core bacteriome here in *P. attenuatus*, was also found in the adult-specific core bacteriome of *C. parvum* [17]. Also, in both *P. attenuatus* and *C. parvum*, no ASV reached 50% prevalence in cercariae, confirming that in both trematodes, this stage may represent a severe bottleneck for the continuation of the bacteriome from the previous life stage (rediae). In other words, in both trematode species, bacteriomes of cercariae may result from the random acquisition of a subset of taxa from the rediae.

The observed differences in bacteriome composition across life stages suggest that the parasite's direct environment (the host or environmental water) constitutes the primary source of bacteria for the parasite's microbial community. Therefore, we might expect that two parasites co-infecting the same tissue in the same host would share a good portion of their bacteria. Yet, we found relatively little sharing of bacteria between co-infecting parasites. Sharing was higher among rediae co-infecting the same snail than at other life stages, which leads us to the hypothesis that the parasite's genotype might influence the composition of its bacteriome. Indeed, all rediae within a snail are usually issued from the same original egg [61], meaning that any two individual rediae taken from a snail are almost certainly clones. In contrast, two adult worms in the same bird eye are almost certainly unrelated. Nevertheless, at all life stages, a large portion of the parasite bacteriome consists of "unique" bacteria not found in co-infecting parasites, in the host, or in the environment. Taxa with low abundance in environmental or host samples might have remained undetected. Inside the parasite, the same bacteria may have found a beneficial environment and become abundant enough to allow detection. To test this theory of environmental filtering, it would be necessary to search specifically for the presence of these rare ASVs in the parasite's direct environment, probably by filtrating larger volumes of environmental water samples and collecting larger host tissue samples.

One of our key findings is the greater consistency of the parasite bacteriome on a geographic scale than on an ontogenetic scale, which contrasts with some studies on free-living organisms [e.g. 18,19]. In other words, the bacteriome of a given life stage (rediae) was much more similar across localities separated by hundreds of kilometres, than the parasite bacteriome among life stages in a single locality. Rediae from different localities have a much higher consistency among the taxa composing their cores. For example, six bacterial families were shared by all localities at a prevalence > 50%. The similarity in bacteriome composition decreased significantly with increasing spatial distance, but only slightly, hinting at mechanisms maintaining its integrity at that particular life stage. Dispersal of the parasite through the highly mobile bird definitive host might contribute to the transport of bacterial taxa symbiotic with the parasite across localities, but, most importantly, it results in a genetic homogenisation of the parasite populations across our sampling localities [28]. Thus, the similar genetic background of rediae may promote their colonisation by certain bacterial families, maybe because they contribute to improve avoidance of host defences or exploitation of host resources; if these bacteria are present in the environment or the snail hosts across all localities, similar bacteriomes would arise in rediae regardless of location. Selection may have favoured certain associations because they benefit all partners, with only certain bacterial taxa being recruited or allowed to proliferate by the parasite during the redial stage. The non-random and comparable microbial assembly observed among rediae from distant localities suggests some predictability and fidelity exist in trematode-microbe interactions, consistent with their functioning as holobionts.

There are several potential sources of bias and of contamination in metabarcoding studies [62]. Estimates of microbial community composition are ultimately dependent on sequencing depth, and whether or not it is sufficient to capture community diversity and complexity. These issues can seriously affect the biological interpretation of metabarcoding data. To assess potential sources of bias and errors during library preparation and sequencing, we have included mock community standards in all library preparations. We followed a conservative approach, removing all features found in blanks and laboratory controls and excluding samples with low sequencing depths, which are known to affect downstream diversity and abundance analyses. In addition, we collected a diversity of environmental control samples and host samples that were used to correct for external contamination of parasite samples. Furthermore, we based most of our estimates of diversity on phylogeny-based metrics that perform better to explore microbial communities based on low sequence coverage [63]. We observed a high proportion of ASVs that were unique to an individual sample, which could indicate high biological variation but could also result from sequencing bias. We assume the former because quality control analyses based on observed and expected microbial community standards (MCS) did not detect bias in extraction and amplification of the different bacteria composing the MCS. Thus, comparisons aiming at controlling for the origin of parasite-associated bacteria and for transmission mode focused on shared ASVs, whereas estimates of community diversity across life stages and from different localities were

also performed at higher taxonomic levels to avoid bias in structure from fine-scale data (ASVs).

In summary, our results suggest that the bacteriome of the trematode *P. attenuatus* varies across its life cycle, with each life stage characterised by a different bacterial community, but that it shows consistency at a particular life stage across populations separated by hundreds of kilometres. Variation in the microbiome composition of hosts and parasites is increasingly acknowledged to be of central importance for our understanding of disease dynamics and evolution [8, 33, 34]. At present, information on the microbiomes of parasitic helminths is very limited; however, as more species are investigated [29, 30], the generality of the patterns found here will soon be testable through comparative analyses.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00248-021-01774-z.

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Author Contribution RP, FJ and NMD conceived and designed the study. FJ, CF and EW conducted the field sampling and the laboratory work. FJ conducted all genetic, bioinformatic and statistical analyses, with input from NMD and RP. FJ and RP co-wrote the manuscript, with input from NMD.

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Data Accessibility Data used in this study is available through the NCBI BioSample Submission Portal as Bioproject ID: PRJNA707308 and as Sequence Read Archive (SRA, https://www.ncbi.nlm.nih.gov/sra/) submission no: SUB9096643.

Declarations

Competing Interests The authors declare no competing interests.

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