Cultivation and Fluorescent in situ hybridization suggest that some shipworm species acquire endosymbiotic bacteria through indirect horizontal transmission

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ABSTRACT

Beneficial microbial symbionts provide essential functions for their host from nutrients to defense against disease. Whether hosts acquire their symbionts directly from parents (vertical transmission) or by sampling from the environment (horizontal transmission) can have dramatic impacts on host adaptability and, in the case of ecosystem engineers, ecosystem health. Wood-boring bivalve mollusks (Teredinidae shipworms) act as ecosystem engineers in marine environments, creating habitat out of submerged wood for fish and invertebrates. Essential to shipworm success is their community of endosymbiotic gill bacteria that produce the enzymes necessary for wood digestion. How shipworms acquire their symbionts, however, remains largely unexplored. Using culturing, fluorescence *in-situ* hybridization, confocal microscopy, and tank experiments, we provide evidence suggesting the mode of symbiont transmission the shipworms for either the shipworm, *Lyrodus pedicellatus* or *Teredo bartschi* or both. Symbiotic bacteria were not detected by cultivation or microscopy in brooding larvae within gravid adults or as veliger

27 larvae collected from the water column, but were observed in adult specimens and juveniles that
28 had begun burrowing into wood. These data suggest that the specimens examined have both
29 aposymbiotic and symbiotic life phases and acquire their symbionts through indirect horizontal
30 transmission. Our findings reveal how the long-term brooders *L. pedicellatus* and/or *T. bartschi*
31 acquire their gill endosymbionts.
32

33 **IMPORTANCE**

34 How eukaryotic hosts acquire their microbial symbionts can have significant consequences for
35 their ability to adapt to varied environments. Although wood-boring bivalve shipworms have
36 diverse reproductive strategies and are found in unique environments across the globe, little is
37 known about how they transmit their essential gill endosymbionts. We used the closely related
38 shipworms, *Lyrodus pedicellatus* and/or *Teredo bartschi* to study how these long-term brooding
39 shipworms acquire their gill endosymbionts. Our work, unlike previous claims for the broadcast
40 spawning species *Bankia setacae* which reportedly transmits its symbionts directly from parent to
41 offspring, suggests that juvenile *L. pedicellatus* and/or *T. bartschi* acquire their symbionts through
42 horizontal transmission rather than directly from their parents. This work reveals the mechanism
43 by which some brooding shipworm species acquire their symbionts, adding to our limited
44 understanding of intracellular symbiont transmission of Teredinidae.
45

46 **INTRODUCTION**

47 Nutritional symbioses allow eukaryotic hosts to exploit otherwise unavailable food
48 sources, expanding the niches available to both symbiotic partners [1]. Many marine ecosystem
49 engineers such as scleractinian corals, hydrothermal vent tubeworms, some deep-sea mussels,
50 and wood-boring shipworms rely on their symbiotic microbes to create structural habitats in their
51 respective ecosystems [1-4]. How these mutualistic associations are established and able to

52 persist across multiple generations has important implications for host fitness and ecosystem
53 health.

54 Theoretical studies indicate that mutualisms strongly favor vertical transmission of
55 symbionts, i.e., direct transmission of symbionts from parents prior to offspring release [5-7].
56 Vertical transmission guarantees the next generation has symbionts and their associated benefits
57 and has been linked with more cooperative symbionts and reduced risk of host exploitation [6].
58 Horizontally transmitted symbioses in which offspring acquire symbionts from the environment in
59 each subsequent generation, however, are wide spread and allow hosts to adapt to new
60 conditions [5]. Hosts that acquire symbionts from the environment can select for partners that
61 confer local advantage [8, 9] and avoid genetic bottlenecks due to reproductive isolation of
62 symbionts through many host generations [6, 7]. In marine environments, transmission of
63 microbial symbionts occurs predominantly horizontally, even in the case of intracellular symbionts
64 [10, 11], likely due to the ability of symbionts to more easily travel from one host to another, as
65 compared to terrestrial environments where desiccation and osmolarity issues are apparent [10,
66 12]. While the mode of symbiont transmission has been of keen interest for many ecosystem
67 engineers [1, 13], little is known about how shipworms acquire their intracellular endosymbiotic
68 bacteria.

69 Dubbed termites of the sea [14], shipworms are marine wood-boring bivalve mollusks from
70 the family Teredinidae that use wood as a source of shelter and food [3, 15, 16]. Shipworms are
71 among the most divergent and diverse bivalve families [17] however, how these ecologically
72 important bivalves digest wood remains unknown. Shipworms harbor dense communities of
73 intracellular gill symbionts that produce cellulolytic enzymes; similarly to the majority of wood-
74 eating animals, these enzymes may allow for wood digestion by the host [2-4]. Cellulase-
75 producing symbionts are localized within the gland of Deshayes, a specialized region within the
76 host gill, rather than in the digestive tract, allowing the host to directly consume wood-derived
77 sugars without competing with their symbionts [18, 19]. Recent work has revealed that many

78 shipworm hosts harbor up to three species that make up the majority of the community, as well
79 as a diverse group of low abundance taxa, although the identity and relative abundance of these
80 taxa vary by host species [20].
81 To date, few studies have explored symbiont transmission in shipworms. Sipe et al [21]
82 studied symbiont distribution in the shipworm *Bankia setacea* and detected symbiont 16S rRNA
83 in the gill, ovary, and egg using PCR amplification, suggesting *B. setacae* acquires their symbionts
84 through vertical transmission. However, whether the same is true for other shipworm species
85 remains unknown. In this work, we explored the mode of symbiont transmission for representative
86 long-term brooding shipworms. Unlike most intracellular symbionts, many shipworm gill symbionts
87 share characteristics of free-living bacteria, do not appear to be obligate, and be cultivated *in vitro*
88 [15, 22, 23], thus making the shipworm symbiosis an intriguing model to explore symbiont
89 transmission. Further, a community of shipworms is currently maintained in aquaria at the Ocean
90 Genome Legacy Center (OGL), allowing for experimental manipulation. Using a combination of
91 Fluorescence *In-Situ* Hybridization and confocal microscopy, culturing, and tank experiments we
92 aimed to describe the mode of symbiont transmission for shipworms in the colony maintained at
93 OGL.
94 At the time that this experimental work was performed, it was thought that the OGL
95 shipworm community was composed only of *Lyrodus pedicellatus*, however the community also
96 appears to contain a second species, *Teredo bartschi.* For this reason, we cannot rule out the
97 possibility that specimens, especially larvae and juveniles, of one or both species were included
98 in the described experiments. However, because *L. pedicellatus* and *T. bartschi* are closely
99 related, it is unlikely that this will alter the conclusions of this work. Nonetheless, we are currently
100 working to verify the results described here for each of these shipworm species independently
101 prior to peer review publication. Until that time, we present our results here, referring to the
102 experimental specimens as members of this mixed species OGL colony.
103

104 **RESULTS**

105 **Bacterial symbionts infect adult *L. pedicellatus* and/or *T. bartschi* gill tissue.** To determine
106 the mode of symbiont transmission in the shipworms in the OGL colony, we first sought to localize
107 bacterial symbionts within adult gill tissue (Fig 1A). Fluorescence in situ hybridization (FISH) was
108 performed on paraffin-embedded 5 um thick sections from whole adult specimens. Experiments
109 were performed with a general bacterial probe (EUB338) that is complementary to a portion of
110 16S rRNA found in all bacteria, and control probe (NON338) which accounts for non-specific
111 binding of EUB338 [24]. These probes were used previously to localize bacterial symbionts in
112 shipworm tissue [19, 25, 26], including *L. pedicellatus* [18]. Specific fluorescent signals were
113 observed in the gills of all adult specimens examined (Fig 1B and 1C, Fig S1). Signal from the gill
114 appears to come primarily from the interlamellar junction (center of the body), rather than the gill
115 filament (Fig 1B and 1C), which is consistent with the previously described location of
116 endosymbiont containing bacteriocytes [18]. All of the adult specimens examined had sections of
117 the gill that contained bright EUB338 signal, and sections without any visible signal. This
118 phenomenon is most likely due to the spatial orientation of the specimen within the paraffin block
119 prior to embedding, as shipworms tend to curl during fixation. Alternatively, variation in signal
120 could indicate variability in the concentration of symbionts along the gill. Hybridization with the
121 NON338 did not generate the florescent signals (Fig S1).

122 These observations are consistent with previous studies and suggest that adult *L.*
123 *pedicellatus* shipworms harbor symbiotic bacteria within their gill tissue [18]. We took two
124 approaches to examine bacterial symbionts across *L. pedicellatus and/or T. bartschi* ontogeny,
125 and thus determine the mode of symbiont transmission for the OGL shipworm colony: 1) quantify
126 bacterial abundance through culturing, and 2) localize bacteria within shipworm tissue using FISH
127 and confocal microscopy.

128

129 **Endosymbiotic bacteria can be cultured from juvenile yet not larval *L. pedicellatus and/or* 130 *T. bartschi* shipworms.** Although many endosymbiotic microbes remain unculturable, a portion 131 of the *L. pedicellatus and/or T. bartschi* gill microbiome can be readily cultured adult specimens 132 and importantly, *Teredinibacter turnerae,* is commonly isolated from adult *T. bartschi* specimens 133 [15, 22, 23]. Therefore, to begin determining bacterial abundance in shipworms of different life 134 stages, we quantified bacterial colony forming units (CFUs) from shipworm tissue using shipworm 135 basal medium (SBM) that is selective for marine cellulolytic bacteria, including shipworm 136 symbionts. Shipworms were collected from the OGL colony at the following life stages: burrowing 137 juveniles, and developed juveniles were extracted from wood, veliger larvae were collected from 138 the surface of wood or the water column, and brooding larvae were dissected from the gill tissue 139 of gravid adults. Specimens were either rinsed with filter sterilized autoclaved sea water (FSASW) 140 five times and dissected/homogenized immediately or flash frozen for 24 hours prior to 141 dissection/homogenization. Brooding larvae and veliger larvae were pooled into groups of 3 142 larvae prior to homogenization. Tissue homogenate was then serially diluted and plated onto SBM 143 agar plates to quantify bacterial abundance across ontogeny. CFUs were not detected in any 144 brooding larvae regardless of sample preparation (Fig 2) and CFUs were detected at the limit of 145 detection (1 CFU at 10^0 dilution) in 2 out of 8 pools (6 of 24 larvae) of FSASW rinsed veliger 146 larvae, yet not any frozen veliger larvae (Fig 2). Burrowing juveniles that had begun initial 147 metamorphosis and minor burrowing into the wood contained approximately 10^5 CFUs per animal, 148 while more developed juveniles that had begun to elongate contained approximately 10^6 CFUs 149 per animal (Fig 2). There was no significant difference in the number of CFUs collected between 150 only rinsed or rinsed and then frozen specimens at the life stages examined.

151 These data yield two important observations: 1) neither brooding nor veliger larvae contain 152 a detectable level of endosymbiotic, culturable bacterial symbionts, and 2) juveniles that have 153 begun go undergo metamorphosis (burrowing and more developed juveniles) contain a 154 substantial community of culturable bacteria. These data are consistent with a hypothesis

155 whereby *L. pedicellatus* and/or *T. bartschi* acquire endosymbiotic bacteria from the environment
156 rather than directly from a parent during gestation.
157
158 **Symbionts can be visualized in juvenile *L. pedicellatus* and/or *T. bartschi* through FISH**
159 **microscopy.** We sought to test the hypothesis that *L. pedicellatus* and/or *T. bartschi* are
160 aposymbiotic in early development by determining the spatial localization of bacterial symbionts
161 within shipworm tissue using FISH and confocal microscopy. Given that our CFU data suggest
162 that shipworms within the OGL colony do not acquire bacterial symbionts until they begin to
163 burrow into wood, we predicted that we would not detect a bacterial signal in brooding or veliger
164 larvae, and only observe a signal in more developed juveniles that had already begun to burrow.
165 We first examined brooding larvae by imaging gravid adults. Although a signal was observed in
166 adult gill lamellae, which is where brooding larvae reside, we were unable to detect a EUB338
167 signal from any brooding larvae themselves (Fig 3A, Fig S2) (n=34), suggesting that these larvae
168 do not harbor endosymbiotic bacteria.
169 We next interrogated veliger larvae that were collected from the water column and the
170 surface of wooden baits, and juvenile shipworms that had just begun to burrow into wooden baits
171 for the presence of a EUB338 bacterial signal. Similar to the brooding larvae, no detectable signal
172 was observed for veliger larvae (Fig 3B, Fig S3) (n=8). However, the majority of more developed
173 larvae (8 out of 10) did contain a bright signal with the EUB388 probe in tissue resembling
174 bacteriocytes within adult gill tissue (Fig 3C, Fig S4). Hybridization with the NON338 negative
175 control probe did not generate a florescent signal (Fig S4). These data suggest that *L. pedicellatus*
176 and/or *T. bartschi* have an aposymbiotic life stage, as brooding and veliger larvae, and acquire
177 their symbionts by the time they have begun metamorphosis and burrowing.
178
179 ***L. pedicellatus* and/or *T. bartschi* larvae acquire bacteria through indirect horizontal**
180 **transmission.** Finally, we sought to determine the environmental source of horizontally acquired

181 bacterial symbionts for juvenile *L. pedicellatus* and/or *T. bartschi* shipworms. Based on the CFU
182 and FISH microscopy data, we predicted that juvenile shipworms acquire their symbionts either
183 from the water column, or the wood on which the settle and burrow. We performed a series of
184 tank experiments in which veliger larvae were collected from adult tanks, rinsed with FSASW, and
185 allowed to settle on either sterile (autoclaved) or nonsterile wooden baits in either FSASW or
186 larva-free water collected from tanks containing adult, larvae-producing *L. pedicellatus* and/or *T.*
187 *bartschi*. We first tested whether larvae could settle and metamorphose in a “sterile” environment,
188 on autoclaved wooden baits in FSASW, and did not observe any settlement or metamorphosis
189 (n=93) (Table 1). We repeated this experiment with filtered water collected from adult tanks and
190 similarly did not observe any settlement or metamorphosis on sterilized baits (n=94) or non-
191 sterilized baits (n=76) (Table 1). We then repeated the experiment with adult tank water, non-
192 sterilized baits, and an additional bait containing non-larvae producing adult shipworms. In this
193 treatment, we observed settlement, burrowing, and initial metamorphosis for 13% of larvae (+/-
194 3% standard deviation) (n=78) (Table 1).

195 Results of these tank experiments yield several important findings: 1) *L. pedicellatus*
196 and/or *T. bartschi* larvae are unable to settle and burrow into wood in a sterile environment, 2)
197 larvae-free water from adult tanks does not induce burrowing, and 3) presence of adult
198 shipworms, whether larvae-producing or not, promotes larval settlement. These findings
199 combined with our culture and FISH microscopy data support a hypothesis whereby *L.*
200 *pedicellatus* and/or *T. bartschi* have an aposymbiotic life stage during early development and
201 horizontally acquire their symbionts prior to or during settlement and early metamorphosis.

202

203 **DISCUSSION**

204 In this work, we sought to determine the mode of transmission for the widely-distributed
205 shipworms, *L. pedicellatus* and/or *T. bartschi*. Previous work on this model shipworm revealed
206 they harbor endosymbiotic, intracellular gill bacteria [15, 19]. These communities provide

207 important cellulase and nitrogenase enzymes to their hosts [3, 15, 16, 27-29] and are relatively
208 simple, composed of *Teredinibacter turnerae* and other gram-negative proteobacteria [15, 23].
209 Using a combination of culturing, confocal microscopy, and tank experiments, our data suggest
210 that *L. pedicellatus* and/or *T. bartschi* do not acquire their endosymbiotic bacteria vertically but
211 rather through horizontal transmission. Our findings support a model whereby brooding larvae in
212 adult gill tissue and veliger larvae are aposymbiotic and acquire their symbionts at some point
213 during settlement on wood and/or as they begin burrowing and metamorphosis.

214 Horizontal acquisition of gill symbionts is consistent with work predicting that *T. turnerae*
215 is a facultative rather than obligate intracellular symbiont. *T. turnerae* lacks many features
216 associated with an obligate intracellular life style such as reduced genome size, skewed GC
217 content, and loss of core metabolic genes [22]. Further, *T. turnerae* has characteristics consistent
218 with a free-living lifestyle including mechanisms to defend against bacteriophage, production of
219 secondary metabolites and the ability to be cultivated *in vitro* without added vitamins or growth
220 factors [20, 22]. The gene content of *T. turnerae* may provide a hint as to its possible
221 environmental niches outside of the host. For example, *T. turnerae* appears to be a specialist for
222 woody plant materials as it has extensive cellulolytic capabilities but lacks enzymes associated
223 with common marine polysaccharides such as agar, alginate, and fucoidan [22]. Given that *L.*
224 *pedicellatus* and/or *T. bartschi* appear to acquire their bacterial symbionts during association with
225 wooden baits, it is possible that larvae acquire *T. turnerae* from the wood surface either prior to
226 or during initial burrowing. Future work will need to explore whether *T. turnerae* are capable of
227 surviving on the surface of submerged wood in the absence of a shipworm host and/or
228 planktonically in the water column.

229 Although we were unable to determine the specific environmental source of horizontally
230 acquired shipworm symbionts, our data suggest that juvenile shipworms may acquire them
231 through indirect horizontal transmission. Indirect horizontal transmission occurs when a symbiont
232 is transferred from a host to the environment and then another host [30], and has been observed

233 in other marine symbioses. For example, in the horizontally acquired symbiosis between the
234 *Euprymna scolopes* squid and bioluminescent bacterium *Vibrio fischeri*, adult squid release
235 symbiotically competent bacteria into the water column daily, both seeding the environment with
236 potential symbionts for the next generation and controlling symbiont growth [31-33]. In corals,
237 which primarily acquire their algal symbionts horizontally [34], proliferating *Symbiodinium* cells
238 are preferentially expelled over nonproliferating cells as a way to control symbiotic populations
239 [35] and may also provide a pool of symbionts for coral recruits. Such a phenomenon may also
240 occur for *L. pedicellatus* and/or *T. bartschi*, possibly serving to control symbiont growth within gill
241 tissue and provide juveniles with a pool of potential symbionts.

242 Our findings contrast previous work describing that a different species of shipworm,
243 *Bankia setacea,* acquires at least one bacterial symbiont through vertical transmission [21].
244 Shipworms are an incredibly diverse group of marine bivalves with unique communities of gill
245 symbionts [2, 15, 20, 36] and varied reproduction strategies from broadcast spawning to brooding.
246 *B. setacea* is a broadcast spawner, releasing gametes into the water column where they fully
247 develop, which enables a broad distribution of larval settlement [17, 37]. In contrast, *L.*
248 *pedicellatus* and *T. bartschi* are a long-term brooders, allowing their larvae to fully develop within
249 brood pouches in the gills [38]; *L. pedicellatus* and *T. bartschi* larvae released into the water
250 column rapidly settle within hours and begin boring into wood and metamorphose within days [39-
251 41]. While vertical transmission ensures juvenile *B. setacea* can settle uncolonized wood,
252 potentially reducing competition between parents and progeny [21], horizontal transmission may
253 increase the diversity of symbionts within a shipworm population and favor selection acting on
254 symbionts to benefit their hosts [42]. Notably, the apparent correlation between reproductive
255 strategy and symbiont transmission mode in shipworms is the inverse of observations of
256 Scleractinian corals [5]. In stony corals horizontal transmission is prevalent in broadcast
257 spawners, allowing highly dispersive larvae that can travel long distances to sample from their
258 local environment, while vertical transmission is common in brooders [43, 44]. Additional studies

259 are required to determine whether this trend is apparent across diverse Teredinidae and to
260 elucidate the ecological and evolutionary benefits of horizontal symbiont transmission for *L.*
261 *pedicellatus and/or T. bartschi*.

262 Because this work focused on the mode of transmission for *L. pedicellatus and/or T.*
263 *bartschi* gill symbionts, the composition of such communities across ontogeny remains unknown.
264 Although we were able to culture bacteria consistent with *T. turnerae* physiology [45] from
265 burrowing and more developed juvenile shipworms, it is unclear whether they are the only
266 member of the community at this stage, or whether the community is more complex. Previous
267 work identified at least four distinct bacterial genotypes, including *T. turnerae,* within adult *L.*
268 *pedicellatus* gill bacteriocytes [15]. Therefore, further investigation should focus on the
269 composition of juvenile *L. pedicellatus and/or T. bartschi* microbiomes throughout morphogenesis
270 and into adulthood, and whether symbiont acquisition is an acute event [46] or continues to occur
271 throughout the shipworm lifespan, as is the case with other marine bivalves [47].

272

273 **METHODS**

274 **Cultivation and collection of *L. pedicellatus and/or T. bartschi.*** A colony of *L. pedicellatus*
275 *and T. bartschi* shipworms were collected from found wood in mangroves in the Banana River
276 north of Kelly Park, Merit Island on January 24, 2020 and on the Pineda Causeway on January
277 25, 2020 and maintained at the Ocean Genome Legacy Center at Northeaster's Marine Science
278 Center in Nahant, MA. Animals were kept between 25 and 27 C in natural seawater from Nahant
279 at a salinity of 31 ppt.

280 Adult animals, burrowing juveniles, and more developed juveniles were removed from
281 wooden baits by hand, transferred to filter sterilized autoclaved sea water (FSASW) (0.2 um pore-
282 size filter) and processed (fixed in 4% paraformaldehyde (4% PFA), dissected, or homogenized)
283 within two hours. Veliger larvae were collected via plastic transfer pipettes, transferred to filter

284 sterilized sea water and processed within two hours. Brooding larvae were dissected from gravid
285 adults, rinsed in FSASW, and processed within two hours.
286
287 **Symbiont culture quantification.** Freshly collected specimens were rinsed with FSASW five
288 times and either processed immediately or frozen at -80°C for at least 24 hours and then
289 processed. Tissue was homogenized using 1.5mL tubes and disposable homogenizer pestles.
290 Tissue homogenate was then serially diluted and plated onto SBM agar plates. Plates were
291 incubated at room temperature and checked daily for colony forming units (CFUs). Brooding and
292 veliger larvae were pooled into groups of three for homogenization and CFU quantification.
293 Treatments where no CFUs were observed are indicated as having the limit of detection, 1 CFU
294 at 10^0 dilution.
295
296 **Shipworm fixation and Fluorescence *in situ* hybridization (FISH) microscopy.** Shipworm
297 specimens were fixed, embedded in paraffin, sectioned, and hybridized as described in Betcher
298 *et al.* [18]. Briefly, freshly collected specimens were fixed in 4% PFA in 0.22 μm filtered seawater
299 and incubated at 4°C for 1-3 hours. Each organism was then washed in a series of increasing
300 ethanol solutions (30%, 50%, and 70% x3) for 10 – 15 minutes. Samples were then decalcified in
301 5% acetic acid in miliQ shaking at 50 rpm overnight at room temperature. Once fully decalcified,
302 samples were again washed in a series of ethanol solutions (30%, 50%, and 70% x3) for 10 - 15
303 minutes. Larval and juvenile samples were then embedded in autoclaved 4% low melt agarose in
304 PBS to prevent shearing during sectioning. Agar blocks were allowed to harden at room
305 temperature for at least 20 minutes, removed from the mold, and fixed in 4% PFA and stored at
306 4°C until paraffin embedding.
307 Paraffin tissue embedding was performed with an automated tissue processor at Beth
308 Israel Deaconess Medical Center, Boston, MA. Paraffin blocks were then mounted onto the

309 microtome, trimmed, sectioned into 5 um thick sections, and mounted onto glass slides and stored
310 at -20°C.
311 Samples were deparaffinized, rehydrated, and hybridized at the Northeastern University
312 Marine Science center. Samples were deparaffinized by incubating slides in a series of xylene
313 and ethanol solutions using Coplins jars for 3 minutes / solution: xylene x2, 1:1 xylene:100%
314 ethanol, 100% ethanol x2, 95% ethanol, 70% ethanol, and 50% ethanol. Slides were then
315 rehydrated with nanopure water. Oligonucleotide probe hybridizations were performed using
316 Bact338 (seq), a general bacterial-targeted 16S rRNA probe, or NON338 (seq) as a negative
317 control. Probes were mixed in a 1:9 volume solution (5 ng/ul probe final concentration) with
318 hybridization buffer (35% formamide, 0.9 M NaCl, 20 mM Tris-HCl (pH 8.0), 0.01 % SDS). Slides
319 were incubated with each probe for 90 minutes at 46°C, rinsed with washing buffer (35%
320 formamide, 0.7 M NaCl, 20 mM Tris-HCl (pH 8.0), 0.01% SDS) and incubated in preheated
321 washing buffer at 48°C for 25 minutes. Slides were then rinsed with ice cold miliQ, allowed to air
322 dry in the dark, covered in one drop of antifade and a cover slip and allowed to cure in the dark
323 at room temperature for 24 hours. Slides were visualized on a Zeiss LSM 800 inverted confocal
324 laser scanning microscope at the Institute for Chemical Imaging of Living Systems at Northeastern
325 University. Images were visualized and processed using ImageJ software.
326
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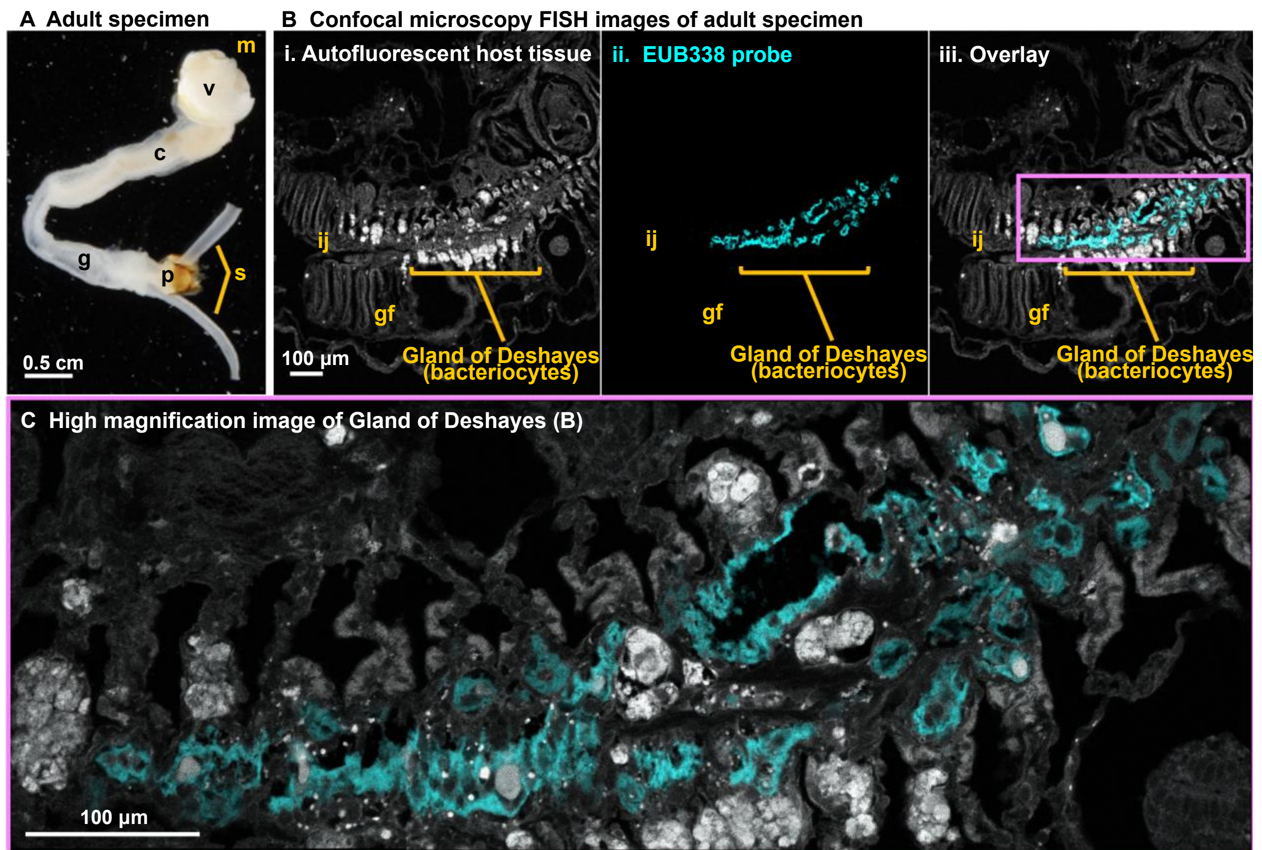
332
333

334 **Figures & Tables**



335

336 **Figure 1. EUB338 can be used to detect intracellular bacteria within adult *L. pedicellatus***
337 **and/or *T. bartschi* Gland of Deshayes.** (A) Adult shipworm specimen from the OGL colony. (B)
338 Confocal microscopy FISH images of a representative adult specimen focused on the gill; each
339 panel shows: i. autofluorescent host tissue, ii. signal from the eubacterial EUB338 probe, and iii.
340 an overlay of i and ii. Control images with the non-specific NON338 probe are shown in
341 supplemental information. (C) High magnification image of gland of Deshayes indicated by the
342 pink box in Biii. Abbreviations are as follows: cecum (c), gill (g), gill filament (gf), interlamellar
343 junction (ij), mouth (m), pallet (p), siphon (s), valve or shell (v).

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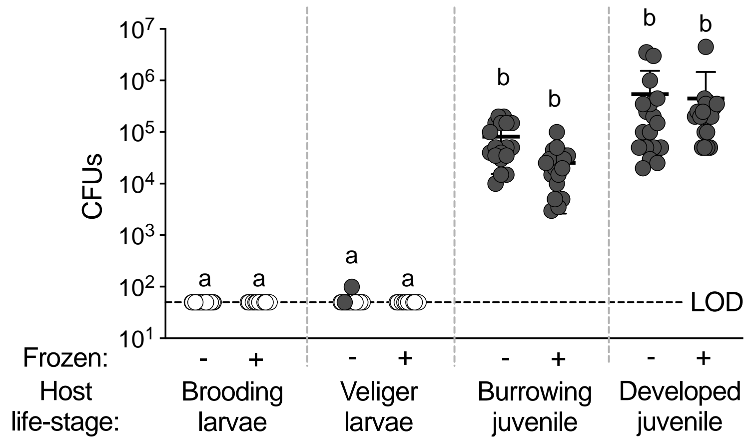

347 **Figure 2. Bacteria can be cultivated from juvenile shipworms.** Total CFUs from larvae that
348 were rinsed with filter-sterilized and autoclaved sea water (-) or rinsed and then frozen for 24
349 hours (+) and plated onto SBM media. Host developmental stage is indicated on x-axis. Each
350 circle represents CFUs for 1 (juvenile) or 3 (larvae) organisms. White circles indicate limit of
351 detection (< 1 CFU from undiluted spot). Each experiment was performed twice with at least 8
352 biological replicates.
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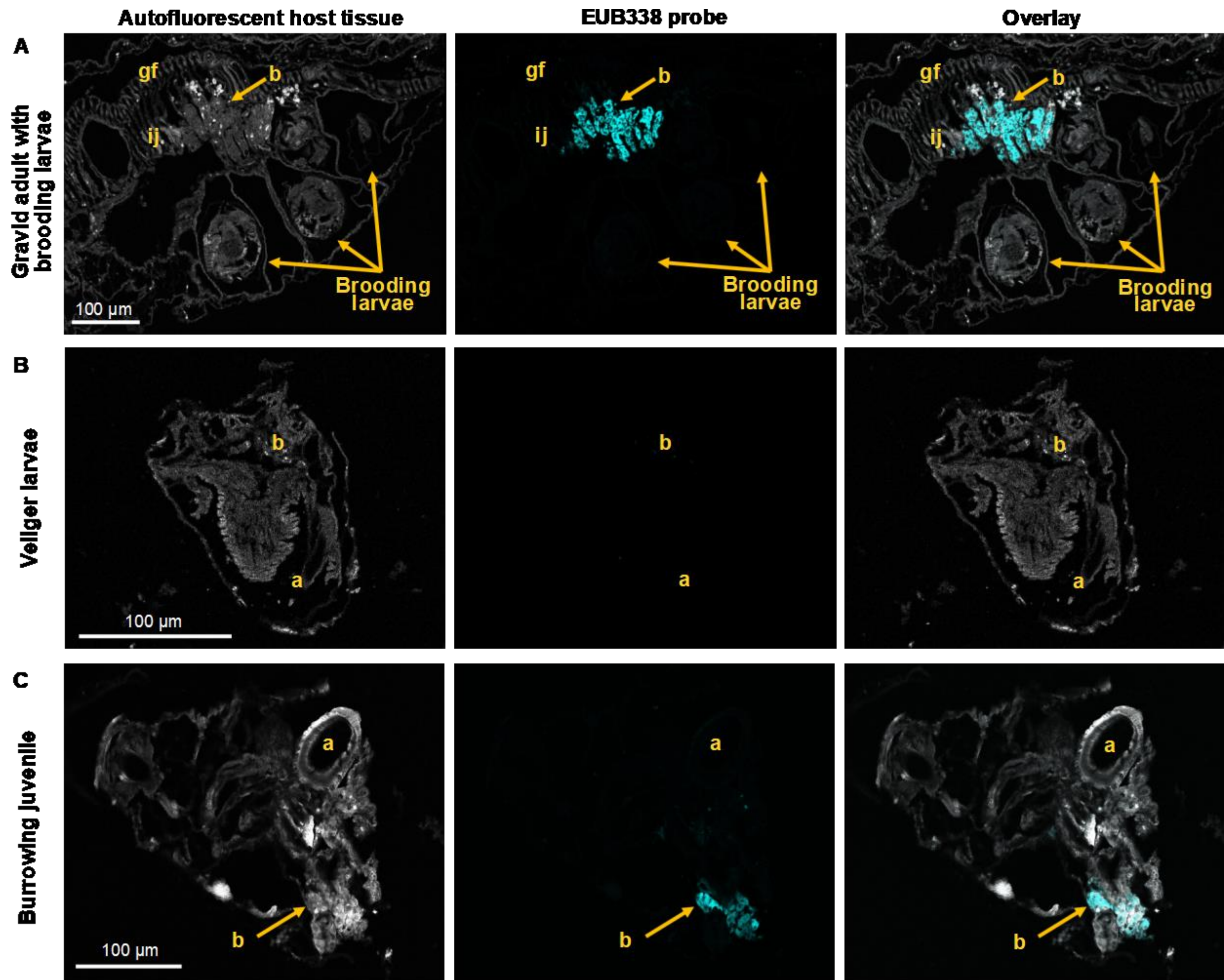

355 **Figure 3. Bacteria are detected in burrowing juvenile shipworms via FISH.** Confocal
356 microscopy images of representative shipworms across ontogeny: A) brooding larvae within
357 gravid adults, B) veliger larvae, and C) burrowing juveniles that have begun metamorphosis; each
358 panel shows: autofluorescent host tissue (left, gray), signal from the eubacterial EUB338 probe
359 (center, cyan), and an overlay of the two (right). Control images with the non-specific NON338
360 probe are shown in supplemental information. At least eight animals were imaged at each life
361 stage. Abbreviations are as follows: adductor (a), bacteriocytes (b), gill filament (gf), interlamellar
362 junction (ij), and pallet (p). Scale bars indicate 100 μm.
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374 **Table 1. Results of settlement experiments in sterile, semi-sterile, and standard conditions.**
375 Veliger larvae were collected from the water column of adult-containing tanks, rinsed with
376 FSASW, and transferred to tank conditions indicated in the first column. Three to four trials of
377 each experiment were performed with at least 21 veliger larvae per trial (total larvae n=341).

Tank Conditions	Trial #	# veliger larvae	# burrowing juveniles
Sterilized baits in FSASW	1	25	0
	2	22	0
	3	21	0
	4	25	0
Sterilized baits in adult tank water	1	25	0
	2	23	0
	3	22	0
	4	24	0
Non-sterilized baits in adult tank water	1	25	0
	2	25	0
	3	26	0
Non-sterilized baits in adult tank water with non -larvae producing adults	1	25	4
	2	26	3
	3	27	3

378

379 **Table 2. Number of *L. pedicellatus* specimens examined via FISH and confocal**
380 **microscopy.** The number of specimens with a detectable EUB338 signal are indicated in the
381 right column; at least two separate experiments were performed for animals at each life stage.

Life Stage	# of specimens	EUB338 signal
Adult	4	4
Brooding larvae	34	0
Veliger larvae	8	0
Burrowing juvenile	10	8

382

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