Running head: *Microbiota composition of Hen Fleas*

Bacterial microbiota composition of a common ectoparasite of cavity-breeding birds, the Hen Flea *Ceratophyllus gallinae*

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Experimental field studies have demonstrated negative fitness consequences of Hen Flea *Ceratophyllus gallinae* infestations for bird hosts, yet it is currently unclear if these negative effects are a direct consequence of flea-induced blood loss or a result of flea-borne pathogen transmission. Here we used a 16S rRNA sequencing approach to characterise the bacterial microbiota community of Hen Fleas collected from Great Tit *Parus major* nests and found that *Brevibacterium* (Actinobacteria), *Staphylococcus* (Firmicutes), *Stenotrophomonas* (Proteobacteria), *Massilia* (Proteobacteria), as well as the arthropod endosymbionts "*Candidatus* Lariskella" and "*Candidatus*

Midichloria" were most abundant. We found evidence for the occurrence of *Staphylococcus* spp. in Hen Fleas, which may cause opportunistic infections in bird hosts, but not of other known pathogens commonly transmitted by other flea species, such as *Bartonella* spp. or *Rickettsia* spp. However, Hen Fleas might transmit other pathogens (e.g. viruses or bacteria that are not currently recognised as bird pathogens) which may contribute to the negative fitness consequences of Hen Flea infestations in addition to direct blood loss or secondary infections of wounds caused by biting fleas.

Keywords: host-parasite interactions, zoonotic diseases, nestboxes, garden birds, next generation sequencing (NGS), microbiome, pathogens, public health, wildlife disease

34 The Hen Flea *Ceratophyllus gallinae* is a common, generalist ectoparasite of cavity- and semi-
35 cavity breeding birds of the Western Palearctic, such as tits (Paridae), sparrows (Passeridae) or
36 flycatchers (Muscicapidae) (Tripet & Richner 1997). They live and reproduce in nesting material
37 and suck blood from nestlings and adults (Tripet & Richner 1997). Experimental manipulations of
38 Hen Flea load in Great Tit nests demonstrates substantial negative fitness consequences of flea
39 infestations for the bird host (e.g. Tschirren *et al.* 2003, Fitze *et al.* 2004). Yet, it is currently
40 unknown whether these negative effects are caused directly by the ectoparasite (i.e. through blood
41 loss) or indirectly through the transmission of flea-borne pathogens.

42 Many members of the flea order Siphonaptera are known vectors for wildlife, pet and / or human
43 pathogens (Bitam *et al.* 2010). In total, there are over 2500 described flea species; the majority
44 parasitise mammals, but about 6% are ornithophilic (Bitam *et al.* 2010). The most famous example
45 of a flea-transmitted pathogen is *Yersinia pestis,* the causative agent of bubonic plague, which is
46 vectored by a range of mammal flea species (Stenseth *et al.* 2008). Other pathogens transmitted by
47 fleas include *Rickettsia* spp., the causative agents of diseases such as murine typhus (Traub 1978)
48 and spotted fever (Pérez-Osorio *et al.* 2008), *Bartonella* spp.*,* the causative agents of diseases such
49 as cat-scratch disease and endocarditis (Chomel *et al.* 2006) or *Coxiella* spp.*,* the causative agents of
50 diseases such as Q fever (Angelakis *et al.* 2014). The vector potential of bird fleas, on the other
51 hand, is largely unknown. However, a previous study on *Ceratophyllus garei* fleas infesting
52 migratory reed warblers and their nests in Slovakia observed a *Rickettsia* spp. prevalence of 31.9%
53 (Sekeyova *et al.* 2012), indicating that bird fleas can also act as vectors. Furthermore, evidence of
54 *Bartonella* spp. infections was found in a range of bird species (Mascarelli *et al.* 2014).

55 Here we characterise the bacterial microbiota of *Ceratophyllus gallinae* fleas and survey the
56 occurrence of bacterial pathogens for which Hen Fleas may act as a vector and which may
57 contribute to the lower fitness of Hen Flea-exposed birds. Assessing the vector-potential of Hen
58 Fleas is not only important for our understanding of the eco-evolutionary dynamics of bird-

ectoparasite interactions, but also for the identification of potential human health risks, as people are exposed to and bitten by Hen Fleas on a regular basis when cleaning out nest boxes in their garden (du Feu 1987).

Methods and materials

Hen Flea sampling

We collected Hen Fleas *Ceratophyllus gallinae* from 22 nests of Great Tits *Parus major* breeding in nestboxes in Zurichbergwald, Switzerland (47°20′08″N, 8°30′01″E) in summer 2016. Fleas were stored in 95% ethanol.

Bacterial microbiota sequencing

DNA isolation was performed in a laminar flow cabinet. Individual fleas (n = 208 Hen Fleas from 22 nests; Table 1) were washed thrice with sterile water, once with 3% hydrogen peroxide and then crushed with a sterile pestle. Fleas from 7-8 nests were then pooled (Table 1) for DNA extraction and 16S sequencing. Nests were randomly allocated to pools. Whole-pool DNA extraction was performed using DNeasy Blood & Tissue kit (Qiagen, Hilden, Germany), following the manufacturer's protocol.

We quantified the bacterial microbiota of Hen Fleas by sequencing the hypervariable V3-V4 region of the 16S rRNA gene. The protocol has been described in detail elsewhere (Aivelo *et al.* 2019). In short, we prepared sequencing libraries following the Earth Microbiome 16S Illumina protocol using the primers 515F and 806R (Apprill *et al.* 2015, Parada *et al.* 2016), with an expected amplicon size of approximately 300 bp. The 16S region was amplified in a first PCR reaction, the

PCR products were visualized on an agarose gel, cut out and purified with MinElute Gel Extraction kit (Qiagen, Hilden, Germany). Illumina Miseq adaptors were then added in second PCR step and the products were purified as described above. 4 nM library pools were then created by mixing equimolar amounts of products, followed by standard normalization protocols. Paired-end sequencing of flea pools and negative controls (n = 5) was performed on a Illumina MiSeq at the Functional Genomic Center Zurich using reagent kit 600cycle v3 (Illumina, San Diego, CA, USA) following the manufacturer's protocol, with a target amplicon length of 250 bp. Overall, we obtained 918 - 23296 amplicons across flea pools.

We analysed the sequence data with *mothur* pipeline (Schloss *et al.* 2009), following MiSeq standard operation procedures (Kozich *et al.* 2013). For sequence alignment, we used aligned SILVA bacterial references (release 132; https://www.arbsilva.de/documentation/release-132/) and grouped operational taxonomic units (OTUs) based on 99% sequence similarity. OTUs were assigned to taxa with the *wang* method (Wang *et al.* 2007) using SILVA taxonomy. We used taxonomic lists in Bitam *et al.* (2010), McElroy *et al.* (2010) and Eisen & Gage (2011) to identify flea-borne pathogens among the OTUs. Endosymbionts were identified by a literature search that combined the taxonomic labels and 'endosymbiont' as search terms in Web of Science (Clarivate Analytics, Philadelphia, PA, USA), following Aivelo *et al.* (2019). Samples were rarefied to lowest amplicon count (1017) to account for amplicon number variation across pools. All OTUs with less than three amplicons across pools were discarded; these OTUs likely represent sequencing errors or very rare bacteria that are unlikely to have major effects on host populations. Rarefaction did not alter the OTU composition of the more deeply sequenced pools 1 and 2. Indeed, Bray-Curtis dissimilarities between the non-rarefied and rarefied pool 1 was 0.14, and 0.09 in pool 2. The main effect of rarefaction was the removal of rare OTUs (from 954 to 58 in pool 1 and from 463 to 68 in pool 2).

To compare the composition of the three sequencing pools, we calculated alpha diversity (Inverse Simpson Index, Simpson 1949), beta diversity (Bray-Curtis dissimilary, Bray & Curtis 1957), and UniFrac distance (Lozupone *et al.* 2011) using the *vegan* package (Oksanen *et al.* 2013) in *R* (*R* Core Team 2013).

Results

After filtering, 95 bacterial OTUs were identified across the three flea pools (mean: 66, range 58 - 73; Suppl. Table 1). 28 OTUs were observed in all three pools and 64 OTUs in two of the three pools (Suppl. Table 1). Inverse Simpson index (range: 7.0-11.8; Table 1), Bray-Curtis dissimilarity index (range: 0.49-0.64; Suppl. Table 2) and weighted UniFrac distances (range: 0.39-0.45; Suppl. Table 2) between pools were comparatively uniform, suggesting that none of the pools is markedly different in composition from the others.

Most of the amplicons (97.7%) belonged to three bacterial phyla: Actinobacteria (24.0%), Firmicutes (29.2%) and Proteobacteria (44.4%) (Fig. 1; Suppl. Table 1). The most common OTUs were *Staphylococcus* (Firmicutes), *Brevibacterium* (Actinobacteria), *Massilia* (Proteobacteria), and *Stenotrophomonas* (Proteobacteria) (Fig. 1; Suppl. Table 1).

No known bird or mammal pathogens were detected in the sequence data. However, we found evidence for the occurrence of the arthropod endosymbionts "*Candidatus* Lariskella", "*Candidatus* Midichloria" (both among the 6 most common OTUs) and *Rickettsiella* in Hen Fleas. No evidence for the occurrence of other arthropod endosymbionts (such as *Wolbachia* spp. or "*Candidatus* Cardinium") was found.

Discussion

We characterised the bacterial microbiota of the Hen Flea *C. gallinae*, a common, nest-based ectoparasite of cavity-nesting birds, using a 16S sequencing approach. In total, we observed 95 different OTUs across sequencing pools, which is broadly in line with the results of previous mammal flea microbiota studies (Pornwiroon *et al.* 2007, Erickson *et al.* 2009, Jones *et al.* 2010, 2015). The phylum Proteobacteria as well as specific bacterial genera, such as *Brevibacterium* and *Stenotrophomonas,* were particularly abundant in Hen Fleas. Interestingly, these groups have also been found to dominate the bacterial communities of mammal flea species (Pornwiroon *et al.* 2007, Erickson *et al.* 2009, Jones *et al.* 2010).

No evidence for the occurrence of flea-transmitted bacterial (bird / mammal) pathogens such as *Bartonella* spp. or *Rickettsia* spp. (Bitam *et al.* 2010, McElroy *et al.* 2010, Eisen & Gage 2011) was found. This contrasts the findings of a previous study in another bird flea, *C. garei*, which observed a high (31%) prevalence of *Rickettsia* spp. (Sekeyova *et al.* 2012). Currently it is unclear if this difference is due to spatial variation in *Rickettsia* spp. prevalence (i.e. Slovakia vs Switzerland) or if *C. gallinae* is a non-competent vector for *Rickettsia* spp. Although studies testing for *Rickettsia* spp. infections in wild birds are still rare, there is some evidence that infections can occur, but that prevalence is generally low (Elfving *et al.* 2010). Evidence of *Bartonella* spp. infection has also been found in several bird species (Mascarelli *et al.* 2014).

Staphylococcus spp., which can cause opportunistic infections (Coates *et al.* 2014), was another particularly common OTU found in Hen Fleas. Such opportunistic infections (e.g. of wounds caused by biting fleas) caused by *Staphylococcus* spp. or other opportunistic pathogens may contribute to the negative fitness effects of Hen Flea infestations on bird hosts (e.g. Tschirren *et al.* 2003, Fitze *et al.* 2004). Other particularly common OTUs across flea pools were the arthropod endosymbionts "*Candidatus*_Lariskella" and "*Candidatus*_Midichloria". Interestingly, however, we found no evidence for the occurrence of the endosymbiont *Wolbachia* spp., which is observed in many flea species (Gorham *et al.* 2006, Jones *et al.* 2015, Lawrence *et al.* 2015; but see Jones *et al.*

155 2010), including the bird flea *C. garei* (Sekeyova *et al.* 2012). It is possible that *Wolbachia* spp. is
156 not truly absent in *C. gallinae* but was simply not detected because of low prevalence (only 208
157 fleas were sequenced in this study). Alternatively, negative interactions among endosymbionts (i.e.
158 competition effects; Aivelo *et al.* 2019) might explain the absence of *Wolbachia* spp. Indeed, a
159 comparative survey of bacterial microbiota of mammalian fleas suggested that co-occurrence of
160 endosymbionts (such as "*Ca.* Lariskella", *Wolbachia* spp., *Spiroplasma* spp. and "*Ca.* Cardinium")
161 within fleas is rare (Jones *et al.* 2015). Thus, the comparably high "*Ca.* Lariskella" abundance in
162 Hen Fleas may impede *Wolbachia* spp. infections.

163 Although only three pools were sequenced in our study, they included more than 200 Hen Fleas
164 from 22 different Great Tit nests. Importantly, despite marked among-pool differences for some
165 OTUs, overall the three independent pools showed remarkable similarities in bacterial microbiota
166 composition, with the range of OTU diversity being small and almost half of the OTUs being
167 present in at least two pools. Furthermore, a comparison of non-rarefied and rarefied pools
168 highlighted that the lower sequencing depth of pool 3 did not bias the results. Together, it suggests
169 that the bacterial communities we describe here for *C. gallinae* are representative.

170 However, for some OTUs there were marked among-pool differences, most prominently for
171 *Massilia* (Proteobacteria), which was very common in pool 1 but rare or absent in the other pools.
172 These differences are likely due to environmental effects (e.g. differences in the composition of the
173 nesting material among nests), which may affect some members of the flea microbiota. Indeed it is
174 typically found that a part of the arthropod microbiota is non-resident (i.e. consists of bacteria that
175 are obtained from the environment) and thus susceptible to environmental influences (Hammer *et*
176 *al.* 2017).

177 Our study has a number of limitations. First, although we did not detect any known bacterial bird or
178 mammal pathogens in our study, we cannot exclude the possibility that Hen Fleas may act as a

vector for them, but that they were not detected because of low prevalence. Also, for most OTUs observed in our study the pathogenic potential is currently unknown. Furthermore, only Hen Fleas from Great Tit nests were analysed and it is possible that Hen Flea microbiota composition might differ depending on host species. Finally, we cannot exclude the possibility that there is spatiotemporal variation in bacterial microbiota composition of Hen Fleas, or in the occurrence of bacterial pathogens. The characterization of seasonal changes in the microbiota composition of Hen Fleas feeding on different hosts, as well as variation in Hen Flea microbiota composition on a large spatial scale would thus be an interesting next step.

In conclusion, we found no evidence that *C. gallinae* fleas harbour known bacterial bird (or mammal) pathogens, such as *Rickettsia* spp. or *Bartonella* spp. (Bitam *et al.* 2010, McElroy *et al.* 2010, Eisen & Gage 2011). The negative fitness consequences of Hen Flea infestations for their bird hosts (Tschirren *et al.* 2003, Fitze *et al.* 2004) are thus most likely caused by blood loss, secondary infections of wounds caused by biting fleas and / or other flea-borne pathogens (such as viruses or bacteria that are not currently recognised as bird pathogens). The lack of (known) human bacterial pathogens in Hen Fleas furthermore suggests that the human health risk of exposure to Hen Fleas during nest box cleaning is likely limited. However, the pathogenic potential of most OTUs identified in our study is currently unknown and Hen Fleas may act as vectors for non-bacterial pathogens. Thus, to prevent exposure to such unidentified flea-borne pathogens, as well as a bad itch, we recommend to clean out nest boxes during a cold winter day when Hen Fleas are inactive.

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209 Data Availability Statement

210 Sequences have been deposited to the NCBI Sequence Read Archive under BioProject

211 PRJNA528393.

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292 Table 1: Number of Great Tit nests and individual Hen Fleas included in the sequencing pools and
293 measures of bacterial microbiota composition of fleas in the three pools.

Pool	Number of nests	Number of fleas	Fleas per nest (mean (range))	Number of OTUs	Inverse Simpson index
1	8	68	8.5 (4-11)	58	7.0
2	7	70	10 (2-17)	68	11.8
3	7	70	10 (3-18)	73	10.8

294

Figure legend

Figure 1: Phylotypic distribution of bacterial OTUs in Hen Fleas (pools FL1-FL3). The most common OTUs are shown separately whereas less common OTUs are grouped by phyla.