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# Pronounced and prevalent intersexuality does not impede the 'Demon Shrimp' invasion

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Crustacean intersexuality is widespread and often linked to infection by sex-distorting parasites. However, unlike vertebrate intersexuality, its association with sexual dysfunction is unclear and remains a matter of debate. The 'Demon Shrimp', *Dikerogammarus haemobaphes*, an amphipod that has invaded continental waterways, has recently become widespread in Britain. Intersexuality has been noted in *D. haemobaphes* but not investigated further. We hypothesise that a successful invasive population should not display a high prevalence of intersexuality if this condition represents a truly dysfunctional phenotype. In addition, experiments have indicated that particular parasite burdens in amphipods may facilitate invasions. The rapid and ongoing invasion of British waterways represents an opportunity to determine whether these hypotheses are consistent with field observations. This study investigates the parasites and sexual phenotypes of *D. haemobaphes* in British waterways, characterising parasite burdens using molecular screening, and make comparisons with the threatened *Gammarus pulex* natives. We reveal that invasive and native populations have distinct parasitic profiles, suggesting the loss of *G. pulex* may have parasite-mediated eco-system impacts. Furthermore, the parasite burdens are consistent with those previously proposed to facilitate biological invasions. Our study also indicates that while no intersexuality occurs in the native *G. pulex*, approximately 50% of *D. haemobaphes* males present pronounced intersexuality associated with infection by the microsporidian *Dictyocoela berillonum*. This unambiguously successful invasive population presents, to our knowledge, the highest reported prevalence of male intersexuality. This is the clearest evidence to date that such intersexuality does not represent a form of debilitating sexual dysfunction that negatively impacts amphipod populations.

## Pronounced and prevalent intersexuality does not impede the 'Demon Shrimp' invasion

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### Abstract

Crustacean intersexuality is widespread and often linked to infection by sex-distorting parasites. However, unlike vertebrate intersexuality, its association with sexual dysfunction is unclear and remains a matter of debate. The 'Demon Shrimp', *Dikerogammarus haemobaphes*, an amphipod that has invaded continental waterways, has recently become widespread in Britain. Intersexuality has been noted in *D. haemobaphes* but not investigated further. We hypothesise that a successful invasive population should not display a high prevalence of intersexuality if this condition represents a truly dysfunctional phenotype. In addition, experiments have indicated that particular parasite burdens in amphipods may facilitate invasions. The rapid and ongoing invasion of British waterways represents an opportunity to determine whether these hypotheses are consistent with field observations. This study investigates the parasites and sexual phenotypes of *D. haemobaphes* in British waterways, characterising parasite burdens using molecular screening, and make comparisons with the threatened *Gammarus pulex* natives. We reveal that invasive and native populations have distinct parasitic profiles, suggesting the loss of *G. pulex* may have parasite-mediated eco-system

24 impacts. Furthermore, the parasite burdens are consistent with those previously proposed to  
25 facilitate biological invasions. Our study also indicates that while no intersexuality occurs in the native  
26 *G. pulex*, approximately 50% of *D. haemobaphes* males present pronounced intersexuality associated  
27 with infection by the microsporidian *Dictyocoela berillonum*. This unambiguously successful invasive  
28 population presents, to our knowledge, the highest reported prevalence of male intersexuality. This is  
29 the clearest evidence to date that such intersexuality does not represent a form of debilitating sexual  
30 dysfunction that negatively impacts amphipod populations.

### 33 Introduction

34 *Dikerogammarus haemobaphes* (Eichwald, 1841), an effective predatory amphipod from the Ponto-  
35 Caspian (Bacela-Spychalska & van der Velde, 2013), has spread through Europe and is now recognised  
36 as an extremely successful invader of British waterways (Green Etxabe & Ford, 2014). *D.*  
37 *haemobaphes*, also known as the 'demon shrimp', invaded the British Isles more recently than the  
38 infamous 'killer shrimp' (*Dikerogammarus villosus*, Sowinsky, 1894) (MacNeil et al., 2010) but is  
39 already more widespread (Fig. 1). Amphipods harbour many parasites that can drastically impact host  
40 populations by influencing the health, behaviour, reproduction and sex determination of their host  
41 (Hatcher & Dunn, 2011; Bacela-Spychalska et al., 2012). The invasive *D. haemobaphes*, therefore,  
42 could not only out compete and prey on native amphipod species, but also introduce parasites into  
43 their new habitats. Screening parasites in invasive and native amphipod species associated with a  
44 rapid and on-going invasion will test hypotheses that particular parasitic burdens impact invasion  
45 success (MacNeil et al., 2003a; Hatcher & Dunn, 2011; Hatcher et al., 2014).

46

47 Some amphipod-infecting parasites maximise their transmission via the host's progeny by converting  
48 males into reproductive females (Ford 2012). Infection by such parasites results in sex-biased  
49 populations (Terry et al., 2004) and, in cases of incomplete conversion, intersexuality, where  
50 individuals present secondary sex characteristics of both genders (Ford 2012). Intersex phenotypes  
51 are found in a range of animals (Matthiessen & Gibbs, 1998; Harris et al., 2011; Hayes et al., 2002),  
52 including crustaceans (Ginsburger-Vogel, 1991; Bishop, 1974; Ford, 2012), where they are linked to  
53 parasitic infection (Li, 2002; Short et al., 2012a) and environmental conditions (Dunn, McCabe &  
54 Adams., 1996), as well the direct (Short et al., 2012b) and indirect (Jacobson et al., 2010) influence of  
55 contaminant exposure. In cases of parasitic infection, an incomplete conversion is thought to occur  
56 due to insufficient parasite burden, suboptimal conditions, or effective host responses (Dunn &  
57 Rigaud 1998; Kelly, Dunn & Hatcher, 2002; Short et al., 2014). Current evidence suggests the impact  
58 of female intersexuality is subtle (Ford et al., 2003; Kelly, Hatcher & Dunn, 2004) or effectively non-  
59 existent (Glazier, Brown & Ford, 2012), and the female intersexuality observed in *D. haemobaphes*  
60 successfully invading Polish waterways (Bacela, Konopacka & Grabowski, 2009) is consistent with  
61 these hypotheses.

62

63 Male intersexuality is widespread in amphipods, however, our understanding of its reproductive  
64 consequences is poorly understood relative to vertebrates (Harris et al., 2011). The extents of  
65 morphological and behavioural changes (McCurdy et al., 2008; Yang, Kille & Ford, 2008) have led to  
66 the suggestion (Yang, Kille & Ford, 2008; Ford 2012) that the impact of crustacean male intersexuality  
67 may be similar to that seen in vertebrates (Harris et al., 2011). Despite some evidence of

68 intersexuality in invasive *D. haemobaphes* (Bacela, Konopacka & Grabowski, 2009), sexual phenotypes  
69 in this species have not been studied, even though notable levels of intersexuality in the  
70 unambiguously successful invading population would reveal considerable insight into the  
71 consequences of intersexuality for wild crustacean populations.

72 This study investigates the sexual phenotypes and parasites of *D. haemobaphes* and the native  
73 *Gammarus pulex* (Linnaeus, 1758) at multiple locations in British waterways to give insights into this  
74 rapidly invading species and expand our understanding of crustacean intersexuality.

75

## 76 **Methods**

77

### 78 **Specimen characterisation**

79 Amphipods were collected from Wallingford Bridge and Bell Weir, U.K. Amphipods were categorised  
80 into species and phenotypes: males, females, intersex males and intersex females. Intersex males  
81 were identified by genital papillae, between pereonite 7 and pleonite 1, in conjunction with  
82 rudimentary oostegites. Intersex females were identified by oostegites in conjunction with secondary  
83 genital papilla/e. Animals from each phenotype were measured from antennal joint to telson to  
84 obtain body length (ImageJ, v1.4u4) and comparisons were made using analysis of variance (ANOVA)  
85 with the post hoc Tamhanes-T2 test (SPSS v21).

86

### 87 **Scanning electron microscopy**

88 Specimens of *D. haemobaphes* were taken through transitional steps (100% ethanol to 100%  
89 hexamethyldisilazane, HMDS) then evaporated to dryness. The dry samples were mounted on SEM

90 stubs, sputter coated with gold-palladium and examined using a scanning electron microscope (JEOL  
91 6060LV) operating in high vacuum mode with a secondary electron detector active at an acceleration  
92 voltage of 10kV. Images were cropped and coloured using Adobe Photoshop (CS5v12).

93

#### 94 **PCR screen**

95 DNA was purified from internal animal tissue (excluding gut) or eggs using the DNeasy Blood and  
96 Tissue Kit (Qiagen, UK). Samples were screened using previously described PCR primers for general  
97 parasites (Table 1). PCR reactions were performed in 25 µl volumes containing 10 ng of DNA as  
98 template, 1 U of Taq polymerase (Promega, UK), 5 µl of 5x PCR buffer, 1.25 mM MgCl<sub>2</sub> and 0.4 mM of  
99 each corresponding primer. Quality of the DNA samples were analysed using the primers 1073F and  
100 18SR (Table 1) which amplified a 867bp product of the host 18S.

101

102

#### 103 **Sequence identification**

104 PCR products were analysed using agarose gel electrophoresis containing 1x GelGreen™ (Cambridge  
105 Bioscience, UK) for the presence of bands potentially representing amplified parasite sequences.  
106 Individual bands were isolated and DNA extracted using the QIAquick Gel Extraction Kit (Qiagen, UK)  
107 and sequenced (Source Bioscience, UK), before a BLAST analysis was performed against sequences  
108 stored in GenBank (NCBI).

109

#### 110 **Results**

#### 111 **Sexual phenotypes**

112 Pronounced male intersex phenotypes were found in *D. haemobaphes* at both sites, with most  
113 specimens displaying well-developed oostegites with visible setae (Fig. 2). Almost half the male  
114 population presented intersex characteristics at both locations and very few cases of female  
115 intersexuality were observed (Fig. 3a). *G. pulex* was only found in conjunction with *D. haemobaphes*  
116 at one sampling site and no intersex phenotypes were found (Fig. 3a). Significant differences were  
117 found in lengths of *D. haemobaphes* phenotypes ( $F = 3.885$ ,  $df = 2$ ,  $p = 0.023$ ) where normal males  
118 ( $14.85\text{mm} \pm 3.65$ ,  $N = 32$ ) are significantly larger ( $p = 0.04$ ) than females ( $13.01\text{mm} \pm 2.29$ ,  $N = 52$ ).  
119 However, there is no significant difference between intersex males ( $13.57\text{ mm} \pm 3.10$ ,  $N = 37$ ) and  
120 either females ( $p = 0.735$ ) or males ( $p = 0.328$ ), therefore forming an intermediate size.

121

122

### 123 **Parasite screening**

124 Screening of *D. haemobaphes* and *G. pulex* populations revealed evidence of infection by several  
125 parasites (Table 2). All *D. haemobaphes* females and intersex males were found infected with *D.*  
126 *berillonum*, with one female weakly infected (Fig. 3b), as previous defined (Yang et al., 2011). The  
127 majority of males were also infected, although more weak infections were found (Fig. 3b). This  
128 pattern of *D. berillonum* infection was consistent at both collection sites and when combined in a  
129 Fishers Exact test (two-tailed) reveal a significant difference in the level of infection between normal  
130 and intersex males ( $p = 0.003$  using strong infections only,  $p = 0.02$ , using weak and strong infections).  
131 To confirm vertical transmission, the broods of ten infected females were also tested and all were  
132 infected by *D. berillonum*. Only one case of weak *D. berillonum* infection was found in *G. pulex* (Fig.  
133 3b).



134

135 **Discussion**

136 Our screen of invasive and native species associated with an extremely successful, and ongoing,  
137 amphipod invasion reveals parasitic-profiles strikingly consistent with hypotheses that particular  
138 parasitic burdens influence the dynamics of biological invasion (MacNeil et al., 2003a; Hatcher &  
139 Dunn, 2011; Hatcher et al., 2014). The native *G. pulex* are infected with a microsporidian of the genus  
140 *Pleistophora*, which include behaviour altering species known to increase the likelihood of predation  
141 on native amphipods and reduce their predatory behaviour when interacting with invaders (MacNeil  
142 et al., 2003a; Fielding et al., 2005). Sequences were also found for an acanthocephalan, most likely  
143 *Echinorhynchus truttae*. This species can both reduce its host's predatory behaviour and increase  
144 vulnerability to predation by fish (Fielding et al., 2003; MacNeil et al., 2003b; Lagrue, Güvenatam &  
145 Bollache, 2013). Consequently, the parasite burden of *G. pulex* may facilitate invasion of *D.*  
146 *haemobaphes* through British waterways by impairing the competitive abilities of the native  
147 population, a scenario consistent with recent experiments and population modelling (MacNeil et al.,  
148 2003a; Haddaway et al., 2012; Hatcher et al., 2014). In contrast, the invasive *D. haemobaphes* was  
149 almost ubiquitously infected by the vertically transmitted microsporidian *Dictyocoela berillonum*. It is  
150 possible the initial invasive population consisted of a small number of infected individuals and the  
151 current infection prevalence represents a parasitic founder-effect. Alternatively, given that parasite  
152 infection is predicted to influence invasion success (MacNeil et al., 2003a; Fielding et al., 2005;  
153 Hatcher et al., 2014) via trait-mediated effects, it is possible the high prevalence of *D. berillonum*  
154 occurs due to a subsequent enhancement in invasive capabilities.

155 The distinct parasitic profiles of *G. pulex* and *D. haemobaphes* may have ecological impacts.  
156 Our results suggest the eradication of native *G. pulex* would lead to the removal of a pleistophoran  
157 microsporidian from the ecosystem potentially capable of causing disease in fish (Lom & Nilsen, 2003)  
158 and an acanthocephalan indistinguishable from *E. truttae* (García-Varela & Nadler, 2005). Although *E.*  
159 *truttae* infection in fish does not appear to cause morbidity (Dorucu et al., 1995), infected amphipods  
160 are more vulnerable to fish predation due to altered habitat usage (MacNeil et al., 2003b; Lagrue,  
161 Güvenatam & Bollache, 2013). Therefore, loss of this parasite may alter prey abundance, even if the  
162 overall amphipod biomass is maintained following the displacement of *G. pulex*.

163 The sexual phenotype survey revealed that while no intersexuality was evident in *G. pulex*, the  
164 invasive *D. haemobaphes* presents striking levels of pronounced male intersexuality, where males  
165 exhibit unambiguous oostegites possessing rudimentary seta, and their size is not significantly  
166 different from males or females. In contrast, the low levels of female intersexuality in *D.*  
167 *haemobaphes* were much like those previously reported in Polish waters (Bacela, Konopacka &  
168 Grabowski, 2009). To our knowledge, this is the highest prevalence of male intersexuality recorded in  
169 an amphipod population (McCurdy et al., 2004; Ford & Fernandes, 2005; Short et al., 2012b; Yang et  
170 al., 2011) and is the first evidence clearly linking *D. berillonum* with amphipod intersexuality (Terry et  
171 al., 2004; Yang et al., 2011). Other *Dictyocoela* species have been linked to both abnormal sexual  
172 phenotypes and female-biased sex ratios (Terry et al., 2004; Short et al., 2012a), however, the lack of  
173 female-bias in *D. haemobaphes* suggests *D. berillonum* is unable to fully convert males in to females.  
174 This could result from sub-optimal environmental conditions impacting the efficacy of conversion or  
175 the consequence of *D. berillonum* infecting an unfamiliar host. Whatever the cause, the *D.*  
176 *haemobaphes* intersexuality is of interest. The association between male intersexuality and sexual

177 dysfunction is, despite recent molecular advances (Short et al., 2014), still poorly understood. The  
178 functional impact of *D. haemobaphes* intersexuality is unclear but must incur some form of cost, even  
179 if the production of non-functional oostegites on intersexes is merely reducing the resources available  
180 for normal growth and reproduction. It is also possible that intersexuality is the outward  
181 manifestation of more serious sexual dysfunction. Lower sperm counts have been reported in  
182 intersex males of *Echinogammarus marinus* (Yang, Kille & Ford, 2008) and in *Corophium volutator*,  
183 females mating with intersex males produce smaller broods (McCurdy et al., 2004). Furthermore,  
184 intersexuality may be associated with behavioural changes. Gammarid amphipods mate after a period  
185 of mate-guarding and a reduced capacity of intersex males to initiate or maintain this behaviour could  
186 also impact reproductive success. The plausibility of such altered behaviours is made more likely given  
187 numerous behavioural changes observed in *C. volutator* intersexes (McCurdy et al., 2008).  
188 Investigation of *D. haemobaphes* reproductive function and behaviour will help determine the extent  
189 of dysfunction associated with the intersexuality.

190

191 Although the observed intersexuality will incur some cost, the fact that such high levels of  
192 pronounced intersexuality has not impeded a successful amphipod invasion is the strongest evidence  
193 to date that crustacean male intersexuality is not, in any meaningful sense, equivalent to vertebrate  
194 male intersexuality, which is commonly associated with serious sexual dysfunction (Jobling et al.,  
195 1998; Harris et al., 2011; Kidd et al., 2011). Furthermore, our findings are consistent with  
196 experimentally generated hypotheses that certain parasitic burdens facilitate biological invasions.

197

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201

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328

329 **Table 1.** Primers used to conduct parasite screen.

330

331 **Table 2.** A screen of parasites using a subsample of the *D. haemobaphes* and *G. pulex* populations  
332 revealed infection by a variety of parasites. Strong infection as defined by previous studies (Yang et  
333 al., 2011).

334

335 **Fig. 1** Recent confirmed reports of *D. haemobaphes* (green triangles) and *D. villosus* (red circles) in UK  
336 waterways (EA – unpublished data January 2014; image courtesy of SE Environment Agency).

337

338 **Fig. 2** External sexual phenotypes. a) Normal female *D. haemobaphes* with only oostegites (green). b)  
339 Intersex male *D. haemobaphes* specimen presenting genital papillae (purple) alongside oostegites  
340 (green) with rudimentary setae. c) Normal male *D. haemobaphes* with only genital papillae (purple).

341

342 **Fig. 3** Frequency of sexual phenotypes and prevalence of *D. berillonum* infection. a) Sexual  
343 phenotypes found in two *D. haemobaphes* populations and *G. pulex*. b) Infection of *D. berillonum*  
344 found in *D. haemobaphes* and *G. pulex* found in both sites (NF=Normal Female, EIF=External Intersex  
345 Female, NM=Normal Male, EIM= External Intersex Male).

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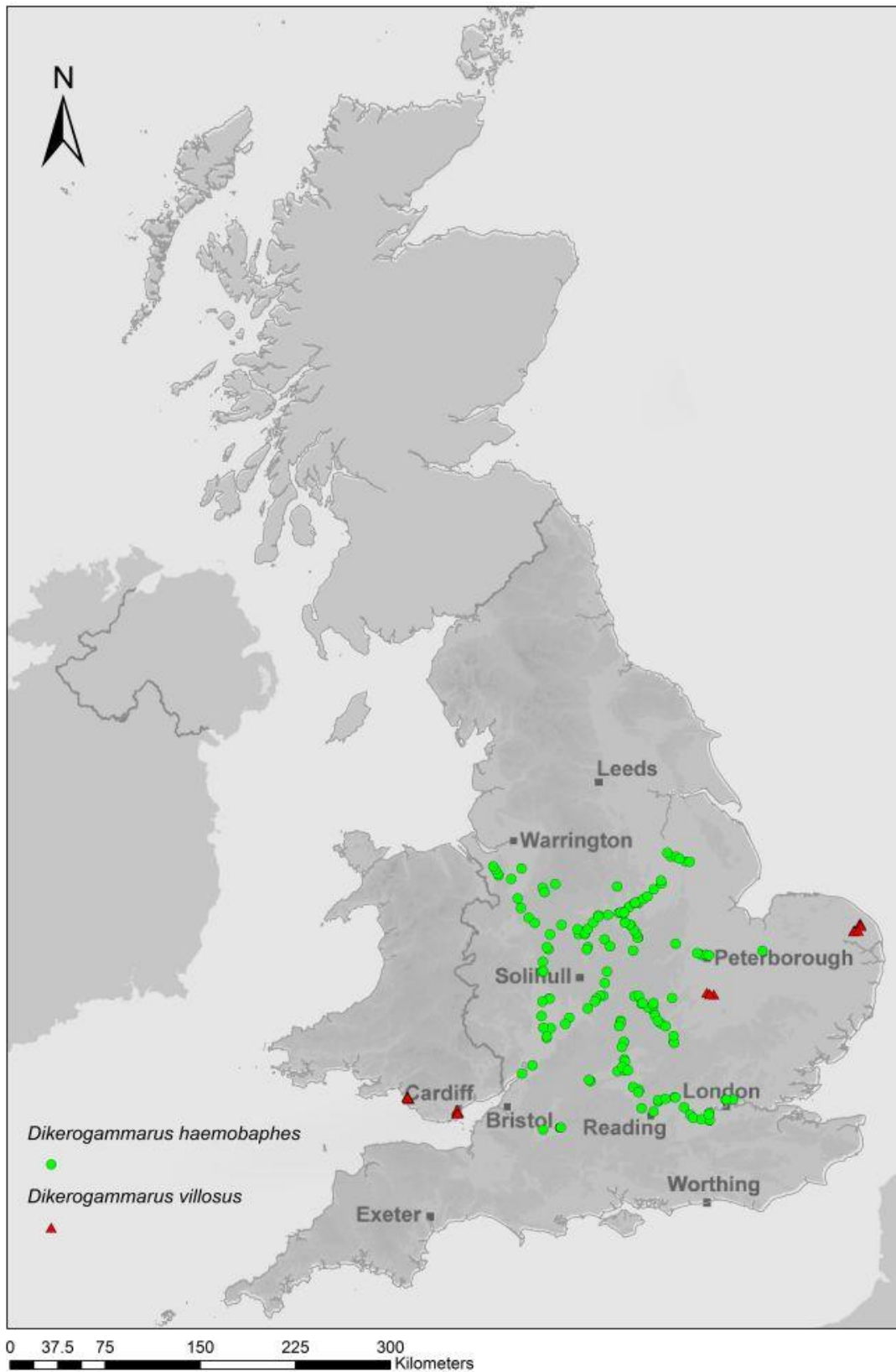
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**Figure 1** (on next page)

Figure 1

Fig. 1 Recent confirmed reports of *D. haemobaphes* (green triangles) and *D. villosus* (red circles) in UK waterways (EA - unpublished data January 2014; image courtesy of SE Environment Agency).

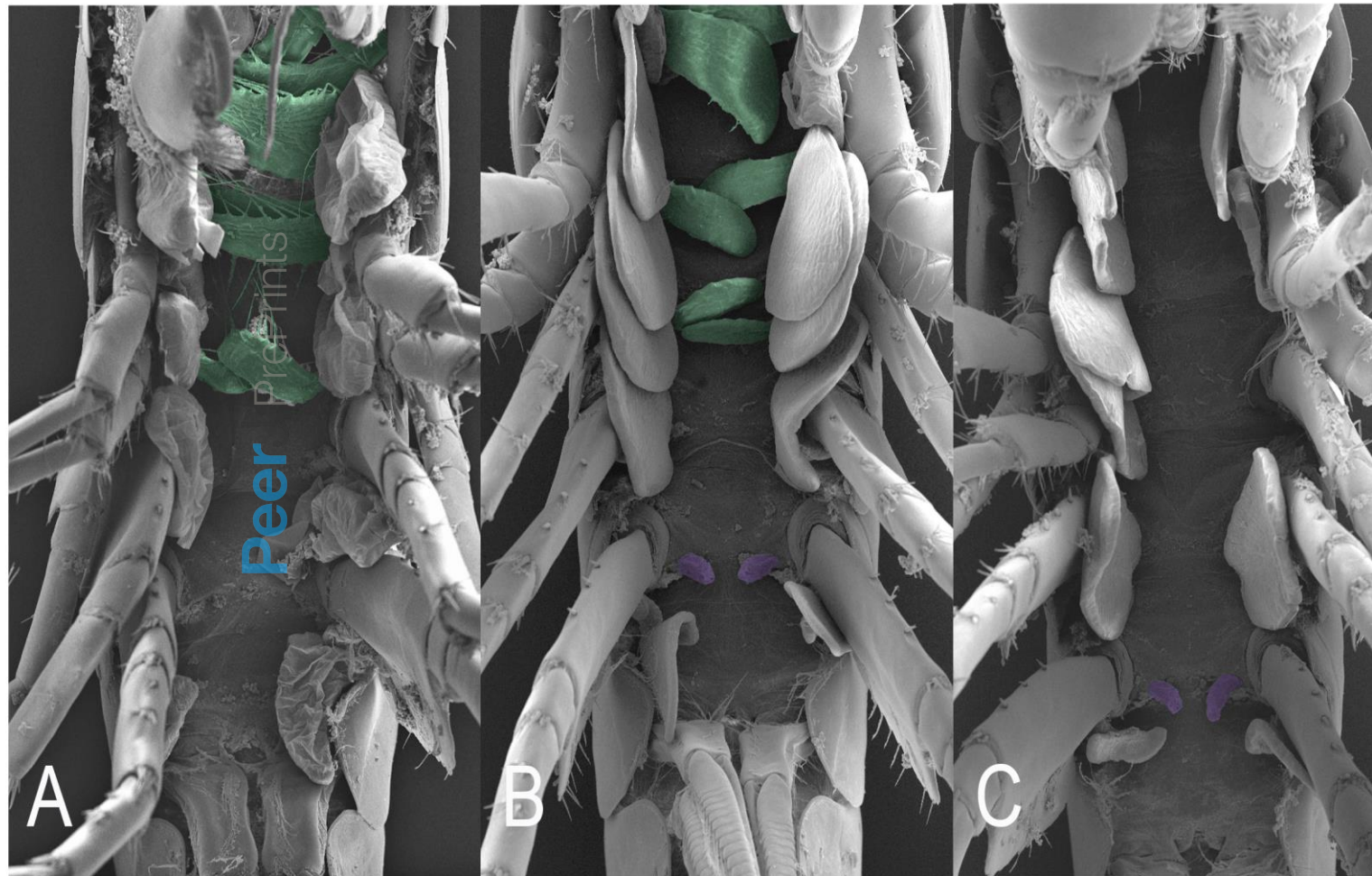




**Figure 2** (on next page)

## Figure 2

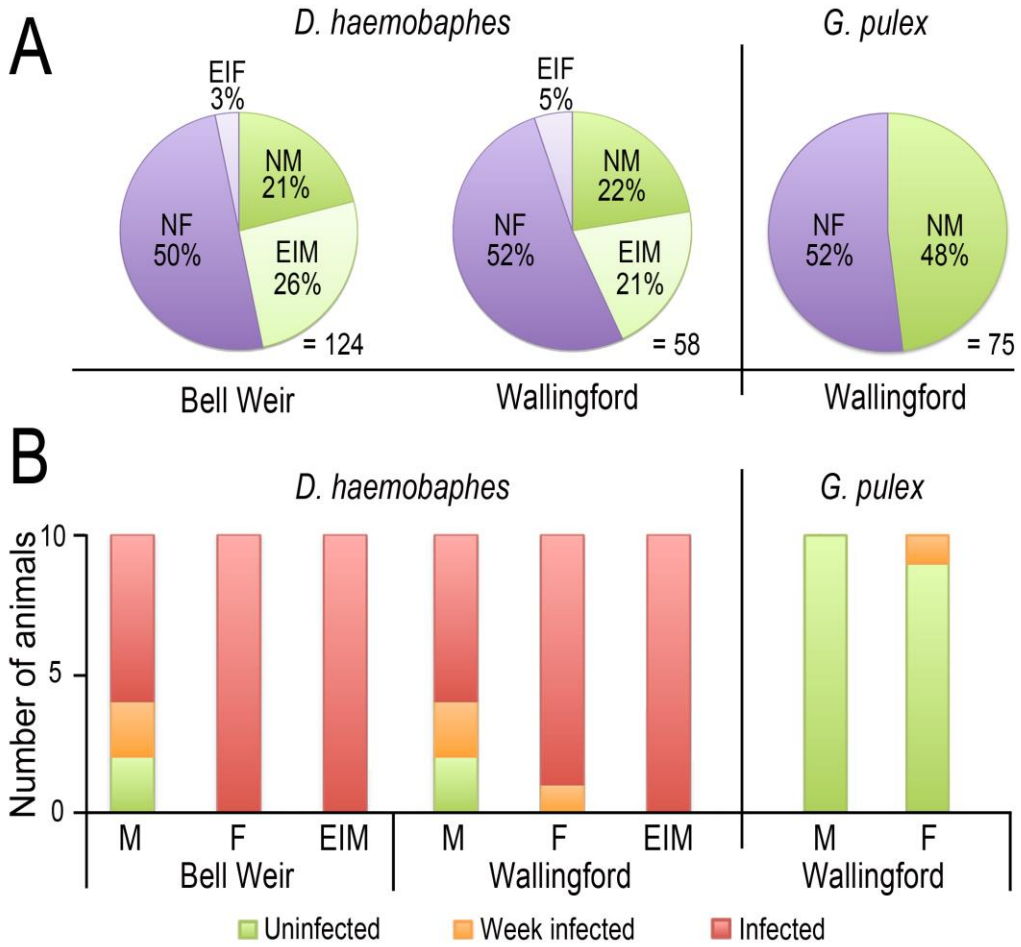
Fig. 2 External sexual phenotypes. a) Normal female *D. haemobaphes* with only oostegites (green). b) Intersex male *D. haemobaphes* specimen presenting genital papillae (purple) alongside oostegites (green) with rudimentary setae. c) Normal male *D. haemobaphes* with only genital papillae (purple).



**Figure 3** (on next page)

## Figure 3

Fig. 3 Frequency of sexual phenotypes and prevalence of *D. berillionum* infection. a) Sexual phenotypes found in two *D. haemobaphes* populations and *G. pulex*. b) Infection of *D. berillionum* found in *D. haemobaphes* and *G. pulex* found in both sites (NF=Normal Female, EIF=External Intersex Female, NM=Normal Male, EIM= External Intersex Male).



**Table 1** (on next page)

Table 1

Target	Primer	Sequence	Reference
Microsporidea 16S	V1f	5'- CACCAGGTTGATTCTGCCTGAC- 3'	Weiss <i>et al.</i> , 1994
	1342AC	5'- ACGGGCGGTGTGTACAAGGTAC AG-3'	Yang <i>et al.</i> , 2011
Acanthocephala 18S	537F	5'-GCCGCGTAATTCCAGCTC-3'	Near, Garey & Nadler, 1998
	1133R	5'-CTGGTGTGCCCTCCGTC-3'	
	1073F	5'-CGGGGGGAGTATGGTTGC-3'	
	18SR	5'- TGATCCTTCTGCAGGTTACCTAC -3'	
	18SF	5'- AGATTAAGCCATGCATGCGTAAG- 3'	
	549R	5'-GAATTACCGCGGCTGCTGG-3'	
Nematode/acanthocephala/apicomplexa	Nem18Slon gF	5'- CAGGGCAAGTCTGGTGCCAGCA GC-3'	Wood <i>et al.</i> , 2013
	Nem18Slon gR	5'- GACTTTCGTTCTTGATTAATGAA- 3'	
Paramyxea	Para18SF3	5'-CTACGGCGATGGCAGGTC-3'	Short <i>et al.</i> , 2012b
	Para18SR3	5'-GGGCGGTGTGTACAAAGG-3'	
<i>Wolbachia</i>	WSPEC-F	5'-CATACTATTCTGAAGGATAG- 3'	Werren & Windsor, 2000
	WSPEC-R	5'-AGCTTCGAGTGAAACCAATTC- 3'	

Table  
1.

Primers used to conduct parasite screen.

**Table 2** (on next page)

Table 2

Table 2. A screen of parasites using a subsample of the *D. haemobaphes* and *G. pulex* populations revealed infection by a variety of parasites. Strong infection as defined by previous studies (Yang et al. 2011).



Amphipod	Phylum of isolated parasite	No of strongly infected animals	Length of ribosomal sequence	Primers used for amplification	GenBank accession of isolated sequence	Closest identity using a BLAST	GenBank accession of closest match	% Identity
<b><i>D. haemobaphes</i></b>	Nematoda	11/60	472bp	537F 1133R	KM486061	<i>Hysterothylacium deardorffoverstreetorum</i>	JF718550	100%
	Microsporidia	51/60	1148bp	V1f 1342AC	KM486059	<i>Dictyocoela berillonum</i>	KF830272	99.9%
<b><i>G. pulex</i></b>	Acanthocephala	3/20	547bp	537F 1133R	KM486063	<i>Echinorhynchus gadi</i> <i>Echinorhynchus truttae</i>	AY830156	98%
	Microsporidia	10/20	1135bp	V1f 1342AC	KM486060	<i>Pleistrophora hippoglossoideos</i> <i>Pleistrophora typicalis</i> <i>Pleistrophora mulleri</i>	EF119339	99.6%
	Apicomplexa	10/20	402bp	537F 1133R	KM486064	<i>Mattesia geminate</i>	AY334568	90.2%

**Table 2.** A screen of parasites using a subsample of the *D. haemobaphes* and *G. pulex* populations revealed infection by a variety of parasites.

Strong infection as defined by previous studies (Yang *et al.*, 2011).