Macrobdella decora: Old World Leech Gut Microbial Community Structure Conserved in a New World Leech

Emily Ann McClure[a], Michael C. Nelson[a*], Amy Lin[a], Joerg Graf[a,b,#]

[a]Department of Molecular and Cell Biology, University of Connecticut, Storrs, Connecticut, U.S.A.

[b]Institute for Systems Genomics, University of Connecticut, Storrs, Connecticut, U.S.A.

Running Head: *Macrobdella decora* Conserved Symbionts

#Address correspondence to Joerg Graf, Joerg.graf@uconn.edu

*Present address: Sema4 Genomics, Branford, Connecticut, U.S.A..

ABSTRACT

Leeches are found in terrestrial, aquatic, and marine habitats on all continents. Sanguivorous leeches have been used in medicine for millennia. Modern scientific uses include studies of neurons, anticoagulants, and gut microbial symbioses. *Hirudo verbana*, the European medicinal leech, maintains a gut community dominated by two bacterial symbionts, *Aeromonas veronii* and *Mucinivorans hirudinis*, which sometimes account for as much as 97% of the total crop microbiota. The highly simplified gut anatomy and microbiome of *H. verbana* make it an excellent model organism for studying gut microbial dynamics. The North American medicinal leech, *Macrobdella decora,* is a hirudinid leech native to Canada and the northern U.S.A. In this study we show that *M. decora* symbiont communities are very similar to those in *H. verbana*. This similarity allowed for an extensive study in which wild caught animals were sampled to determine effects of geographic separation, time of collection, and feeding on the microbiome. Through 16S V4 rRNA deep sequencing we show that: i) the *M. decora* gut and bladder microbial communities are distinct, ii) the *M. decora* gut community is affected by feeding and long periods of starvation, and iii) geographic separation does not appear to affect the overall gut microbial community structure. We propose that *M. decora* is a replacement for *H. verbana* for studies of wild-caught animals and offer evidence for the conservation of annelid symbionts. Successful culturing and comparison of dominant symbionts from *M. decora* and *H. verbana* will provide the ability to assess host-symbiont co-evolution in future work.

IMPORTANCE

47 Building evidence implicates the gut microbiome in regulating animal digestion, nutritional
48 acquisition, immune regulation, development, and even mood regulation. Because of the
49 difficulty of assigning causative relationships in complex gut microbiomes a simplified model
50 for testing hypotheses is necessary. Previous research in *Hirudo verbana* has suggested this
51 animal as a highly simplified and tractable animal model of gut symbioses. Our data show that
52 *Macrobdella decora* may work just as well as *H. verbana* without the drawback of being an
53 endangered organism and with the added convenience of easy access to field-caught specimens.
54 The similarity of the microbial community structure of species from two different continents
55 reveals the highly-conserved nature of the microbial symbionts in sanguivorous leeches and
56 confirms the medicinal leech as a highly simplified, natural animal model in which to study gut
57 symbioses.
58
59
60 **INTRODUCTION**
61 Leeches are a diverse animal group capable of surviving in freshwater, marine, and terrestrial
62 environments. They are found on all continents and oceans on planet Earth (1, 2). Records of
63 humans applying leeches medicinally survive from populations as far back as ancient Egypt (3,
64 4), resulting in the name medicinal leech, *Hirudo medicinalis* Linnaeus, 1758. As our
65 understanding of hirundinid taxonomy improved, *Hirudo medicinalis* was subdivided into
66 additional species including *H.verbana* Carena 1820 and *H.orientalis* Utevsky & Trontelj, 2005
67 (5, 6). Since 2004 in the United States, only *H. medicinalis* and *H. verbana* are approved for use
68 as a medical device and must be shipped from suppliers in Europe (5). Although it shares the
69 same common name, the North American medicinal leech, *Macrobdella decora*, was rarely used

for blood letting. No mechanical or pharmaceutical product has yet been able to replicate the reduction of venous congestion achieved by the medical application *of Hirudo* leeches (briefly reviewed in (7)). This results in a continued need for medicinal leeches and a better understanding of their biology.

As approved medical devices, the natural feeding habits of leeches are exploited to reduce venous congestion and improve blood circulation in affected patients. To make the most out of unpredictable encounters with prey, hirudinid leeches consume up to five times their body weight in one feeding and can go up to 6-12 months between feedings (1). After initial attachment, the leech stimulates blood flow in the prey by secreting vasodilators and a number of anticoagulant peptides including hirudin and orthologues (8-11). The gastrointestinal system of *H. verbana* is highly simplified and consists of a pharynx, crop, and intestinum (1, 7). Once consumed, excess ions and water are rapidly removed from the blood meal to form a highly viscous intraluminal fluid (ILF) in the crop (7, 12). ILF remains in the crop over long periods of time before it slowly passes into the intestinum where it is digested (7).

The gut microbiome of medicinal leeches is especially simple when compared to common mammalian gastrointestinal models. Previous research described an ILF microbiota in *H. verbana* dominated by *Aeromonas veronii* and *Mucinivorans hirudinis* (9, 13-17). Subsequent studies revealed the presence of additional *Aeromonas* spp in *H. verbana* and other hirudinid leeches (18-20). *Clostridial* species have also been detected in culture-independent studies of the ILF of *H. verbana* (14, 21) and *H. orientalis* (20). Several functions for the dominant crop microbiota have been proposed including providing essential nutrients to the host (9, 22, 23),

93 preventing other bacteria from colonizing, inhibiting putrefaction of the ILF (24), aiding the
94 host's immune responses (25, 26), or initiating the digestion of erythrocytes (27).
95
96 In most animals, the diversity of the microbiome increases along the length of the digestive tract
97 and similar findings have been reported for *Hirudo* spp. The intestinum contains Alpha-,
98 Gamma-, and Delta-proteobacteria, *Fusobacteria*, *Firmicutes,* and Bacteroidetes as well as
99 *Aeromonas* and *Mucinivorans* (14). A number of closely related hirudiniform leech species have
100 also tested positive for *Aeromonas* and Bacteroidetes from the digestive tract (8, 19, 20). The
101 composition of the microbial community among the different *Hirudo* species studied is similar in
102 both crop and intestinum (14, 17, 19, 20).
103
104 Unlike humans but similar to other annelids (28, 29) the bladder of *H. verbana* is colonized by a
105 number of microbial species (30). Sequence analysis and fluorescence *in situ* hybridization
106 micrographs of the *H. verbana* bladder show a stratified community consisting of *Ochrobactrum,*
107 *Bdellovibrio, Niabella,* and *Sphingobacterium* (30). The difference in bladder microbiome when
108 compared to the ILF or intestinum indicates that *H. verbana* microbiomes are body site specific
109 and suggests a selection process that regulates the composition of these communities.
110
111 Much less is known about the gut microbiome of *M. decora*. Prior studies attempting to
112 characterize the *M. decora* gut microbiome relied on aerobic culturing methods or sequencing
113 total ILF DNA with primers specific for *Aeromonas* or Bacteroidetes symbionts. These studies
114 revealed that the *Aeromonas* species associated with *M. decora* was *A. jandaei* (31) and the
115 Bacteroidetes species was most similar to uncultured and unidentified species in a clade with

116 *Rikenella, Mucinivorans,* and *Alistipes* (8). In this study, we strived for a more complete
117 understanding of the *M. decora* gut microbiome through culture-independent 16S rRNA V4 deep
118 sequencing with confirmation by fluorescence *in situ* hybridization (FISH). This procedure was
119 also performed on *H. verbana* to compare microbial communities between the two host species
120 using identical techniques.
121
122 In this work we describe the microbiota of the ILF, bladder, and intestinum in wild and
123 laboratory-maintained specimens of the North American medicinal leech, *Macrobdella decora*.
124 Comparison of core and common microbial operational taxonomic units (OTUs) from *M. decora*
125 to those of the well-described *H. verbana* provides insights about the level of conservation of the
126 microbiomes between distantly-related and geographically-isolated sanguivorous leeches.
127
128 **RESULTS**
129 Leech Organs Contain Distinct Microbial Communities
130 The microbial communities of three organs from two leech species (*Hirudo verbana* and
131 *Macrobdella decora*) were analyzed in this study: the intestinum, crop, and bladder (Table S1).
132 Due to their small size, for the intestinum and bladder the entire organ was homogenized, while
133 for the crop only the ILF was collected. Total DNA was extracted from the samples and the V4
134 region of the 16S rRNA gene was amplified, sequenced using an Illumina MiSeq, and analyzed
135 to determine the community composition using Qiime 1.9 .1 (32) and R 3.6.0. Of all parameters
136 tested, the leech host species had the greatest effect on microbiome composition
137 (PERMANOVA: $F=316.77$, $R^2=0.53$, $p=0.001$) (Figure 1A). This difference diminished when
138 performing the same analysis at a higher taxonomic level. For example, when the

PERMANOVA was performed at the order level (88% sequence identity) the variation accounted for by host species still had the greatest effect on microbiome composition, but that effect decreased to only 25% (PERMANOVA: $F=95.79$, $R^2=0.25$, $p=0.001$) (Figure 1B). This suggests that although the specific genera within the microbial community have changed between leech hosts, the general physiological functions performed by the microbiome have likely been conserved. An additional 47% of the variation between samples was not accounted for by host leech species alone and other factors are important in determining the community composition.

In *H. verbana* samples, the sampled organ had the greatest effect on microbiome composition (PERMANOVA: $F=57.56$, $R^2=0.29$, $p=0.001$) with feeding in the laboratory accounting for 18% of variation (PERMANOVA: $F=3.90$, $R^2=0.18$, $p=0.001$), the supplier of *H. verbana* animals accounting for 5% of variation (PERMANOVA: $F=19.71$, $R^2=0.05$, $p=0.001$), and the shipment date of *H. verbana* accounting for 7% of variation (PERMANOVA: $F=6.63$, $R^2=0.07$, $p=0.001$). This confirms previous results that the bladder and digestive tract have very different microbiomes (14, 30) the microbial composition changes as a result of feeding (15). In addition, these data suggests that leech suppliers and date of shipment affect the observed ILF microbiome of *H. verbana*.

For this study, we obtained the *H. verbana* from two suppliers: one located in Germany that sold field-caught animals and one in France that sold farm-bred animals. Treatments before shipping, including prolonged starvation time to reduce abundance of human pathogens and potentially feeding animals antibiotic-contaminated blood (33), are additional sources of influence on

microbiome variation accounted for but not uniquely discernable within the confounding influences clustered under the 'supplier' variable (18). Laboratory feeding in conjunction with sampled organ or animal source accounted for an additional 7% of variation (PERMANOVA: $F=3.82$, $R^2=0.03$, $p=0.001$ and PERMANOVA: $F=3.94$, $R^2=0.04$, $p=0.001$ respectively). This left ~35% of variation unaccounted for by these four variables.

In *M. decora*, the sampled organ had the greatest effect on microbiome composition (PERMANOVA: $F=26.47$, $R^2=0.19$, $p=0.001$) with the month of animal collection accounting for 17% of variation (PERMANOVA: $F=9.23$, $R^2=0.17$, $p=0.001$), feeding in the laboratory accounting for 6% of variation (PERMANOVA: $F=2.85$, $R^2=0.06$, $p=0.001$), and animal source accounting for only 5% of variation (PERMANOVA: $F=4.37$, $R^2=0.05$, $p=0.001$). This mirrors the results from *H. verbana* in that the bladder and digestive tract have very different microbiomes, a change in microbial composition occurs as a result of feeding, and animal collection site and time also affect the observed ILF microbiome of *M. decora*.

Some variables additionally exhibited statistical significance when evaluated as a group. Sampled organ in conjunction with the month that the animals were collected accounted for an additional 15% of variation (PERMANOVA: $F=5.16$, $R^2=0.15$, $p=0.001$). The animal collection month in conjunction with feeding accounted for a final 4% of variation (PERMANOVA: $F=4.10$, $R^2=0.04$, $p=0.001$). This left ~34% of variation unaccounted for by these four variables. Although the microbial differences between organs and after feeding were expected from previous studies of the *Hirudo* microbiome (14, 15, 19, 20, 30), it was surprising to discover the large role that collection month played in affecting the *M. decora* microbiome.

185

186 Deep Sequencing of *H. verbana* ILF Microbiome Reveals Rare Community Members

187 16S rRNA V4 deep sequencing of the *H. verbana* ILF identified the same two dominant taxa as
188 reported previously from 454 pyrosequncing of the 16S rRNA V6 region (21), 16S rRNA V3-V4
189 deep sequencing (17), and 16S rRNA clone library analysis (14) (Table 1). The ILF microbiota
190 of *H. verbana* has been described as being dominated by *Mucinivorans* and *Aeromonas* with
191 occasional Clostridial spp (14, 21). In the 36 animals tested, *Mucinivorans* and *Aeromonas*
192 together accounted for 30.6 – 99.8% (median = 81.8%) of the sequences from the ILF (Table 1)
193 with *Proteiniclasticum* making up an additional 0 – 63.4% (median when present = 10.8%,
194 present in 69% of animals) and *Fusobacterium* making up an additional 0 – 28.3% (median when
195 present = 11.5%, present in 47% of animals) (Table S2). The *Proteiniclasticum* OTU was
196 previously reported in the ILF 16S rRNA gene clone libraries of *Hirudo orientalis*, a closely
197 related leech species (20). The *Fusobacterium* OTU was previously reported in 16S clone
198 libraries from the intestinum of *H. verbana* (14). The increase in Clostridial sequences observed
199 in this study compared to previous studies is likely due to the improved DNA extraction
200 techniques, which included a bead-beating step specifically optimized to increase clostridial cell
201 lysis and therefore detection (34).

202

203 In addition to the four most abundant taxa, common sequences (found in at least 70% of
204 samples) in the *H. verbana* ILF included three Bacteroidetes, two *Bacteroides* (0 – 13%, median
205 when present = 1.6%) and one *Millionella-like* OTU (0 – 2%, median when present = 0.4%); two
206 *Proteocatella* (0 – 14.9%, median when present = 2.2%); and one *Desulfovibrio* (0 – 8.7%,
207 median when present = 0.6%) (Table 1 and S2). Finding these additional OTUs in such a high

208 percentage of animals is likely due to the much greater sequencing depth of Illumina technology
209 as compared to those previously used. The additional *Bacteroides* and *Millionella-like* OTUs
210 have not been described before in *H. verbana*. However, other researchers have previously noted
211 that, although dominant, *Mucinivorans* may not be the only Bacteroidales associated with *Hirudo*
212 leeches (14, 19, 35).
213
214 Sequences belonging to other taxa noted by previous researchers were also found in the 36 *H.*
215 *verbana* at average concentrations below 1.5% of the total sequences from the ILF community:
216 Firmicutes: *Erysipelothrix* (max = 5.1%, 17% of animals), *Vagococcus* (max = 1.2%, 17% of
217 animals), and *Enterococcus* (max = 0.7%, 17% of animals); Alphaproteobacteria: *Ochrobactrum*
218 (max = 0.4%, 6% of animals); Deltaproteobactera: *Desulfovibrio-like* (max = 5.9%, 39% of
219 animals) and *Desulfovibrio* (0.1%, 3% of animals); Gammaproteobacteria: *Proteus* (max =
220 16.1%, 44% of animals) and *Morganella* (max = 5.2%, 53% of animals); Bacteroidetes:
221 *Pedobacter* (max = 0.6%, 8% of animals) (14, 20, 21) (Table S2).
222
223 The low concentrations and intermittent presence of many of these previously identified bacteria
224 highlights the importance of greater sequencing depth and a greater number of specimens tested.
225 Our analysis of the microbial community in the ILF of *H. verbana* confirms that *Aeromonas* and
226 *Mucinivorans* comprise the core microbiome with Clostridial species also dominant when
227 present. Our analysis also reveals a more diverse rare *H. verbana* ILF microbiome than
228 previously described as well as community members that are present in >70% of the animals.
229
230 <u>*M. decora* ILF Microbiome is Similar to *H. verbana* ILF Microbiome</u>

One unique feature of the analysis of *H. verbana* is that these animals were obtained from leech farms that breed captive animals or maintain field-caught animals for months in captivity before shipping. Analyzing the microbiome of field-caught *M. decora,* would allow us to assess if the diversity of the microbiome could be greater in animals captured in the wild. The composition of the ILF microbiota from the 52 sampled *M. decora* was very similar to that of *H. verbana* in that it is dominated by *Bacteroides*, *Aeromonas*, and four Clostridiales spp (Table 1 and S2). *Aeromonas* and *Bacteroides* sequences made up 16 – 73.5% (median = 51%) of the *M. decora* ILF microbiota while the Clostridiales spp accounted for another 22 – 98%. A *Bacteroides-like* OTU was present in *H. verbana* ILF at 0 – 2.1% of sequences (63% of animals) and sequences from only one of these Clostridiales species (*Alkaliphilus-like)* had been reported before from the *H. verbana* intestinum (14) (Table 1 and S2). To our knowledge, the remaining three Clostridiales species (*Papillibacter-like, Clostridium,* and *Butyricicoccus*) have never been identified from any site in *Hirudo* species.

Sequences from common OTUs (found in at least 70% of samples) in the *M. decora* ILF included one additional Bacteroidaceae and three Clostridiales (Table S2). These four OTUs made up an additional 0.6 – 12.8% of the ILF microbial community (median = 4.6%). The presence of more Clostridiales spp in the ILF microbiota is the primary cause for the observed increased alpha diversity in *M. decora* over *H. verbana* (Figure 2A). An increase in alpha diversity between *H. verbana* and *M. decora* ILF microbiota may have been a result of a 'zoo effect' in comparing farmed (*H.verbana*) to newly captured (*M. decora*) animals, an effect that has been well documented in mammals (36). This hypothesis will be addressed below when comparing the effect of feeding on the microbiota.

254

255 Microbiome of the Intestinum is Similar to that of the ILF

256 The intestinum microbiota from nine *H. verbana* samples was dominated by sequences from four
257 OTUs: *Insolitispirillum-like* (0.2 – 34.6%, median = 25.4%), *Aeromonas* (2.3 – 24.7%, median =
258 16.6%), *Mucinivorans* (2 – 51.3%, median = 9.3%), and *Desulfovibrio* (1.7 – 15.3%, median =
259 4.1%) (Table 1 and S2). *Aeromonas* and *Mucinivorans* were also dominant members of the ILF
260 community. Sequences from the *Insolitispirillum-like* and *Desulfovibrio* OTUs have been
261 previously identified in the intestinum of *H. verbana* and *H. orientalis* (19, 20). The difference in
262 microbial community between the *H. verbana* ILF and intestinum was marked by changes in the
263 relative abundance of sequences from both dominant and common OTUs: *Insolitispirillum-like*
264 (16.9 $\log_2$ fold change, $p < 0.001$), *Rikenella-like* (14.5 $\log_2$ fold change, $p < 0.001$),
265 *Aquaspirillum-like* (13.8 $\log_2$ fold change, $p < 0.001$), *Mucinivorans* (-2.6 $\log_2$ fold change, $p <$
266 0.001) and *Proteocatella* (-11 $\log_2$ fold change, $p < 0.001$). The change in microbial composition
267 between the ILF and intestinum may be directly related to the differences in organ functions
268 between the two (erythrocyte storage vs meal digestion respectively (1)). The almost doubling of
269 OTUs that were present in the intestinum versus those found in the ILF is consistent with
270 previous reports (14, 19) of an increase in alpha diversity between the two organs in *Hirudo* spp
271 (Figure 2B) and confirms that microbial colonization of the ILF is closely regulated.

272

273 In contrast, in *M. decora*, the alpha diversity between ILF and intestinum samples did not change
274 significantly (Figure 2). The dominant OTUs of both ILF and intestinum from *M. decora* were
275 *Aeromonas, Bacteroides*, *Butyricicoccus,* and *Alkaliphilus-like* (Table 1 and S2). In the
276 intestinum (45 animals sampled), these OTUs accounted for 52.9 – 96.7% of the total sequences

277 while in the ILF, these OTUs accounted for 73.2 – 98.8% of the sequences (Table 1 and S2).
278 Sequences from common OTUs (found in at least 70% of samples) in the *M. decora* intestinum
279 included additional *Bacteroides*, *Proteocatella-like, Desulfovibrio,* and two *Alkaliphilus-like*
280 OTUs. An explanation for the observation that the microbiome of *M. decora* ILF is not as
281 reduced as that observed in *H. verbana* may a result of maintaining or raising the animals in a
282 farm.
283
284 The dominant *Alkaliphilus*-like OTU from *M. decora* was previously found in the *H. verbana*
285 intestinum (14) but was detected only in three intestinum and three bladder samples from *H.*
286 *verbana* processed in this study. All of the other OTUs common in the *M. decora* intestinum
287 were not found in *H. verbana* samples. In the intestinum, like in the ILF, OTUs defined to the
288 genus level are not the same but when identified to the order level community composition is
289 conserved.
290
291 <u>*H. verbana* Bladder Community Contains Three Core OTUs</u>
292 16S rRNA V4 deep sequencing of bladders from ten *H. verbana* animals revealed a more
293 complex community than had been previously reported through RFLP and 16S rRNA gene
294 sequencing (30). Kikuchi et al reported the *H. verbana* bladder contains a stratified community
295 including Alphaproteobacteria *(Ochrobactrum),* Betaproteobacteria *(Comamonas-like &*
296 *Sterolibacterium-like),* Deltaproteobacteria *(Bdellovibrio),* and Bacteroidetes *(Niabella &*
297 *Sphingobacterium)* with *Ochrobactrum, Comamonas-like, Bdellovibrio, Niabella,* and
298 *Sphingobacterium* found in > 90% of animals tested (30). The deep sequencing carried out in the
299 current study on bladders from ten animals expanded these results by identifying

Alphaproteobacteria *(Ochrobactrum, Aminobacter, Ensifer, Insolitispirillum-like, Phreatobacter-like),* Betaproteobacteria *(Ramlibacter, Pelomonas, Variovorax, Acidovorax, Ralstonia),* Deltaproteobacteria *(Bdellovibrio-like* and *Desulfovibrio),* Bacteroidetes *(Niabella, Pedobacter,* and *Flavobacterium),* and *Spirochaetes (Spirochaeta-like)* in *H. verbana* bladders although only *Ochrobactrum, Ramlibacter* (previously *Comamonas-like*), *Bdellovibrio-like,* and *Pedobacter* were recovered frequently enough (in ≥ 90% of samples) to be considered part of the core (Table 2).

The increase in OTUs identified in the *H. verbana* bladder was likely due to sequencing three times as many animals as in the previous study as well as sequencing to a much greater depth. The expanded community contains sequences from an additional four Alphaproteobacteria, three Betaproteobacteria, one Deltaproteobacteria, and one Spirochaete. Comparing the 16S rRNA V4 sequences to the 16S rRNA sequences produced by Kikuchi et al, indicates that the *Ochrobactrum, Ramlibacter*, *Niabella,* and *Pedobacter* (previously *Sphingobacterium*) OTUs are the same between the two studies (Table S2). However, the previously-identified *Sterolibacterium-like* and *Bdellovibrio* OTUs did not have any similar sequences in the new dataset. The increased number of identified OTUs in conjunction with the small number of OTUs identified as core suggests that there is large variation in the minor members of the bladder communities between individual animals. Further research would be required to determine if this large variation is also apparent between bladders of an individual animal.

<u>*M. decora* Bladder Community</u>

322 The 16S rRNA V4 sequencing of *M. decora* bladder from 20 animals identified sequences
323 belonging to *Alphaproteobacteria (Ochrobactrum, Ensifer, Rhizobium, Rhizobium-like,*
324 *Azospirillum, Phreatobacter-like, Sphingomonas, Rhodopseudomonas),* Betaproteobacteria
325 *(Ramlibacter, Methylopumilus-like, Bacteriovorax-like, Pandoraea,* and a Rhodocyclaeae sp),
326 and *Deltaproteobacteria (Bdellovibrio, Cystobacter-like, Desulfovibrio,* and *Bacteriovorax-like).*
327 Of these identified taxa, only *Ramlibacter, Methylopumilus, Phreatobacter-like,* and
328 *Azospirillum* were considered core (Table 2). The *Ramlibacter, Ochrobactrum,* and
329 *Sphingobacterium* OTUs sequenced from *M. decora* are the same as those identified from *H.*
330 *verbana* bladders (30) (Table S2) and suggest a conservation of bladder symbionts at the genus
331 level (97% sequence similarity) despite geographic and evolutionary separation.
332
333 Surprisingly, *Aeromonas*, *Bacteroides,* and Clostridial species were also detected in the bladder
334 sequences from *M. decora* and *Aeromonas*, *Mucinivorans,* and Clostridial species were identified
335 in the bladder sequences from *H. verbana*. Two possibilities seem likely to be responsible for
336 this observation: (i) ILF symbionts are also found in the bladder and nephridia or (ii)
337 contamination from the ILF occurred during dissection, PCR, or sequencing (37). The
338 contamination seemed especially likely in animals that had been recently fed as the filled crop is
339 easily punctured during dissection. In addition, previous data from *H. verbana* bladder
340 communities suggested that it was unlikely that these two dominant ILF taxa were present in the
341 bladder (30).
342
343 In an effort to confirm the 16S V4 rRNA deep sequencing results, FISH was used to detect
344 specific taxa in the bladder. Unexpectedly, low levels of Aer66-binding cells were detected in

cells lining the *M. decora* bladder/nephridia of animals 4 and 7 days after feeding (Figure 3). While this observation confirms the deep sequencing results, it raises questions to the origin of these *Aeromonas* cells. A SILVA probeCheck (38) of the Aer66 probe confirms that it should be specific for *Aeromonadaceae*, interestingly, there is only a single base pair mismatch to the *Ochrobactrum* sequence. Thus, it remains unclear if this probe is binding to the highly abundant *Ochrobactrum* or *Aeromonas*. Because the Bacteroidetes probe used in this study also targets *Pedobacter* and *Flavobacterium* and may cross-react with *Phreatobacter-like*, we cannot conclusively determine whether the Bacteroidetes symbiont exists within the *M. decora* bladder.

Physically Stratified Bacterial Community in *M. decora* Bladders

FISH imaging of *M. decora* bladders suggests that most Alphaproteobacteria (*Ochrobactrum* and *Phreatobacter-like*) occur intracellularly in the epithelial cells lining the interior of the bladder, while Rhizobiaceae (*Aminobacter* and *Ensifer*) occur in the matrix of the bladder contents. Betaproteobacteria (*Methylopumilus-like* and *Ramlibacter*) occur in close association with the *M. decora* bladder epithelial cells (Figure 3). This layered community is consistent with localizations reported previously in *H. verbana* bladders (30). Together, this data confirms that the *M. decora* bladder community composition and distribution are similar to those of *H. verbana* despite differences in specific OTUs and suggests an evolutionary conservation between these hosts and their symbionts.

Seasonal Changes Affect the Wild ILF Microbiome

The *M. decora* animals used in this study allowed us to evaluate how a wild leech's microbiome changes during the year. Animals collected during warm months (June-September) had similar

ILF and intestinum microbiomes. However, animals collected in October (the beginning of cold months) contained significantly different ILF microbiomes from those collected in April (end of cold months) or during warm months ($p = 0.003$) (Figure 4A). Those collected in April showed the least variation between individuals.

Among intestinum samples, those from animals collected in April were significantly different from those collected in October or during warm months ($p = 0.003$ and $p = 0.009$ respectively) (Figure 4B). However, the difference in intestinum microbiome between animals collected in warm months were not significantly different from those collected in October ($p = 0.123$). The seasonal difference in the microbiome composition was detected over multiple years with animals collected during warm months being more similar to those collected in warm months of other years than to those from animals collected in April or October of the same year.

Feeding Affects the ILF Bacterial Community

Sanguivorous leeches are opportunistic ectoparasites that consume several times of their body weight in a single feeding and rest for months between feeding events (7). Following a feeding event, the abundance of *Mucinivorans* and *Aeromonas* in the *H. verbana* ILF increases (13, 15)). Monitoring of bacterial communities after a laboratory-administered, sterile sheep blood meal in *M. decora* revealed a significant decrease in alpha diversity in ILF by 2 days after feeding (DaF) that rebounded by 30-90 DaF (Figure 6). At 2 and 4 DaF the communities in the ILF were significantly different from those at any other time after feeding ($p = 0.009$ and $p = 0.003$ respectively). A similar change in community was observed in *H. verbana* where *Mucinivorans* and *Aeromonas* populations increased after feeding (15). Interestingly, *M. decora* seemed to be

391 divided into two groups, those that recovered quickly (by 4 – 7 DaF) and those that recovered
392 more slowly (> 7 DaF). These groups can be seen in the dichotomy in the violin plot of Bray-
393 Curtis distances presented in Figure 6B.
394
395 By 30 DaF, the alpha diversity and common members of the ILF communities of both *H.*
396 *verbana* and *M. decora* returned to levels indistinguishable from those in unfed animals (Figure
397 5 and 6). This would suggest that a single blood meal and maintenance in the artificial lab
398 environment was not sufficient to significantly change the composition of the gut community.
399 However, it should be noted that animals maintained for longer than 90 days before sampling did
400 appear to have a reduced variation between individuals (data not shown). This would suggest
401 that a zoo effect is minimal, although a slight decrease in biodiversity from reduction in the rare
402 microbiome members should be anticipated, as has been described in mammals (39). The
403 abundance of sequences from dominant members of the gut microbial community overall
404 appeared to return to levels comparable to those of unfed animals within 30 DaF (Figure 5).
405
406 The dominant *Bacteroides* and *Aeromonas* symbionts identified by deep sequencing were
407 additionally observed through FISH imaging. Immediately after capture, the microbial
408 population in the *M. decora* crop was below the limit of detection for FISH, although rare
409 *Aeromonas* cells were occasionally found (Figure 7). After feeding a blood meal the microbial
410 population increased at 4 and 7 DaF (Figure 7) with Bacteroidetes forming large microcolonies
411 by 4 DaF and *Aeromonas* pelagically spread throughout the crop by 7 DaF. A similar pattern
412 was previously observed and quantified in *H. verbana* (15). Interestingly, some *Aeromonas* at 7
413 DaF were found associated with eukaryotic cells within the crop. These cells are likely

414 circulating immune cells from the leech host. Phagocytosis of *Aeromonas* by leech hemocytes
415 was previously observed in *H. verbana* when the *Aeromonas* strain lacked a functioning T3SS
416 but not for the wild type (40).
417
418 Feeding Does not Affect the Intestinum Bacterial Community
419 Unlike the bacterial community present in the ILF, the intestinum bacterial community appears
420 to be much more stable. No significant change between any of the days after feeding were
421 observed ($p \geq 0.15$). This suggests that growth of any single member of the community is
422 matched by other members. More likely, the intestinum may be a more constant environment
423 with a regular inflow and outflow that is not as much affected by sporadic feeding events. It is
424 also possible that the intestinum serves as a reservoir from which new blood meals may be
425 seeded with symbionts after ingestion.
426
427 Geographic Location
428 Because *M. decora* were collected from multiple ponds in nature, we were able to compare the
429 microbial communities of animals from the same species but distant geographic locations. There
430 was no significant difference between microbial communities in the ILF of *M. decora* from CT,
431 MA, NY, and VT ($p \geq 0.222$) (Figure s1). Statistical analyses on geographic effects were
432 performed only on samples from MA and CT due to the lower sample number for samples from
433 NY and VT.
434
435 The microbiome from the intestinum of MA animals was significantly different from that of CT
436 animals ($p = 0.006$), but interestingly the ILF microbiome was indistinguishable between the two

leech populations (Figure s1). The difference in microbial communities between the ILF and intestinum was significant in animals from MA ($p = 0.006$), but not in animals from CT ($p = 0.084$), and was associated with a decrease in *Alkaliphilus-like* ($p = 0.022$) and increase in *Papillibacter-like* ($p = 0.001$) OTUs. In the ILF, these OTUs make up ~ 8.2% of the total ILF sequences while in the intestinum they make up less than 1.6%. This difference between ILF and intestinum microbiota is almost imperceptible in animals from CT. The slight differences in Clostridiales species between MA and CT animals suggests a site-specific variation in less abundant community members within *M. decora*, however the site-specific dominant symbionts and core community remained the same and indicate a conservation of symbionts despite geographic separation.

DISCUSSION

In this study we have used deep sequencing of the V4 region of the 16S rRNA gene and FISH to characterize the microbiome from three organs in the North American medicinal leech, *Macrobdella decora*. At the taxonomic level of order, the microbiomes described from wild-caught *M. decora* appeared very similar to those from *Hirudo verbana*. However, at the genus

460 level, the microbiomes were easily differentiated between the two host leech species. This is
461 similar to findings in other animal models where often the host-specific genera show an
462 evolutionary pattern that mimics that of the host's evolution (41-45).
463
464 In both leech species, the gut microbial communities are dominated by *Aeromonas,*
465 Bacteroidales, and Clostridiales species. This is consistent with previous findings that
466 Bacteroidetes and *Aeromonas* are common but not obligate in the gut of animals and insects fed
467 on blood (46-51). Siddall et al. have shown that many sanguivorous leeches maintain a gut
468 symbiosis with *Aeromonas* and Bacteroidetes species (8). Because of the difficulty of
469 distinguishing *Aeromonas* species based solely on 16S sequencing data (52, 53), we are unable to
470 determine whether the *Aeromonas* species from *M. decora* is the same as that from *H. verbana*.
471
472 In-depth genetic analyses of an *Aeromonas veronii* strain isolated from *H. verbana* has shown
473 that the ability to grow in blood is not sufficient for leech gut colonization, but rather that a
474 number of colonization factors such as secretion systems (54), carbon starvation response (55),
475 oxidative stress response inhibition (7), and heme acquisition processes (56) are critical in
476 enhancing leech gut colonization. New studies comparing genomic variation between
477 *Aeromonas* strains isolated from sanguivorous leech species to those of non host-associated
478 environmental strains may be used to further identify genes critical for host colonization and
479 persistence.
480
481 In addition to confirming a similar core and common microbiome between the two leech hosts,
482 our study also identified a rich rare microbiome. Previous research in a variety of environments

has concluded that although a relatively small number of OTUs may dominate samples, low-abundance populations may be responsible for driving changes in observed phylogenetic diversity (57, 58). Rare species may have a significant role in metabolic cycles and may be a hidden driver of ecosystem functioning (59). Because dominant and core microbes are easier to identify and predict, rare microbes may be overlooked keystone species responsible for regulating the community's development and function (59). In our study, the ability to examine an increased number of animals, to examine three body sites from each animal, and to sequence to a much greater depth resulted in the detection of an increased level of inter-animal variations in the less abundant microbiota and suggest a greater diversity in the rare microbiome than previously predicted.

We were also able to observe the effect of geographic isolation on the less abundant members of the gut microbial community in *M. decora*. The very slight but statistically significant differences in community composition between animals from MA and CT indicated that the relationship with dominant symbionts is consistently conserved despite geographic separation of ~100 km. It is possible that this is due to a small sample size as we compared 13 unfed *M. decora* from MA and seven from CT and previous studies of synthetic and natural microbial communities found that insufficient sampling could yield an artificial difference between geographically separated communities (60, 61). As has been noted previously in the termite gut (58), the difference in community composition is based on minor members of the community and is reminiscent of a similar pattern observed in community differences between suppliers of *H. verbana* (data not shown). We therefore hypothesize that the conserved members of the

505 community have been evolutionarily maintained in hirudinid leeches despite species-
506 diversification and continent-dividing events.
507
508 Not only was the core gut microbiome similar between *M. decora* and *H. verbana*, but also the
509 dynamic response of these symbionts in response to feeding a blood meal. FISH imaging
510 confirmed similar colonization patterns of the ILF of *Aeromonas* and Bacteroidetes from *H.*
511 *verbana* and *M. decora*. Although part of the decrease in alpha diversity in the leech ILF
512 microbiome post blood meal consumption may be due to innate immune properties of the blood
513 meal (62), in a number of vertebrate species it has also been shown that extreme feeding or
514 fasting events do result in decreased alpha diversity (63, 64). In *H. verbana*, it has been
515 suggested that the *Aeromonas* symbiont may provide additional antimicrobial peptides
516 responsible for restricting bacterial colonization in addition to host-produced antimicrobial
517 peptides (65). Because the core community and post-feeding community dynamics are similar in
518 *M. decora*, it is reasonable to assume that a similar process may also occur in this host species.
519
520 While our laboratory-maintained animals were tracked for 30-90 days after feeding, in the wild
521 leeches are thought to go for even longer periods of time between feedings as the animals are
522 inactive during the cold months. This extended period between feedings likely resulted in a
523 starved phenotype in animals collected in April. Our results are consistent with the long
524 starvation period occurring during the cool months affecting the gut microbial community of *M.*
525 *decora* and the relative nimiety of the warm months where feedings may occur regularly. The
526 observed seasonal effect may also be driven by the sequencing of transient environmental

527 microbes from within the leech crop, as has been observed in a recent limited survey of wild *H.*
528 *verbana* (17).
529
530 Changes in gut microbial populations as a result of irregular feeding events has been observed in
531 other animals too. Other researchers have observed Clostridial populations increase rapidly in
532 response to feeding while Bacteroidales numbers increase at a slower rate and high levels of
533 Bacteroidales or Rikenellaceae are indicative of long periods between feedings in avian,
534 reptilian, and mammalian hosts (63, 64, 66). In our experiments, a higher numbers of
535 Bacteroidales sequences were found in the ILF of *M. decora* collected in April when compared
536 to those collected in June-October. The proportion of Bacteroidales sequences additionally
537 decreased in leeches immediately after feeding (when the number of Clostridiales sequences
538 increased). FISH imaging did indicate that Bacteroidales increase in number four days after
539 feeding, suggesting that the change in sequence ratio is due to a combination of rapid replication
540 of Clostridiales and a much slower replication of Bacteroidales. This consistency with
541 community dynamics observed in other animal models then suggests that leeches may be good
542 models to predict microbial changes due to intermittent feedings or extended periods of
543 starvation.
544
545 In addition to the conservation of *Aeromonas*, Bacteroidetes, and Clostridial gut symbionts, the
546 bladder taxa *Comamonadaceae, Ochrobactrum,* and *Nubsella* appear to have also been
547 evolutionarily maintained within Hirudiniform leech bladders. They are common in annelid
548 bladders as are *Bordetella, Methylophilus, Achromobacter, Variovorax, Azospirillum,*
549 *Mesorhizobium, Phyllobacterium, Rhizobium, Desulfovibrio, Pedobacter, Spirochaeta* (28). The

550 taxa from the bladders of both *H. verbana* and *M. decora* were very similar to those found in
551 other annelids, which include Alphaproteobacteria *(Ochrobactrum, Azospirillum,* Brucellaceae,
552 Rhizobiaceae, Rhodospirillaceae, & Bradyrhizobiaceae), Betaproteobacteria (Comamonadaceae,
553 Methylophilaceae, & Rhodocyclaceae) and Bacteroidetes *(Pedobacter)* (28, 29). This
554 observation suggests an even greater conservation than that observed in digestive symbionts as
555 the conservation has occurred at the genus level in medicinal leeches and at the family level over
556 multiple evolutionary events in the annelids.
557
558 The *Ramlibacter* (previously *Comamonas-like*), *Ochrobactrum,* and *Pedobacter* (previously
559 Sphingobacterium) OTUs sequenced from *M. decora* are the same as those identified from *H.*
560 *verbana* bladders (30). However, the *Pedobacter* sequenced in this study does not seem to be the
561 same that Ott isolated from the mucus castings of adult *H. verbana* (67). This may indicate a
562 difference between the bacteria on the outside of the leech and those adapted to colonize the
563 bladder of the leech, as has been observed in the specialization of *Aeromonas* strains inside the
564 leech gut (27, 54-56, 68). Further research is required to determine how these two leech species
565 are able to maintain such specific bladder symbioses and by what means the symbionts are
566 efficiently transmitted between animals.
567
568 Based on the deep-sequencing absence of *Niabella, Pedobacter, Ramlibacter*, *Ensifer,*
569 *Phreatobacter,* and *Aminobacter* sequences in ILF and intestinum samples, the low abundance of
570 *Ochrobactrum* sequences in ILF samples (max = 0.4%), and the absence of any ALF968-,
571 Rhiz1244-, or Bet42a-binding cells in FISH images (data not shown) we hypothesize that these
572 genera are specific to the bladder. Presence of these OTUs in an ILF sample might be due to

573 contamination during dissection or a diseased state of the animal. This has occurred in previous
574 studies where bladder-specific symbionts were found in the ILF and intestinum of 3 wild *H.*
575 *verbana* (17) and *Pedobacter* and *Ochrobactrum* were cultured from intestinum samples (14,
576 69). The fact that, in the Worthen study, *Pedobacter* and *Ochrobactrum* clones were only
577 recovered from recently-fed animals supports the possibility of sample contamination during
578 dissection (recently fed animals have larger bladders). Because of the friability of leech tissue
579 and the examples of bladder symbionts appearing in ILF and intestinum samples, careful
580 dissection technique is critical to reduce contamination during sample acquisition.
581
582 Our research has identified a conservation of gut and bladder symbioses in medicinal leech
583 species from Europe and North America. While the community structure of *H. verbana* and *M.*
584 *decora* microbiomes have specific similarities, the differences that exist between the two can be
585 exploited in the future to gain a better understanding of host/symbiont co-evolution. Not only are
586 the gut symbionts conserved, but their dynamics in response to a blood feeding are similar as
587 well. From our data we can hypothesize that the gut microbiome composition is affected by
588 periods of starvation and torpidity. In *H.verbana*, the simple gut community helps to digest the
589 blood meal (7) and protect the host from invading bacteria (65). It is likely that the microbial
590 communities in the gut of *M. decora* perform very similar functions. Future work should focus
591 on isolating dominant members of both symbioses to compare conserved metabolic and
592 colonization abilities and to test for host species specialization. Through understanding
593 molecular and mechanistic studies enabled by simple systems, especially in evolutionarily
594 conserved biological processes and metabolic capabilities, one can form predictions that are
595 more challenging to test in more complex communities.

METHODS

Animals

M. decora were collected from ponds located in Storrs, CT (41°49'3.074"N, 72°15'32.704"W), Groton, MA (42°35'26.993"N, 71°32'25.63"W), Caroga, NY (43°11'28.684"N, 74°28'10.026"W), and Mount Snow, VT (42°58'25.3"N, 72°55'47.9"W). If sacrificed within 1 week of collection, animals were maintained in native pond water.

H. verbana were purchased from Leeches U.S.A. (Westbury, NY U.S.A.) and BBEZ (Biebertal, Germany) and maintained as described below.

Husbandry

Leeches were maintained in circular tanks (up to 10 animals/tank) in an environmental chamber with 12/12h day/night cycles at 25/23°C respectively. Sterile dilute instant ocean (DIO) consisting of 34 mg/L instant ocean salts (Aquarium Systems, Inc Mentor, OH U.S.A.) in nanopure water was changed weekly or when the animals vomited or an animal in the tank died. Tanks contained a minimum of one autoclaved rock.

Tanks were cleaned by completely emptying the old water and performing multiple complete, small volume, water changes until water remained clear for at least 20 min after the last change. Tanks were then filled to previous level and returned to environmental chamber.

Feeding

618 Animals were fed as described previously (4). Sheep blood with heparin anticoagulant was
619 purchased from Lampire Biological Laboratories (Pipersville, PA U.S.A.). Animals were fed in
620 groups of 1-3 animals on sterile 50 mL Falcon tubes containing 30 mL sheep blood, warmed to
621 37°C, and covered with Parafilm (American National Can, Greenwich, CT U.S.A.). All animals
622 were allowed to feed to satiety and then let sit in sterile DIO for ~30 min before handling after
623 feeding.
624
625 Dissection
626 Animals were removed from the tank and the anterior end was tied with string to inhibit
627 regurgitation before narcotizing in 70% ethanol (13). Animal was then treated with RNase away
628 and rinsed with molecular biology grade water. An additional string was tied around to bissect
629 the animal into anterior and posterior fractions.
630 Bladder: A small primary incision was made immediately lateral to the dorsal lateral dextral
631 stripe and immediately posterior to the second string. Bladders were identified and carefully
632 dissected so as to minimize contamination from ILF. Bladders were briefly rinsed in sterile
633 phosphate buffered saline, PBS, then placed in sterile bead-beating tube. Each sample consisted
634 of 1-3 bladders from the same leech. In this manuscript, the term 'bladders' includes bladders
635 and the attached nephridia.
636 ILF: To access the crop contents, a secondary central incision was made immediately below the
637 second string. Approximately 100 μL ILF was collected as it was released and placed in a sterile
638 bead-beating tube. In animals with especially low volumes of ILF, a sterile pipette tip was used
639 to gently scrape the inside of the crop.

Intestinum: To access the intestinum, a second incision was made immediately lateral to the anus. The intestinum was carefully dissected from the anus and consisted of ≥1cm intestinum with contents. Samples were placed in bead-beating tubes or 1.5 mL microcentrifuge tubes and snap frozen in liquid nitrogen before storage at -80°C.

Tissue Fixation and Embedding

After bladder, ILF, and intestinum sample were collected a fully bissecting incision was made immediately posterior to the second string. The anterior portion of the animal was placed in methacarn (6:3:1 methanol:chloroform:acetic acid) (70). Tissues in methacarn were stored at 4°C with rocking. Regular fixative changes were made when the fixative was no longer clear. Tissues were dissected and a final fixative change was performed with fresh anhydrous methacarn before incubating for one additional week, 4°C with rocking. The anhydrous methacarn was replaced with anhydrous methanol and the tissues stored at 4°C until embedding. Tissues were cleared with a decreasing methanol:xylene series then embedded in Paraplast Plus® (Millipore Sigma, St. Louis, MO U.S.A.).

Fluorescence *In-Situ* Hybridization (FISH)

Paraffin-embedded tissues were sectioned at 6-8 μm using a microtome and placed on poly-L-lysine-coated slides. Sections on slides were cleared with xylene then rehydrated with an ethanol:water series. Slides were bleached for 8-12 h using 2% hydrogen peroxide and a standard fluorescent light bulb (with concomitant cooling over an ice bath). 0.54M NaCl, 12mM Tris-Cl, 30% formamide, and 1.2% sodium doecyl sulfate with 1μM Cy3 probe, 1μM Cy5 probe, and 3μM Eub338. Slides were observed using a Nikon A1R microscope (laser wavelengths 405nm

(DAPI), 488nm (Alexa488), 558nm (Cy3), 640nm (Cy5)) and images processed using ImageJ 1.51s (71).

Bead-beating DNA Extraction

Modified version of previous protocol published by Yu et al (72). 300 μL cell lysis buffer was added to sample in bead tube containing 0.1mm and 0.5mm zirconia/silica beads (BioSpec Products, OK, U.S.A.) beads. Samples were beaten for 90 sec and briefly centrifuged before transferring supernatant to clean 1.5 mL microcentrifuge tube. An additional 150 μL lysis buffer was added to the bead tube and sample was beaten again 90 sec. The supernatant previously removed was returned to bead tube and incubated for 15min at 56°C with 5 sec vortexing every 4 min. The sample was briefly centrifuged before adding 100 μL 10 M ammonium acetate at 4°C and vortexed 15 sec before incubating for 10 min on ice. The sample was centrifuged for 10min at 16 000 x g. The supernatant was transferred to clean 1.5mL MCF tube and to which 350 μL ethanol at 4°C was added before vortexing for 10 sec and transferring the supernatant to a QIAamp Mini spin column (Qiagen Germantown, MD U.S.A.). The sample was process as recommended in the QIAamp DNA Mini Handbook and eluted with 10 mM Tris-Cl, pH 8.5. The eluted DNA concentration was measured using Qubit™ dsDNA HS Assay Kit (Thermo Fisher Scientific Carlsbad, CA U.S.A.). Stored at -20°C.

MasterPure DNA Extraction

MasterPure complete DNA extraction without column purification into 50μL TE was performed according to manufacturer's protocol (Epicentre Madison, WI U.S.A.). No significant difference was found between the two extraction methods (PERMANOVA: $p \geq 0.23$).

686

687 Sequencing and Initial OTU Picking

688 Extracted DNA samples were analyzed by amplifying the V4 hypervariable region of the 16S
689 ribosomal RNA (rRNA) gene using primers designed in (73). PCR reactions were prepared as in
690 (58) and sequenced using an Ilumina MiSeq (Illumina San Diego, CA U.S.A.) with custom
691 sequencing primers added to the reagent cartridge (73) and sequenced 2 × 250bp. The sequence
692 data was deposited in the NCBI SRA under project ID PRJNA544194.

693

694 Resulting community sequences were processed using MacQiime as outlined in (58) using a
695 GreenGenes reference library (2013-08 release). Sequences not clustered were identified using
696 the Ribosomal Database Project (74) to the lowest possible taxonomic level.

697

698 Bacterial Community Analysis

699 Resulting sequencing data was analyzed using MacQiime and R as described below. Complete
700 coding is available in supplemental materials and via GitHub:
701 https://github.com/joerggraflab/McClureE_Md2019/. (32, 75)

702

703 Sample Selection: Samples with 1) fewer than 10,000 reads or 2) fewer than 3 OTUs were
704 excluded from analysis.

705

706 Positive Controls: Two types of positive control were prepared and sequenced. 1) Amplification
707 of a dilution series of a ZymoBIOMICS Microbial Community DNA Standard (Zymo Research,
708 Irvine, CA U.S.A.), 2) Some samples were amplified multiple times with different PCR primers

709 and on different runs to confirm the reproducibility of the data (data not shown). For samples
710 amplified and sequenced multiple times, the sample with the most reads was used for analysis.
711
712 Negative Controls: Two types of negative controls were prepared and sequenced. 1) Reagent
713 controls were prepared by performing the DNA extraction procedure using the same reagents
714 without any sample. The resulting DNA yields for these reagent controls after extraction were
715 always below the limit of detection for the Qubit dsDNA High-Sensitivity Assay. 2) Negative-
716 PCR controls were prepared by performing V4-specific PCR amplification on molecular biology
717 grade water. The resulting reactions produced no bands when analyzed with the QIAxcel DNA
718 Fast Analysis cartridge (Qiagen Germantown, MD U.S.A.).
719
720 After sequencing, each negative control contained less than 2,000 reads. Negative controls were
721 combined and compared to identify contaminating OTUs using maxNeg and meanNeg. maxNeg
722 was defined as the maximum count of a single OTU found in a negative control and was
723 determined to be 379 reads. meanNeg was defined as the mean count of all OTUs when found in
724 negative controls and was determined to be 4 reads. OTUs were first restricted by requiring that
725 each OTU be present in at least 1 sample with a read count >= maxNeg. This resulted in a
726 dataset consisting of 158 OTUs.
727
728 Contaminating OTUs were further identified through the use of the decontam package (76).
729 After removal of contaminants identified by decontam OTUs, the dataset consisted of 157 OTUs,
730 indicating that most contaminant OTUs were found at counts <= maxNeg and so were not
731 considered as community members.

732

733 After initial trimming, all samples contained a minimum of 10,000 reads (maximum = 375,834,
734 minimum=10,287, median=62,015).

735

736 Effect of Extraction Method: A PERMANOVA analysis using the Bray-Curtis metric was
737 performed through the adonis function of the vegan package (77) to determine the effect of
738 extraction method. No significant difference was found between the two extraction methods
739 (PERMANOVA: $p \geq 0.23$).

740

741 Effect of Leech Species: A PERMANOVA analysis using the Bray-Curtis metric was performed
742 through the adonis function of the vegan package (77) to determine the effect of leech species.
743 This analysis was performed twice 1) with taxa split into original OTUs as determined by Qiime
744 and 2) with taxa agglomerated at the order level (using tax_glom function of the phyloseq (75)
745 package).
746 Non-metric Multidimensional Scaling (NMDS) plots were prepared using the distance, ordinate,
747 and plot_ordination function of the phyloseq package (75). Distances were calculated with the
748 Unifrac method (78).
749 Variation within *H. verbana* and *M. decora* was evaluated separately with a PERMANOVA
750 analysis using the Bray-Curtis metric through the adonis function of the vegan package (77).
751 Sample groups were probed for the effect of sampled organ, feeding, animal supplier/source, and
752 shipment/collection date.

753

754 Core and Common OTUs: The average read count of a single OTU in any negative control was
755 calculated to be 4 (meanNeg). This number was then used to calculate a conservative estimate
756 for the minimum fraction of a sample that an OTU must compose to be considered present in that
757 sample. It was assumed that any true OTU would contain a read count greater than or equal to
758 meanNeg, so in the smallest sample with 10,287 total reads, an OTU must contain at least 4
759 reads or .03% of the total community in order to be considered present. The Core community
760 was defined to consist of only those OTUs present in at least 90% of samples in a group. The
761 Common community was defined to consist of only those OTUs present in at least 70% of
762 samples in a group.
763
764 Alpha Diversity: Alpha diversity was calculated using the Shannon metric in the plot_richness
765 function of the phyloseq package (75). Alpha diversity was calculated for ILF and intestinum
766 samples separately.
767
768 Effect of *M. decora* Collection Month: A PERMANOVA analysis using the Bray-Curtis metric
769 was performed through the adonis function of the vegan package (77) to determine the effect of
770 collection month/season on *M. decora* samples. This analysis was performed twice 1) with ILF
771 samples and 2) with intestinum samples.
772 NMDS plots were prepared using the distance, ordinate, and plot_ordination function of the
773 phyloseq package (75). Distances were calculated with the Unifrac method (78).
774
775 Plotting gut community over time: For each time point after feeding, taxa were assessed for
776 presence (≥ 0.1% of total reads) or absence. At any time point, if the taxon was determined to be

present in ≤ 1 samples, it was considered absent from that time point. Ggplot2 was used to produce a stat_boxplot with whiskers at 1.5x interquartile range.

ACKNOWLEDGEMENTS

This research was supported by NIH R01 GM095390 to J. Graf, P. Visscher, and H. Morrison, NSF 1710511 to J. Graf and V. Cooper and the University of Connecticut. We would like to thank R. Rubinstein for help with Python code used to prepare tables and the Microbial Resources and Services Facility of the University of Connecticut for sequencing the samples. J. Graf is a leech microbiology consultant for the German leech farm Biebertaler Blutegelzucht GmbH, Biebertal, Germany, and the company does not direct or approve J. Graf's research and publications.

REFERENCES

1. Sawyer RT. 1986. Leech biology and behavior. Clarendon Press, Oxford.
2. Brinkmann A, Jr. 1947. Two new antarctic leeches. Nature 160:756.
3. Whitaker IS, Rao J, Izadi D, Butler PE. 2004. Historical Article: *Hirudo medicinalis*: ancient origins of, and trends in the use of medicinal leeches throughout history. Br J Oral Maxillofac Surg 42:133-7.
4. Graf J. 2000. The symbiosis of *Aeromonas* and *Hirudo medicinalis*, the medicinal leech. ASM News 66:147-153.
5. Siddall ME, Trontelj P, Utevsky SY, Nkamany M, Macdonald KS. 2007. Diverse molecular data demonstrate that commercially available medicinal leeches are not *Hirudo medicinalis*. Proc Biol Sci 274:1481-1487.

6. Utevsky SY, Trontelj P. 2005. A new species of the medicinal leech (Oligochaeta, Hirudinida, Hirudo) from Transcaucasia and an identification key for the genus Hirudo. Parasitol Res 98:61-6.

7. Marden JN, McClure EA, Beka L, Graf J. 2016. Host Matters: Medicinal Leech Digestive-Tract Symbionts and Their Pathogenic Potential. Front Microbiol 7:1569.

8. Siddall ME, Min GS, Fontanella FM, Phillips AJ, Watson SC. 2011. Bacterial symbiont and salivary peptide evolution in the context of leech phylogeny. Parasitology 138:1815-27.

9. Rio RVM, Attardo GM, Weiss BL. 2016. Grandeur Alliances: Symbiont Metabolic Integration and Obligate Arthropod Hematophagy. Trends in Parasitology 32:739-749.

10. Min GS, Sarkar IN, Siddall ME. 2010. Salivary transcriptome of the North American medicinal leech, *Macrobdella decora*. J Parasitol 96:1211-21.

11. Whitaker IS, Oboumarzouk O, Rozen WM, Naderi N, Balasubramanian SP, Azzopardi EA, Kon M. 2012. The efficacy of medicinal leeches in plastic and reconstructive surgery: a systematic review of 277 reported clinical cases. Microsurgery 32:240-50.

12. Wenning A, Zerbst-Boroffka I, Bazin B. 1980. Water and salt excretion in the leech. J Comp Physiol B 139:97-102.

13. Graf J. 1999. Symbiosis of *Aeromonas veronii* biovar sobria and *Hirudo medicinalis*, the medicinal leech: a novel model for digestive tract associations. Infect Immun 67:1-7.

14. Worthen PL, Gode CJ, Graf J. 2006. Culture-independent characterization of the digestive-tract microbiota of the medicinal leech reveals a tripartite symbiosis. Appl Environ Microbiol 72:4775-4781.

15. Kikuchi Y, Graf J. 2007. Spatial and temporal population dynamics of a naturally occurring two-species microbial community inside the digestive tract of the medicinal leech. Appl Environ Microbiol 73:1984-1991.

16. Nelson M, Bomar L, Maltz M, Graf J. 2015. *Mucinivorans hirudinis* gen. nov., sp. nov., an anaerobic, mucin-degrading bacterium isolated from the digestive tract of the medicinal leech, *Hirudo verbana*. Int J Syst Evol Microbiol 65:990-5.

17. Neupane S, Modry D, Pafčo B, Zurek L. 2019. Bacterial Community of the Digestive Tract of the European Medicinal Leech (*Hirudo verbana*) from the Danube River. Microb Ecol doi:10.1007/s00248-019-01349-z.

18. Beka L, Fullmer MS, Colston SM, Nelson MC, Talagrand-Reboul E, Walker P, Ford B, Whitaker IS, Lamy B, Gogarten JP, Graf J. 2018. Low-Level Antimicrobials in the Medicinal Leech Select for Resistant Pathogens That Spread to Patients. mBio 9.

19. Laufer AS, Siddall ME, Graf J. 2008. Characterization of the digestive-tract microbiota of *Hirudo orientalis*, a european medicinal leech. Appl Environ Microbiol 74:6151-4.

20. Whitaker IS, Maltz M, Siddall ME, Graf J. 2014. Characterization of the digestive tract microbiota of *Hirudo orientalis* (medicinal leech) and antibiotic resistance profile. Plast Reconstr Surg 133:408e-418e.

21. Maltz MA, Bomar L, Lapierre P, Morrison HG, McClure EA, Sogin ML, Graf J. 2014. Metagenomic analysis of the medicinal leech gut microbiota. Front Microbiol 5:151.

22. Nogge G. 1981. Significance of symbionts for the maintenance of an optimal nutritional state for successful reproduction in hematophagous arthropods. Parasitology 82:101-104.

23. Manzano-Marin A, Oceguera-Figueroa A, Latorre A, Jimenez-Garcia LF, Moya A. 2015. Solving a bloody mess: B-vitamin independent metabolic convergence among

gammaproteobacterial obligate endosymbionts from blood-feeding arthropods and the leech *Haementeria officinalis*. Genome Biol Evol 7:2871-84.

24. Rio RVM, Anderegg M, Graf J. 2007. Characterization of a catalase gene from *Aeromonas veronii*, the digestive-tract symbiont of the medicinal leech. Microbiol 153:1897-1906.

25. Husnik F. 2018. Host–symbiont–pathogen interactions in blood-feeding parasites: nutrition, immune cross-talk and gene exchange. Parasitology 145:1294-1303.

26. Silver AC, Graf J. 2011. Innate and procured immunity inside the digestive tract of the medicinal leech. Inv Survival Journal 8:173-178.

27. Maltz M, Graf J. 2011. The type II secretion system is essential for erythrocyte lysis and gut colonization by the leech digestive tract symbiont *Aeromonas veronii*. Appl Environ Microbiol 77:597-603.

28. Davidson SK, Powell R, James S. 2013. A global survey of the bacteria within earthworm nephridia. Mol Phylogenet Evol 67:188-200.

29. Viana F, Paz LC, Methling K, Damgaard CF, Lalk M, Schramm A, Lund MB. 2018. Distinct effects of the nephridial symbionts *Verminephrobacter* and Candidatus *Nephrothrix* on reproduction and maturation of its earthworm host *Eisenia andrei*. FEMS Microbiol Ecol 94.

30. Kikuchi Y, Bomar L, Graf J. 2009. Stratified bacterial community in the bladder of the medicinal leech, *Hirudo verbana*. Environ Microbiol 11:2758-70.

31. Siddall ME, Worthen PL, Johnson M, Graf J. 2007. Novel role for *Aeromonas jandaei* as a digestive tract symbiont of the North American medicinal leech. Appl Environ Microbiol 73:655-8.

868 32. Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, Fierer
869 N, Peña AG, Goodrich JK, Gordon JI, Huttley GA, Kelley ST, Knights D, Koenig JE,
870 Ley RE, Lozupone CA, McDonald D, Muegge BD, Pirrung M, Reeder J, Sevinsky JR,
871 Turnbaugh PJ, Walters WA, Widmann J, Yatsunenko T, Zaneveld J, Knight R. 2010.
872 QIIME allows analysis of high-throughput community sequencing data. Nat Methods
873 7:335-336.
874 33. Sartor C, Bornet C, Guinard D, Fournier PE. 2013. Transmission of *Aeromonas*
875 *hydrophila* by leeches. Lancet 381:1686.
876 34. Ge H, Jensen PD, Batstone DJ. 2011. Temperature phased anaerobic digestion increases
877 apparent hydrolysis rate for waste activated sludge. Water Res 45:1597-1606.
878 35. Ott BM, Rickards A, Gehrke L, Rio RV. 2014. Characterization of shed medicinal leech
879 mucus reveals a diverse microbiota. Front Microbiol 5:757.
880 36. Alexiev A, Oliverio AM, Prest TL, Korpita TM, McKenzie VJ, Song SJ, Di Fiore A,
881 Seguin-Orlando A, Feh C, Mendelson JR, Sanders J, Knight R, Delsuc F, Amato KR,
882 Metcalf JL, Orlando L, Kowalewski M, Avenant NL, Link A. 2017. The Effects of
883 Captivity on the Mammalian Gut Microbiome. Integr Comp Biol 57:690-704.
884 37. Nelson MC, Morrison HG, Benjamino J, Grim SL, Graf J. 2014. Analysis, optimization
885 and verification of Illumina-generated 16S rRNA gene amplicon surveys. PLoS ONE
886 9:e94249.
887 38. Pruesse E, Quast C, Knittel K, Fuchs BM, Ludwig W, Peplies J, Glöckner FO. 2007.
888 SILVA: a comprehensive online resource for quality checked and aligned ribosomal
889 RNA sequence data compatible with ARB. Nucleic Acids Res 35:7188-7196.

890 39. Kohl KD, Dearing MD. 2014. Wild-caught rodents retain a majority of their natural gut
891 microbiota upon entrance into captivity. Environ Microbiol Reports 6:191-5.
892 40. Silver AC, Kikuchi Y, Fadl AA, Sha J, Chopra AK, Graf J. 2007. Interaction between
893 innate immune cells and a bacterial type III secretion system in mutualistic and
894 pathogenic associations. Proc Natl Acad Sci USA 104:9481-9486.
895 41. Clark MA, Moran NA, Baumann P, Wernegreen JJ. 2000. Cospeciation between bacterial
896 endosymbionts (*Buchnera*) and a recent radiation of aphids (*Uroleucon*) and pitfalls of
897 testing for phylogenetic congruence. Evolution 54:517-25.
898 42. Moeller AH, Li Y, Mpoudi Ngole E, Ahuka-Mundeke S, Lonsdorf EV, Pusey AE,
899 Peeters M, Hahn BH, Ochman H. 2014. Rapid changes in the gut microbiome during
900 human evolution. Proc Natl Acad Sci USA doi:10.1073/pnas.1419136111:201419136.
901 43. Chandler JA, Morgan Lang J, Bhatnagar S, Eisen JA, Kopp A. 2011. Bacterial
902 Communities of Diverse *Drosophila* Species: Ecological Context of a Host–Microbe
903 Model System. PLOS Genetics 7:e1002272.
904 44. Brune A, Dietrich C. 2015. The Gut Microbiota of Termites: Digesting the Diversity in
905 the Light of Ecology and Evolution. Annu Rev Microbiol 69:145-166.
906 45. Roeselers G, Mittge EK, Stephens WZ, Parichy DM, Cavanaugh CM, Guillemin K,
907 Rawls JF. 2011. Evidence for a core gut microbiota in the zebrafish. ISME J 5:1595-608.
908 46. Wang Y, Gilbreath TM, Kukutla P, Yan G, Xu J. 2011. Dynamic gut microbiome across
909 life history of the malaria mosquito *Anopheles gambiae* in Kenya. PLoS ONE 6:e24767.
910 47. Aksoy S. 2000. Tsetse--A haven for microorganisms. Parasitol Today 16:114-8.
911 48. Strand MR. 2018. Composition and functional roles of the gut microbiota in mosquitoes.
912 Curr Opin Insect Sci 28:59-65.

913 49. Zepeda Mendoza ML, Xiong Z, Escalera-Zamudio M, Runge AK, Thézé J, Streicker D,
914 Frank HK, Loza-Rubio E, Liu S, Ryder OA, Samaniego Castruita JA, Katzourakis A,
915 Pacheco G, Taboada B, Löber U, Pybus OG, Li Y, Rojas-Anaya E, Bohmann K,
916 Carmona Baez A, Arias CF, Liu S, Greenwood AD, Bertelsen MF, White NE, Bunce M,
917 Zhang G, Sicheritz-Pontén T, Gilbert MPT. 2018. Hologenomic adaptations underlying
918 the evolution of sanguivory in the common vampire bat. Nat Ecol Evol 2:659-668.
919 50. Michel AJ, Ward LM, Goffredi SK, Dawson KS, Baldassarre DT, Brenner A, Gotanda
920 KM, McCormack JE, Mullin SW, O'Neill A, Tender GS, Uy JAC, Yu K, Orphan VJ,
921 Chaves JA. 2018. The gut of the finch: uniqueness of the gut microbiome of the
922 Galápagos vampire finch. Microbiome 6:167-167.
923 51. Siddall ME, Barkdull M, Tessler M, Brugler MR, Borda E, Hekkala E. 2019. Ideating
924 iDNA: Lessons and limitations from leeches in legacy collections. PLOS ONE
925 14:e0212226.
926 52. Colston SM, Fullmer MS, Beka L, Lamy B, Gogarten JP, Graf J. 2014. Bioinformatic
927 genome comparisons for taxonomic and phylogenetic assignments using *Aeromonas* as a
928 test case. mBio 5:e02136.
929 53. Morandi A, Zhaxybayeva O, Gogarten JP, Graf J. 2005. Evolutionary and Diagnostic
930 Implications of Intragenomic Heterogeneity in the 16S rRNA Gene in *Aeromonas*
931 Strains. J Bacteriol 187:6561-6564.
932 54. Silver AC, Rabinowitz NM, Kuffer S, Graf J. 2007. Identification of *Aeromonas veronii*
933 genes required for colonization of the medicinal leech, *Hirudo verbana*. J Bacteriol
934 189:6763-72.

935 55. Bomar L, Graf J. 2012. Investigation into the physiologies of *Aeromonas veronii in vitro*
936 and inside the digestive tract of the medicinal leech using RNA-seq. Biol Bull 223:155-
937 66.
938 56. Maltz M, LeVarge B, Graf J. 2015. Identification of iron and heme utilization genes in
939 *Aeromonas* and their role in the colonization of the leech digestive tract. Front Microbiol
940 6:763.
941 57. Sogin ML, Morrison HG, Huber JA, Welch DM, Huse SM, Neal PR, Arrieta JM, Herndl
942 GJ. 2006. Microbial diversity in the deep sea and the underexplored "rare biosphere".
943 Proc Natl Acad Sci USA 103:12115-12120.
944 58. Benjamino J, Lincoln S, Srivastava R, Graf J. 2018. Low-abundant bacteria drive
945 compositional changes in the gut microbiota after dietary alteration. Microbiome 6:86.
946 59. Jousset A, Bienhold C, Chatzinotas A, Gallien L, Gobet A, Kurm V, Küsel K, Rillig MC,
947 Rivett DW, Salles JF, van der Heijden MGA, Youssef NH, Zhang X, Wei Z, Hol WHG.
948 2017. Where less may be more: how the rare biosphere pulls ecosystems strings. The
949 ISME Journal 11:853-862.
950 60. Meyer KM, Memiaghe H, Korte L, Kenfack D, Alonso A, Bohannan BJM. 2018. Why
951 do microbes exhibit weak biogeographic patterns? The ISME Journal 12:1404-1413.
952 61. Woodcock S, Curtis TP, Head IM, Lunn M, Sloan WT. 2006. Taxa-area relationships for
953 microbes: the unsampled and the unseen. Ecol Lett 9:805-12.
954 62. Indergand S, Graf J. 2000. Ingested blood contributes to the specificity of the symbiosis
955 of *Aeromonas veronii* biovar sobria and *Hirudo medicinalis*, the medicinal leech. Appl
956 Environ Microbiol 66:4735-4741.

957 63. Vasai F, Brugirard Ricaud K, Bernadet MD, Cauquil L, Bouchez O, Combes S, Davail S.
958 2014. Overfeeding and genetics affect the composition of intestinal microbiota in *Anas*
959 *platyrhynchos* (Pekin) and *Cairina moschata* (Muscovy) ducks. FEMS Microbiol Ecol
960 87:204-16.
961 64. Costello EK, Gordon JI, Secor SM, Knight R. 2010. Postprandial remodeling of the gut
962 microbiota in Burmese pythons. The ISME journal 4:1375-1385.
963 65. Tasiemski A, Massol F, Cuvillier-Hot V, Boidin-Wichlacz C, Roger E, Rodet F, Fournier
964 I, Thomas F, Salzet M. 2015. Reciprocal immune benefit based on complementary
965 production of antibiotics by the leech *Hirudo verbana* and its gut symbiont *Aeromonas*
966 *veronii*. Sci Rep 5:17498.
967 66. Carey HV, Walters WA, Knight R. 2013. Seasonal restructuring of the ground squirrel
968 gut microbiota over the annual hibernation cycle. Am J Physiol Regul Integr Comp
969 Physiol 304:R33-R42.
970 67. Ott BM, Beka L, Graf J, Rio RV. 2015. Draft Genome Sequence of *Pedobacter* sp. Strain
971 Hv1, an Isolate from Medicinal Leech Mucosal Castings. Genome Announc 3.
972 68. Silver AC, Williams D, Faucher J, Horneman AJ, Gogarten JP, Graf J. 2011. Complex
973 evolutionary history of the *Aeromonas veronii* group revealed by host interaction and
974 DNA sequence data. PLoS One 6:e16751.
975 69. Mumcuoglu KY, Huberman L, Cohen R, Temper V, Adler A, Galun R, Block C. 2010.
976 Elimination of symbiotic *Aeromonas* spp. from the intestinal tract of the medicinal leech,
977 *Hirudo medicinalis*, using ciprofloxacin feeding. Clin Microbiol Infect 16:563-7.

978 70. Puchtler H, Waldrop FS, Meloan SN, Terry MS, Conner HM. 1970. Methacarn
979 (methanol-Carnoy) fixation. Practical and theoretical considerations. Histochemie 21:97-
980 116.
981 71. Schneider CA, Rasband WS, Eliceiri KW. 2012. NIH Image to ImageJ: 25 years of
982 image analysis. Nat Methods 9:671-675.
983 72. Yu Z, Morrison M. 2004. Improved extraction of PCR-quality community DNA from
984 digesta and fecal samples. Biotechniques 36:808-12.
985 73. Kozich JJ, Westcott SL, Baxter NT, Highlander SK, Schloss PD. 2013. Development of a
986 Dual-Index Sequencing Strategy and Curation Pipeline for Analyzing Amplicon
987 Sequence Data on the MiSeq Illumina Sequencing Platform. Appl Environ Microbiol
988 79:5112-5120.
989 74. Larsbrink J, Rogers TE, Hemsworth GR, McKee LS, Tauzin AS, Spadiut O, Klinter S,
990 Pudlo NA, Urs K, Koropatkin NM, Creagh AL, Haynes CA, Kelly AG, Cederholm SN,
991 Davies GJ, Martens EC, Brumer H. 2014. A discrete genetic locus confers xyloglucan
992 metabolism in select human gut Bacteroidetes. Nature doi:10.1038/nature12907.
993 75. McMurdie PJ, Holmes S. 2013. phyloseq: An R Package for Reproducible Interactive
994 Analysis and Graphics of Microbiome Census Data. PLOS ONE 8:e61217.
995 76. Davis NM, Proctor DM, Holmes SP, Relman DA, Callahan BJ. 2018. Simple statistical
996 identification and removal of contaminant sequences in marker-gene and metagenomics
997 data. Microbiome 6:226.
998 77. Oksanen J, Blanchet FG, Friendly M, Kindt R, Legendre P, McGlinn D, Minchin PR,
999 O'Hara RB, Simpson GL, Solymos P, Stevens MHH, Szoecs E, Wagner H. 2019. vegan:

1000 Community Ecology Package. R package version 2.5-5, https://CRAN.R-
1001 project.org/package=vegan.
1002 78. Lozupone C, Knight R. 2005. UniFrac: a New Phylogenetic Method for Comparing
1003 Microbial Communities. Appl Environ Microbiol 71:8228-8235.

1004

1005

1006 TABLES

1007 Table 1. Average percent of total 16S rRNA V4 sequences of core and common OTUs in ILF
1008 and intestinum samples from *Hirudo verbana* and *Macrobdella decora*

1009

		Hirudo verbana		***Macrobdella decora***		**Accession No.**
Phylum	**Genus***	**ILF†**	**Intestinum**	**ILF**	**Intestinum**	
Bacteroidetes	Bacteroides	1.37%	0.38%	39.82%	40.48%	SAMN11823292
	Mucinivorans-like	0%	8.89%	0%	0%	SAMN11823282
	Millionella-like	0.35%	9.79%	0%	0.65%	SAMN11823299
	Mucinivorans	58.20%	16.83%	0%	0.00%	SAMN11823269
Clostridia	Alkaliphilus-like	0%	1.11%	20.42%	28.62%	SAMN11823295
	Clostridium	0%	0%	2.78%	0.50%	SAMN11823294
	Papillibacter-like	0%	0%	2.71%	1.15%	SAMN11823296
	Butyricicoccus	0%	0%	18.51%	6.70%	SAMN11823293
Alphaproteobacteria	Insolitispirillum-like	0%	21.02%	0%	1.67%	SAMN11823298
Deltaproteobacteria	Desulfovibrio	0.71%	6.15%	0.62%	1.27%	SAMN11823279
Gammaproteobacteria	Aeromonas	18.07%	15.88%	8.10%	9.15%	SAMN11823291

1010

1011

1012

* OTUs determined at the 97% confidence level. Those OTUs indicated as "-like" indicate the most closely-related genus listed in RDP.
† Core (present in ≥ 90% of samples) OTUs are highlighted in green. Common (present in ≥ 70% of samples) OTUs are highlighted in yellow. Core and common OTUS were determined for each species of leech and sampled organ individually.

Table 2. Presence of bladder OTUs in total 16S rRNA V4 sequences from *Macrobdella decora* and *Hirudo verbana* bladder samples.

Class	Genus	GenBank Accession No.	*Hirudo verbana*		*Macrobdella decora*	
			Median‡	Max§	Median	Max
Alphaproteobacteria	Ochrobactrum	SAMN11823287	15.46%	94.99%	0%	12%
	Ensifer	---	0.18%	13.18%	0.37%	13.14%
	Aminobacter	---	0.40%	2.92%	N.D.	N.D.
	Azospirillum	---	N.D.	N.D.	15.13%	28.86%
	Azospirillum	SAMN11823283	N.D.	N.D.	19.08%	45.92%
	Phreatobacter-like	SAMN11823288	0.86%	9.52%	3.23%	21.54%
Betaproteobacteria	Methylopumilus-like	SAMN11823285	N.D.	N.D.	6.68%	17.40%
	Ramlibacter	SAMN11823289	20.81%	75.62%	20.94%	38.93%
Deltaproteobacteria	Bdellovibrio-like	SAMN11823266	2.22%	20.09%	N.D.	N.D.
Bacteroidetes	Niabella	SAMN11823286	0.63%	9.20%	N.D.	N.D.
	Pedobacter	SAMN11823267	18.48%	44.24%	N.D.	N.D.

‡ Median percentage of total 16S V4 rRNA sequences, when OTU is present. Core (present in ≥ 90% of samples) OTUs are highlighted in green.
§ Maximum percentage of total 16S V4 rRNA sequences.

Supplemental Table 1. Number of samples collected of each sample type for this study. Only one sample of each sample type was collected per animal, however multiple sample types were often collected from the same animal. A total of 36 *Hirudo verbana* and 52 *Macrobdella decora* animals were used in this study.

	ILF		**Intestinum**		**Bladder**	
Da1F[**]	***M. decora***	***H. verbana***	***M. decora***	***H. verbana***	***M. decora***	***H. verbana***
0	17	10	15	2	8	2
1	6	–	6	–	2	–
2	2	6	2	–	–	1
4	6	4	9	–	4	–
7	9	8	9	–	3	–
30	7	–	4	–	2	–
90+	5	8	–	7	1	9

Supplemental Table 2. See Excel

[**] Days after first laboratory-administered, sheep blood meal

Supplemental Table 3. FISH probes used in this study

Probe	Nucleotide sequence (5′-3′)	Reference	Target Organism(s)
ALF968	GGTAAGGTTCTGCGCGTT	Neef (1997)	Aminobacter, Insolitispirillum-like, Ochrobactrum, Phreatobacter-like, Sphingomonas
BET42a	GCCTTCCCACWTCGTTT	Manz *et al*. (1992)	Achromobacter, Acidovorax, Pelomonas, Ramlibacter, Variovorax
CF319a	TGGTCCGTGTCTCAGTAC	Manz *et al*. (1996)	Bacteroides, Flavobacterium, Pedobacter, Mucinivorans, (some) Phreatobacter-like, (some) Bdellovibrio
AER66	CTACTTTCCCGCTGCCGC	Kampfer (1996)	Aeromonas
Rhiz1244	TCGCTGCCCACTGTCACC	Thayanukul (2010)	Aminobacter, Ensifer, Phreatobacter-like, (some) Ocrobactrum
Eub338	GCTGCCTCCCGTAGGAGT	Amann *et al*. (1990)	(most) Eubacteria

1059

1060

1061

1062

1063

1064 FIGURES

1065

1066 Figure 1. Unifrac-calculated NMDS plot of leech-associated microbiota showing that the
1067 microbiota is host species-specific at the genus level, but not at higher taxonomic levels. Two
1068 leech species were sampled (*Hirudo verbana* = orange and *Macrobdella decora* = cyan) at three
1069 organ sites (ILF = circle, intestinum = triangle, bladder = cross). A) When OTUs were calculated
1070 at 97% sequence similarity (~Genus), 53% of the total variation between samples is described by
1071 separating the two leech host species. B) When OTUs were calculated at 88% sequence
1072 similarity (~Order), only 25% of the total variation between samples is described by separating
1073 the two leech host species. Ellipses were calculated at 95% confidence.

1074

1075 Figure 2. Shannon Alpha-diversity showing the microbiome of *Macrobdella decora* ILF is more
1076 diverse than that of *Hirudo verbana* but the opposite is true of the intestinum microbiomes.
1077 Calculations for leech A) ILF and B) intestinum samples. All samples were collected before
1078 feeding or greater than 28 days after feeding. Alpha diversity does not change between the ILF
1079 and intestinum of wild-caught *M. decora* (grey) but increases in farm-raised *H. verbana* (white).

1080

Figure 3. False-color FISH micrographs of *Macrobdella decora* bladder samples. From left to right, columns contain images with probes DAPI (blue – Eukaryotic DNA), Eub338 (green – Eubacteria), CF319a (white : Bacteroidetes – *Mucinivorans, Bacteroides, Niabella, Pedobacter*), (red), and composite. From top to bottom, the fourth (red) column contains images with probes Alf968 (Alphaproteobacteria – *Ochrobactrum, Azospirillum, Phreatobacter-like*), Bet42a (Betaproteobacteria – *Methylopumilus-like, Ramlibacter*), Rhiz1244 (Rhizobiales – *Ochrobactrum, Phreatobacter-like*), Aer66 *(Aeromonas)*. The *M. decora* bladder is colonized in a stratified manner with intracellular *Azospirillum*, epithelial-associated Betaproteobacteria, and *Phreatobacter-like* and *Niabella* in the matrix. *Aeromonas* (red arrows) is present associated with eukaryotic cells, suggesting that they have been carried here by leech hemocytes and are not normal flora for the bladder. Bars = 10μm.

Figure 4. Unifrac-calculated NMDS plot of wild-caught *Macrobdella decora* - associated microbiota shows that month of collection affects gut microbiome. Animals were collected in three seasons: late cold (April - light blue), warm (June - pink, July - red, August - brown, and September - brown), and early cold (October - dark blue). A) ILF samples. ILF microbiota from animals collected in October were significantly different from that of animals collected in April or warm months. B) Intestinum samples. Intestinum microbiota from animals collected in April were significantly different from that of animals collected in warm months or October. Ellipses drawn at 95% confidence interval.

Figure 5. Box and Whisker plot of leech-associated gut microbiota showing that the prevalence of taxa in the ILF appears to be greatly affected within ~48h of a blood meal, while the

prevalence of taxa in the intestinum maintains relative stability. The prevalence of 9 core gut taxa from deep-sequenced microbiomes of A) *Hirudo verbana* ILF, B) *Macrobdella decora* ILF, and C) *M. decora* intestinum were assessed at 7 time points after a blood meal (0, 1, 2, 4, 7, 30, and 90+ days after feeding). See Supplemental Table 1 for number of samples analyzed at each time point.

Figure 6. *Macrobdella decora* ILF diversity changes with time after feeding. A) Shannon diversity index calculates a drop in ILF microbiome diversity by 2 days after feeding (DaF) that rebounds by 7 – 30 DaF. B) Bray-curtis distance calculation shows that ILF microbiomes at 2 – 7 DaF are significantly different from those of unfed animals (0 DaF) and that the community rebounds at 30 – 90 DaF. Note the appearance of two sub-populations of ILF samples that i) appears to rebound by ~4 DaF and ii) appears to require > 7 DaF to rebound.

Figure 7. False-color FISH micrograph of *Macrobdella decora* ILF. From left to right, columns contain images with probes DAPI (blue – Eukaryotic DNA), Eub338 (green – Eubacteria), CF319a (white : Bacteroidetes – *Mucinivorans, Bacteroides*), Aer66 (red – *Aeromonas*), and composite. From top to bottom, the rows contain images from animals sacrificed 0 days (wild-caught animals), 4 days , and 7 days after a laboratory-administed sterile blood meal. Blue arrows indicate eukaryotic cells (most likely leech hemocytes), green arrows indicate notable bacteria, white arrows indicate Bacteroidetes microcolonies, and red arrows indicate *Aeromonas*. Background fluorescence is from crop contents, especially the blood meal at 4 and 7 days. Few bacteria are present in the crop of wild-caught, unfed animals. Bacteroidetes colony expansion occurs by 4 days after feeding, while *Aeromonas* prevalence increases by 7 days after feeding.

1127 Other bacteria are present at 4 days after feeding, however their numbers appear to be
1128 overwhelmed by Bacteroidetes and *Aeromonas* growth at 7 days after feeding. In animals 7 days
1129 after feeding, *Aeromonas* occasionally are found associated with hemocytes. Bars = 10μm.
1130
1131 Sup Figure 1. Unifrac-calculated NMDS plot of wild-caught *Macrobdella decora* - associated
1132 microbiota. Animals were collected from four states of the Northeastern U.S.A.: Connecticut
1133 (orange), Massachusetts (cyan), New York (green), and Vermont (black). A) ILF samples show
1134 no significant difference between ILF of *M. decora* from CT, MA, NY, and VT ($p \geq 0.222$).
1135 B) Intestinum samples from CT and MA are significantly different from each other ($p = 0.006$).
1136 Ellipses drawn at 95% confidence interval.
1137
1138

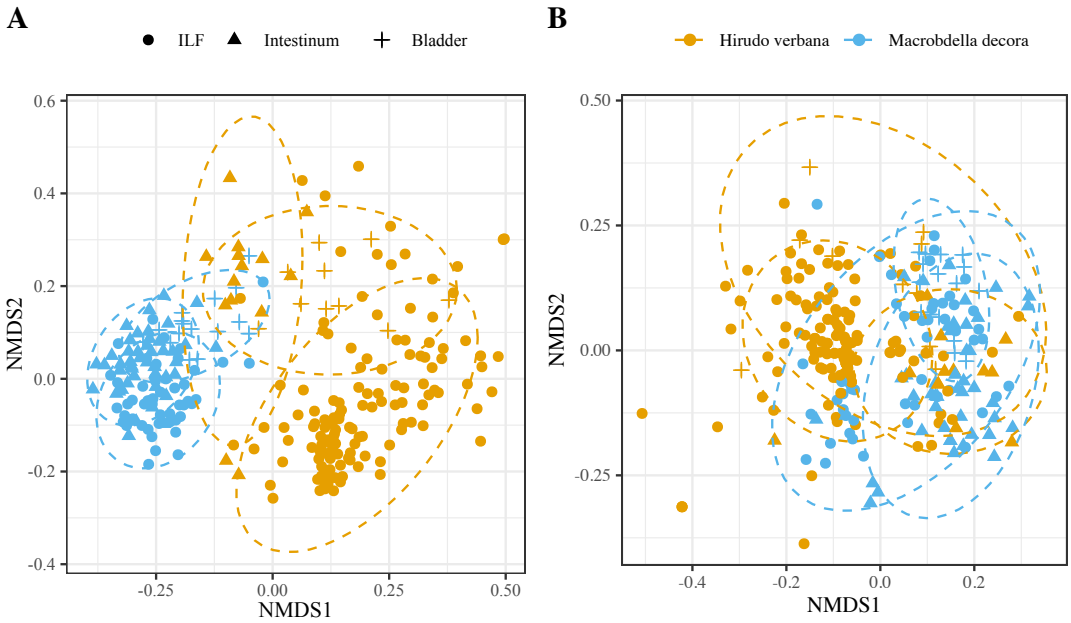

Figure 1: Unifrac-calculated NMDS plot of leech-associated microbiota showing that the microbiota is host species-specific at the genus level, but not at higher taxonomic levels. Two leech species were sampled (*Hirudo verbana* = orange and *Macrobdella decora* = cyan) at three organ sites (ILF = circle, intestinum = triangle, bladder = cross). A) When OTUs were calculated at 97% sequence similarity (~Genus), 53% of the total variation between samples is described by separating the two leech host species. B) When OTUs were calculated at 88% sequence similarity (~Order), only 25% of the total variation between samples is described by separating the two leech host species. Ellipses calculated at 95% confidence.

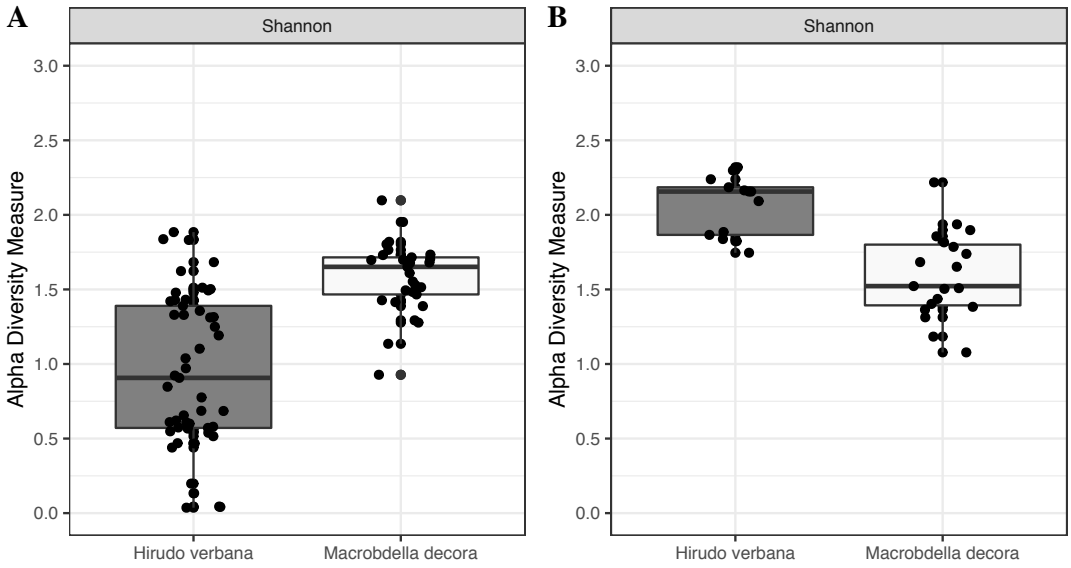

Figure 2: Shannon Alpha-diversity showing the microbiome of *Macrobdella decora* ILF is more diverse than that of *Hirudo verbana* but the opposite is true of the intestinum microbiomes. Calculations for leech A) ILF and B) intestinum samples. All samples were collected before feeding or greater than 28 days after feeding. Alpha diversity does not change between the ILF and intestinum of wild-caught *M. decora* (grey) but increases in farm-raised *H. verbana* (white).

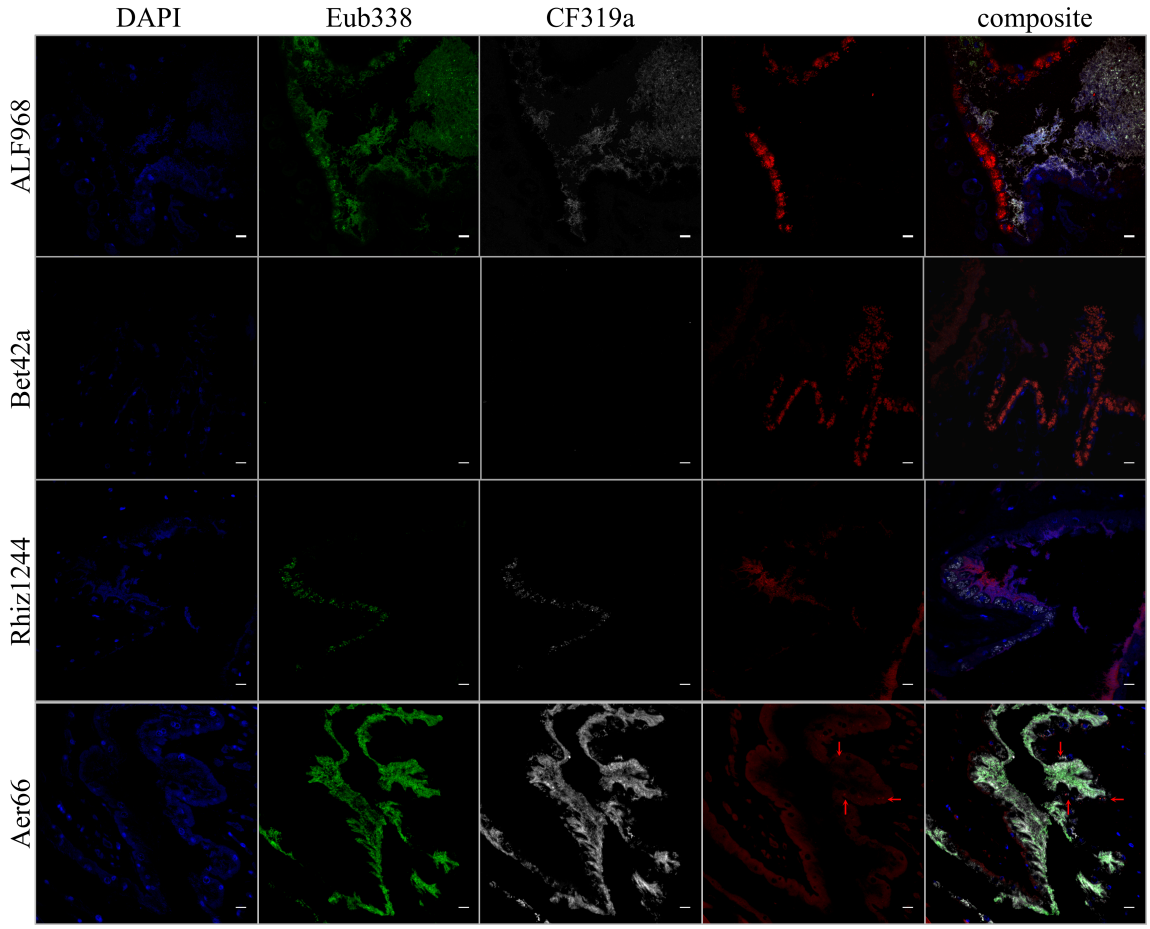

Figure 3: False-color FISH images of *Macrobdella decora* bladder samples. From left to right, columns contain images with probes DAPI (blue - Eukaryotic DNA), Eub338 (green - Eubacteria), CF319a (white : Bacteroidetes - *Mucinivorans*, *Bacteroides*, *Niabella*, *Pedobacter*), (red), and composite. From top to bottom, the fourth (red) column contains images with probes ALF968 (Alphaproteobacteria - *Ochrobactrum*, *Azospirillum*, *Phreatobacter*-like), Bet42a (Betaproteobacteria - *Methylopumilus*-like, *Ramlibacter*), Rhiz1244 (Rhizobiales - *Ochrobactrum*, *Phreatobacter*-like), Aer66 (*Aeromonas*). The *M. decora* bladder is colonized in a stratified manner with intracellular *Azospirillum*, epithelial-associated Betaproteobacteria, and *Phreatobacter*-like and *Niabella* in the matrix. *Aeromonas* (red arrows) is present associated with eukaryotic cells, suggesting that they have been carried here by leech hemocytes and are not normal flora for the bladder. Bars = 10μm.

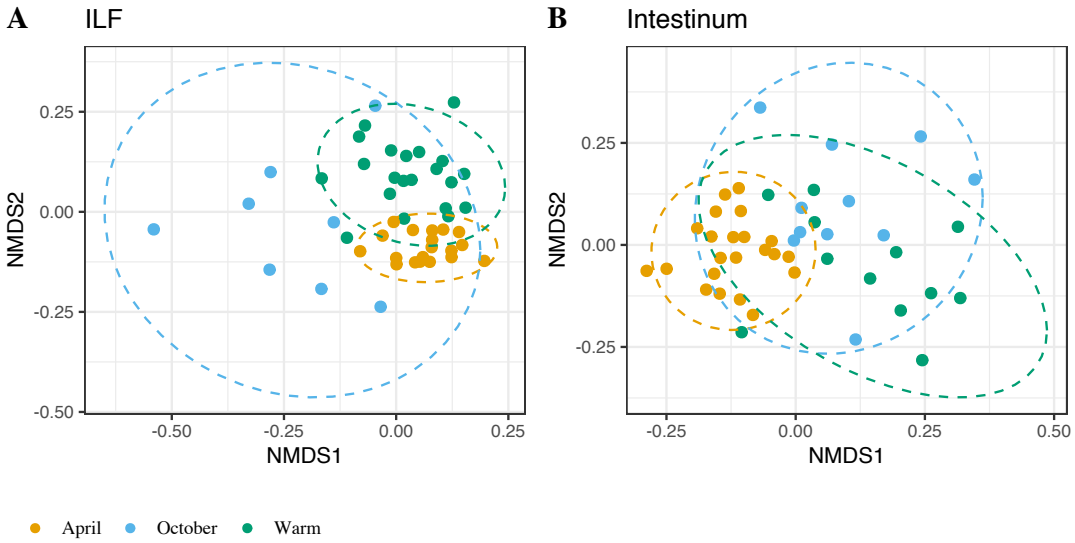

Figure 4: Unifrac-calculated NMDS plot of wild-caught *Macrobdella decora* - associated microbiota shows that month of collection affects gut microbiome. Animals were collected in three seasons: late cold (April - light blue), warm (June - pink, July - red, August - brown, and September - brown), and early cold (October - dark blue). A) ILF samples. ILF microbiota from animals collected in October were significantly different from that of animals collected in April or warm months. B) Intestinum samples. Intestinum microbiota from animals collected in April were significantly different from that of animals collected in warm months or October. Ellipses drawn at 95% confidence interval.

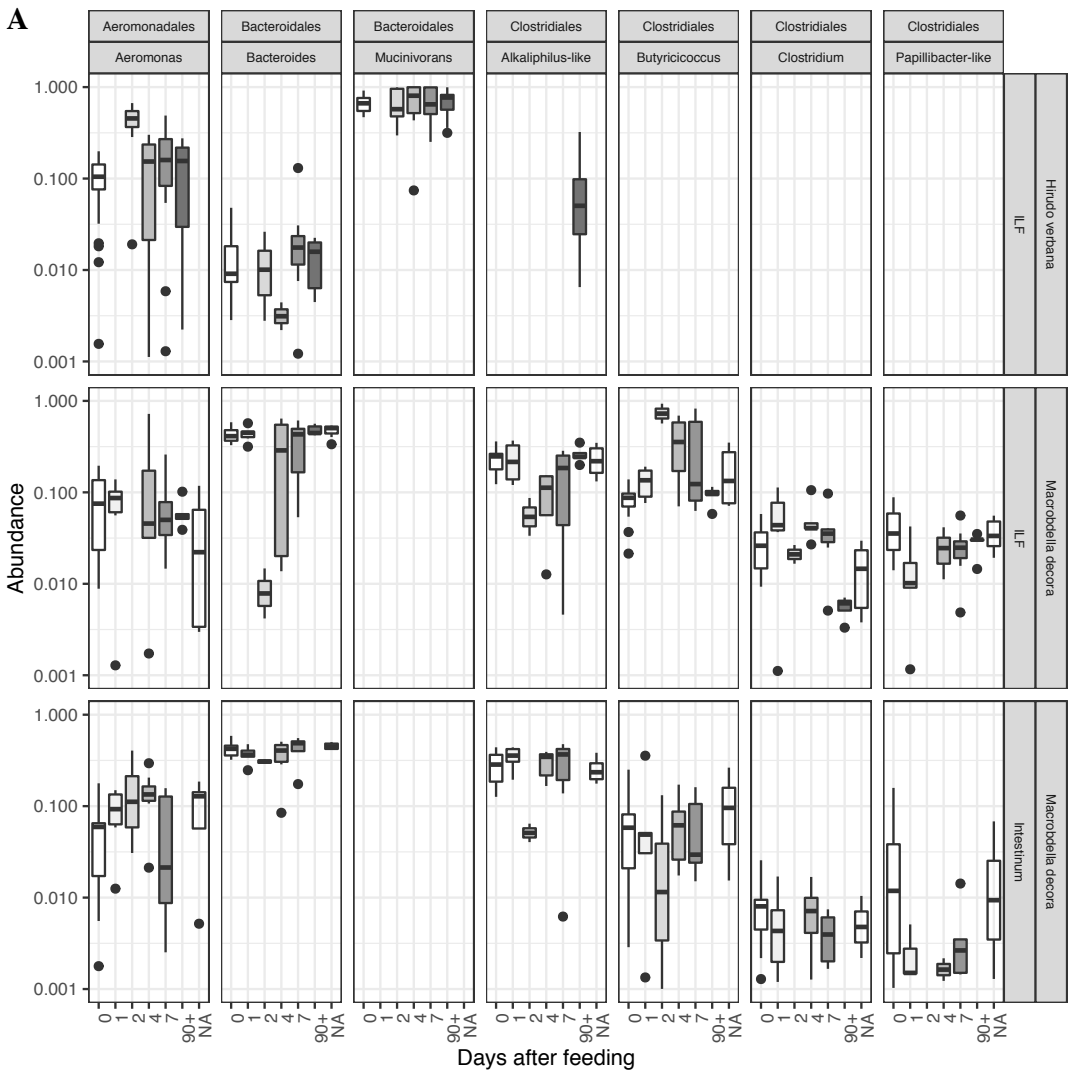

Figure 5: Box and Whisker plot of leech-associated gut microbiota showing that the prevalence of taxa in the ILF appears to be greatly affected within ∼48h of a blood meal, while the prevalence of taxa in the intestinum maintains relative stability. The prevalence of 9 core gut taxa from deep-sequenced microbiomes of A) *Hirudo verbana* ILF, B) *Macrobdella decora* ILF, and C) *M. decora* intestinum were assessed at 7 time points after a blood meal (0, 1, 2, 4, 7, 30, and 90+ days after feeding). See Supplemental Table 1 for number of samples analyzed at each time point.

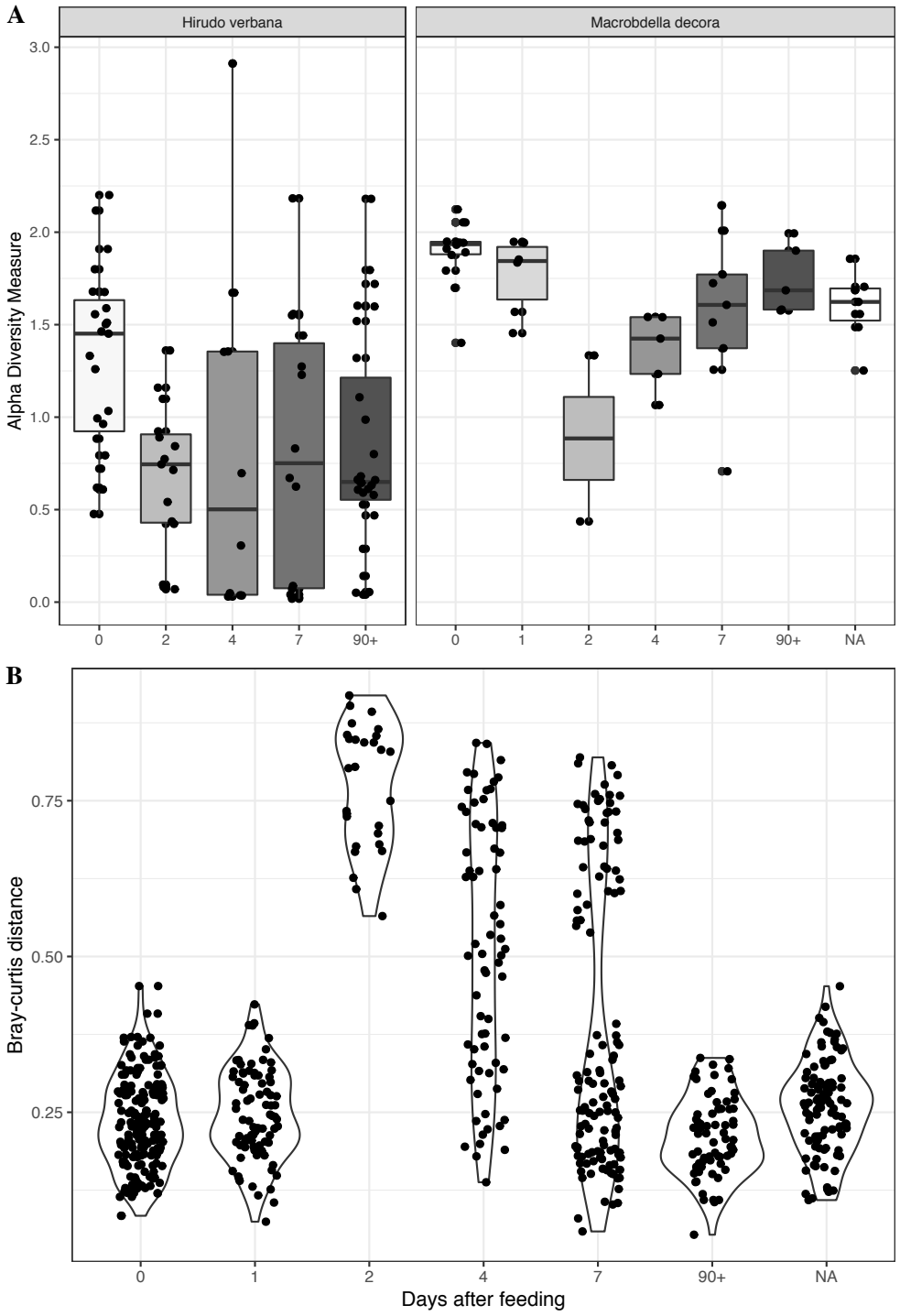

Figure 6: *Macrobdella decora* ILF diversity changes with time after feeding. A) Shannon diversity index calculates a drop in ILF microbiome diversity by 2 days after feeding (DaF) that rebounds by 7–30 DaF. B) Bray-curtis distance calculation shows that ILF microbiomes at 2–7 DaF are significantly different from those of unfed animals (0 DaF) and that the community rebounds at 30–90 DaF. Note the appearance of two sub-populations of ILF samples that i) appears to rebound by $\sim$4 DaF and ii) appears to require $>$ 7 DaF to rebound.

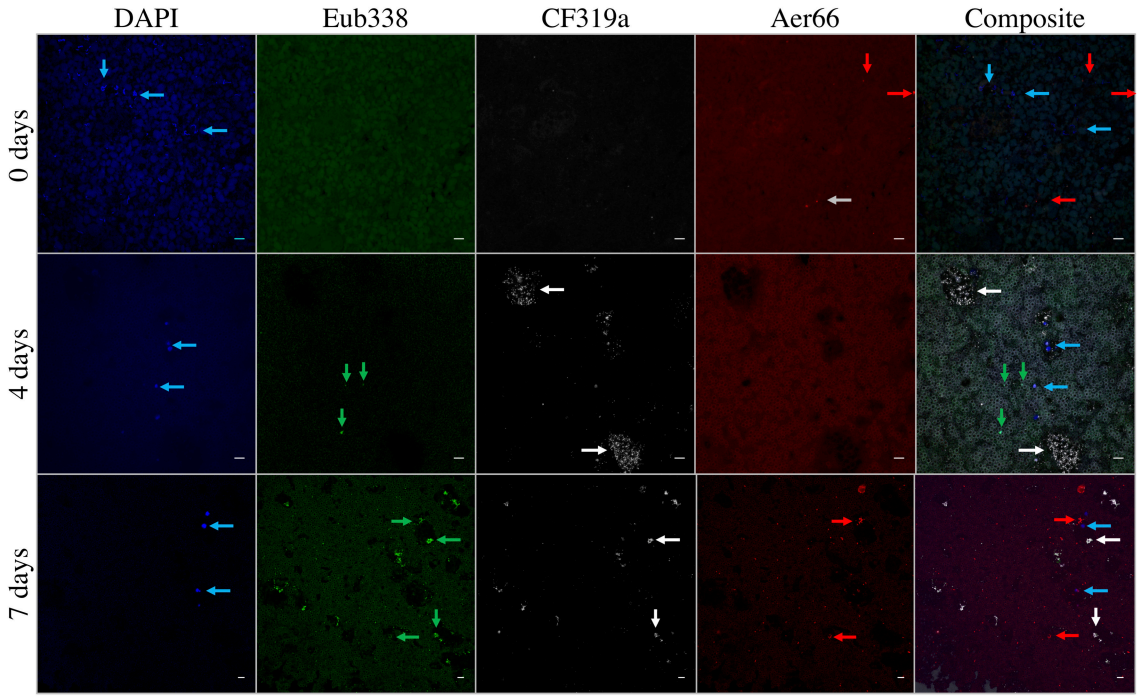

Figure 7: False-color FISH images of *Macrobdella decora* ILF. From left to right, columns contain images with probes DAPI (blue - Eukaryotic DNA), Eub338 (green - Eubacteria), CF319a (white : Bacteroidetes - *Mucinivorans*, *Bacteroides*), Aer66 (red - *Aeromonas*), and composite. From top to bottom, the rows contain images from animals sacrificed 0 days (wild-caught animals), 4 days , and 7 days after a laboratory-administed sterile blood meal. Blue arrows indicate eukaryotic cells (most likely leech hemocytes), green arrows indicate notable bacteria, white arrows indicate Bacteroidetes microcolonies, and red arrows indicate *Aeromonas*. Background fluorescence is from crop contents, especially the blood meal at 4 and 7 days. Few bacteria are present in the crop of wild-caught, unfed animals. Bacteroidetes colony expansion occurs by 4 days after feeding, while *Aeromonas* prevalence increases by 7 days after feeding. Other bacteria are present at 4 days after feeding, however their numbers appear to be overwhelmed by Bacteroidetes and *Aeromonas* growth at 7 days after feeding. In animals 7 days after feeding, *Aeromonas* occasionally are found associated with hemocytes. Bars = 10μm.