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

Amaia Green Etxabe, Stephen Short, Tim Flood, Tim Johns, Alex Ford

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. 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.

1 **Pronounced and prevalent intersexuality does not impede the**

2 **'Demon Shrimp' invasion**

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Abstract

11 Crustacean intersexuality is widespread and often linked to infection by sex-

12 distorting parasites. However, unlike vertebrate intersexuality, its association

13 with sexual dysfunction is unclear and remains a matter of debate. The

14 'Demon Shrimp', *Dikerogammarus haemobaphes*, an amphipod that has

15 invaded continental waterways, has recently become widespread in Britain.

16 Intersexuality has been noted in *D. haemobaphes* but not investigated

17 further. We hypothesise that a successful invasive population should not

18 display a high prevalence of intersexuality if this condition represents a truly

19 dysfunctional phenotype. Experiments have indicated that particular parasite

20 burdens in amphipods may facilitate invasions. The rapid and ongoing

21 invasion of British waterways represents an opportunity to determine

22 whether these hypotheses are consistent with field observations. This study

23   investigates the parasites and sexual phenotypes of *D. haemobaphes* in

24   British waterways, characterising parasite burdens using molecular

25   screening, and make comparisons with the threatened *Gammarus pulex*

26   natives. We reveal that invasive and native populations have distinct

27   parasitic profiles, suggesting the loss of *G. pulex* may have parasite-mediated

28   eco-system impacts. Furthermore, the parasite burdens are consistent with

29   those previously proposed to facilitate biological invasions. Our study also

30   indicates that while no intersexuality occurs in the native *G. pulex*,

31   approximately 50% of *D. haemobaphes* males present pronounced

32   intersexuality associated with infection by the microsporidian *Dictyocoela*

33   *berillonum*. This unambiguously successful invasive population presents, to

34   our knowledge, the highest reported prevalence of male intersexuality. This is

35   the clearest evidence to date that such intersexuality does not represent a

36   form of debilitating sexual dysfunction that negatively impacts amphipod

37   populations.

38   **Introduction**

39   *Dikerogammarus haemobaphes* (Eichwald, 1841), an effective predatory

40   amphipod from the Ponto-Caspian (Bacela-Spychalska and van der Velde

41   2013), has spread through Europe and is now recognised as an extremely

42   successful invader of British waterways (Green Etxabe and Ford 2014). *D.*

43   *haemobaphes,* also known as the 'demon shrimp', invaded the British Isles

44   more recently than the infamous 'killer shrimp' (*Dikerogammarus villosus*,

45   Sowinsky, 1894) (MacNeil et al. 2010) but is already more widespread (Fig. 1).

46   Amphipods harbour many parasites that can drastically impact host

47   populations by influencing the health, behaviour, reproduction and sex

48   determination of their host (Hatcher and Dunn 2011; Bacela-Spychalska et

49   al. 2012). The invasive *D. haemobaphes*, therefore, could not only out

50   compete and prey on native amphipod species, but also introduce parasites

51   into their new habitats. Screening parasites in invasive and native amphipod

52   species associated with a rapid and on-going invasion will test hypotheses

53   that particular parasitic burdens impact invasion success (MacNeil et al.

54   2003a; Hatcher and Dunn 2011; Hatcher et al. 2014).

55

56   Some amphipod-infecting parasites maximise their transmission via the

57   host's progeny by converting males into reproductive females (Ford 2012).

58   Infection by such parasites results in sex-biased populations (Terry et al.

59   2004) and, in cases of incomplete conversion, intersexuality, where

60   secondary sex characteristics of both genders occur on an individual (Ford

61   2012). Intersex phenotypes are found in a range of animals (Matthiessen and

62   Gibbs 1998; Harris et al. 2011; Hayes et al. 2002), including crustaceans

63   (Ford 2012), where they are linked to parasitic infection (Short et al. 2012a)

64   and environmental conditions (Dunn et al. 1996), as well the direct (Short et

65   al. 2012b) and indirect (Jacobson et al. 2010) influence of contaminant

66   exposure. In cases of parasitic infection, an incomplete conversion is thought

67   to occur due to insufficient parasite burden, suboptimal conditions, or

68   effective host responses (Dunn and Rigaud 1998; Kelly et al. 2002; Short et

69   al. 2014). Current evidence suggests the impact of female intersexuality is

70   subtle (Ford et al. 2003; Kelly et al. 2004) or effectively non-existent (Glazier

71   et al. 2012), and the female intersexuality observed in *D. haemobaphes*

72   successfully invading Polish waterways (Bacela et al. 2009) is consistent with

73   these hypotheses.

74   Male intersexuality is also widespread in amphipods, however, our

75   understanding of its reproductive consequences is poorly understood relative

76   to vertebrates (Harris et al. 2011). The extents of morphological and

77   behavioural changes (McCurdy et al. 2008; Yang et al. 2008) have led to the

78   suggestion (Yang et al. 2008; Ford 2012) that the impact of crustacean male

79   intersexuality may be similar to that seen in vertebrates (Harris et al. 2011).

80   Despite some evidence of intersexuality in invasive *D. haemobaphes* (Bacela

81   et al. 2009), sexual phenotypes in this species have not been studied, even

82   though notable levels of intersexuality in the unambiguously successful

83   invading population would reveal considerable insight into the consequences

84   of intersexuality for wild crustacean populations.

85   This study investigates the sexual phenotypes and parasites of *D.*

86   *haemobaphes* and the native *Gammarus pulex* (Linnaeus, 1758) at multiple

87   locations in British waterways to give insights into this rapidly invading

88   species and expand our understanding of crustacean intersexuality.

89   **Methods**

Specimen characterisation

90

91 Amphipods were collected from Wallingford Bridge and Bell Weir, U.K.

92 Amphipods were categorised into species and phenotypes: males, females,

93 intersex males and intersex females. Intersex males were identified by

94 genital papillae, between pereonite 7 and pleonite 1, in conjunction with

95 rudimentary oostegites. Intersex females were identified by oostegites in

96 conjunction with secondary genital papilla/e. Animals from each phenotype

97 were measured from antennal joint to telson to obtain body length (ImageJ,

98 v1.4u4) and comparisons were made using analysis of variance (ANOVA) with

99 the post hoc Tamhanes-T2 test (SPSS v21).

Scanning electron microscopy

100

101 Specimens of *D. haemobaphes* were taken through transitional steps (100%

102 ethanol to 100% hexamethyldisilazane, HMDS) then evaporated to dryness.

103 The dry samples were mounted on SEM stubs, sputter coated with gold-

104 palladium and examined using a scanning electron microscope (JEOL 6060LV)

105 operating in high vacuum mode with a secondary electron detector active at

106 an acceleration voltage of 10kV. Images were cropped and colourised using

107 Adobe Photoshop (CS5v12).

PCR screen

108

109 DNA was purified from internal animal tissue (excluding gut) or eggs using

110 the DNeasy Blood and Tissue Kit (Qiagen, UK). Samples were screened using

111 previously described PCR primers for general parasites (Table 1). PCR

112 reactions were performed in 25 μl volumes containing 10 ng of DNA as

113 template, 1 U of Taq polymerase (Promega, UK), 5 μl of 5x PCR buffer 1.25

114 mM MgCl$_2$ and 0.4 mM of each corresponding primer.

Sequence identification

116 PCR products were analysed using agarose gel electrophoresis containing 1x

117 GelGreen□ (Cambridge Bioscience, UK) for the presence of bands potentially

118 representing amplified parasite sequences. Individual bands were isolated

119 and DNA extracted using the QIAquick Gel Extraction Kit (Qiagen, UK) and

120 sequenced (Source Bioscience, UK), before a BLAST analysis was performed

121 against sequences stored in GenBank (NCBI).

Results

Sexual phenotypes

124 Pronounced male intersex phenotypes were found in *D. haemobaphes* at both

125 sites, with most specimens displaying well-developed oostegites with visible

126 setae (Fig. 2). Almost half the male population presented intersex

127 characteristics at both locations and very few cases of female intersexuality

128 were observed (Fig. 3a). *G. pulex* was only found in conjunction with *D.*

129 *haemobaphes* at one sampling site and no intersex phenotypes were found

130 (Fig. 3a). Significant differences were found in lengths of *D. haemobaphes*

131 phenotypes (f = 3.885, df = 2, p = 0.23) where normal males (N = 32) are

132 significantly larger (p = 0.04) than females (N = 52). However, there is no

133   significant difference between intersex males (N = 37) and either females (p

134   = 0.735) or males (p = 0.328).

135   **Parasite screening**

136   Screening of *D. haemobaphes* and *G. pulex* populations revealed evidence of

137   infection by several parasites (Table 2). All *D. haemobaphes* females and

138   intersex males were found infected with *D. berillonum*, with one female

139   classed as weakly infected (Fig. 3b), as previous defined (Yang et al. 2011).

140   The majority of males were also infected, although more weak infections

141   were found (Fig. 3b). This pattern of *D. berillonum* infection was consistent at

142   both collection sites and when combined in a Fishers Exact test (two-tailed)

143   reveal a significant difference in the level of infection between normal and

144   intersex males (p = 0.003 using strong infections only, p = 0.02, using weak

145   and strong infections). To confirm vertical transmission, the broods of ten

146   infected females were also tested and all were infected by *D. berillonum*.

147   Only one case of weak infection was found in *G. pulex* (Fig. 3b).

148   **Discussion**

149   Our screen of invasive and native species associated with an extremely

150   successful, and ongoing, amphipod invasion reveals parasitic-profiles

151   strikingly consistent with hypotheses that particular parasitic burdens

152   influence the dynamics of biological invasion (MacNeil et al. 2003a; Hatcher

153   and Dunn 2011; Hatcher et al. 2014). The native *G. pulex* are infected with a

154   microsporidian of the genus *Pleistophora*, which include behaviour altering

155 species known to increase the likelihood of predation on native amphipods
156 and reduce their predatory behaviour when interacting with invaders
157 (MacNeil et al. 2003a; Fielding et al. 2005). Sequences were also found for
158 an acanthocephalan, most likely *Echinorhynchus truttae*. This species can
159 both reduce its host's predatory behaviour and increase vulnerability to
160 predation by fish (Fielding et al. 2003; MacNeil et al. 2003b; Lagrue et al.
161 2013). Consequently, the parasite burden of *G. pulex* may facilitate invasion
162 of *D. haemobaphes* through British waterways by impairing the competitive
163 abilities of the native population, a scenario consistent with recent
164 experiments and population modelling (MacNeil et al. 2003a; Haddaway et
165 al. 2012; Hatcher et al. 2014). In contrast, the invasive *D. haemobaphes* was
166 almost ubiquitously infected by the vertically transmitted microsporidian
167 *Dictyocoela berillonum*. It is possible the initial invasive population consisted
168 of a small number of infected individuals and the current infection prevalence
169 represents a parasitic founder-effect. Alternatively, given that parasite
170 infection is predicted to influence invasion success (MacNeil et al. 2003a;
171 Fielding et al. 2005; Hatcher et al. 2014) via trait-mediated effects, it is
172 possible the high prevalence of *D. berillonum* occurs due to a subsequent
173 enhancement in invasive capabilities.

174 The distinct parasitic profiles of *G. pulex* and *D. haemobaphes* may
175 have ecological impacts. Our results suggest the eradication of *G. pulex*
176 would lead to the removal of a pleistophoran microsporidian from the
177 ecosystem potentially capable of causing disease in fish (Lom and Nilsen
178 2003) and an acanthocephalan indistinguishable from *E. truttae* (García-

179 Varela and Nadler 2005). Although *E. truttae* infection in fish does not appear

180 to cause morbidity (Dorucu et al. 1995), infected amphipods are more

181 vulnerable to fish predation due to altered habitat usage (MacNeil et al.

182 2003b; Lagrue et al. 2013). Therefore, loss of this parasite may alter prey

183 abundance, even if the overall amphipod biomass is maintained following the

184 displacement of *G. pulex*.

185   The sexual phenotype survey revealed that while no intersexuality was

186 evident in *G. pulex*, *D. haemobaphes* presents striking levels of pronounced

187 male intersexuality, where males exhibit unambiguous oostegites possessing

188 rudimentary seta and are significantly smaller than normal males. In

189 contrast, the low levels of female intersexuality in *D. haemobaphes* were

190 much like those previously reported in Polish waters (Bacela et al. 2009). To

191 our knowledge, this is the highest prevalence of male intersexuality recorded

192 in an amphipod population (McCurdy et al. 2004; Ford and Fernandes 2005;

193 Short et al. 2012b; Yang et al. 2011) and is the first evidence clearly linking

194 *D. berillonum* with amphipod intersexuality (Terry et al. 2004; Yang et al.

195 2011). Other *Dictyocoela* species have been linked to both abnormal sexual

196 phenotypes and female-biased sex ratios (Terry et al. 2004; Short et al.

197 2012a), however, the lack of female-bias in *D. haemobaphes* suggests *D.*

198 *berillonum* is unable to fully convert males in to females. This could result

199 from sub-optimal environmental conditions impacting the efficacy of

200 conversion or the consequence of *D. berillonum* infecting an unfamiliar host.

201 Whatever the cause, *D. haemobaphes* is of interest, as the association

202 between male intersexuality and sexual dysfunction is, despite recent

203 molecular advances (Short et al. 2014), still poorly understood.

204 The fact that high levels of pronounced intersexuality has not impeded a

205 successful amphipod invasion is the strongest evidence to date that

206 crustacean male intersexuality is not, in any meaningful sense, equivalent to

207 vertebrate intersexuality, which is commonly associated with serious sexual

208 dysfunction. Furthermore, our findings are consistent with experimentally

209 generated hypotheses that certain parasitic burdens facilitate biological

210 invasions.

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

357 **Table 2.** A screen of parasites using a subsample of the *D. haemobaphes*

358 *and G. pulex* populations revealed infection by a variety of parasites. Strong

359 infection as defined by previous studies (Yang et al. 2011).

360 **Fig. 1** Recent confirmed reports of *D. haemobaphes* (green triangles) and *D.*

361 *villosus* (red circles) in UK waterways (EA – unpublished data January 2014;

362 image courtesy of SE Environment Agency).

363 **Fig. 2** External sexual phenotypes. a) Normal female *D. haemobaphes* with

364 only oostegites (green). b) Intersex male *D. haemobaphes* specimen

365 presenting genital papillae (purple) alongside oostegites (green) with

366 rudimentary setae. c) Normal male *D. haemobaphes* with only genital

367 papillae (purple).

368 **Fig. 3** Frequency of sexual phenotypes and prevalence of *D. berillonum*

369 infection. a) Sexual phenotypes found in two *D. haemobaphes* populations

370 and *G. pulex*. b) Infection of *D. berillonum* found in *D. haemobaphes* and *G.*

371 *pulex* found in both sites (NF=Normal Female, EIF=External Intersex Female,

372 NM=Normal Male, EIM= External Intersex Male).

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).
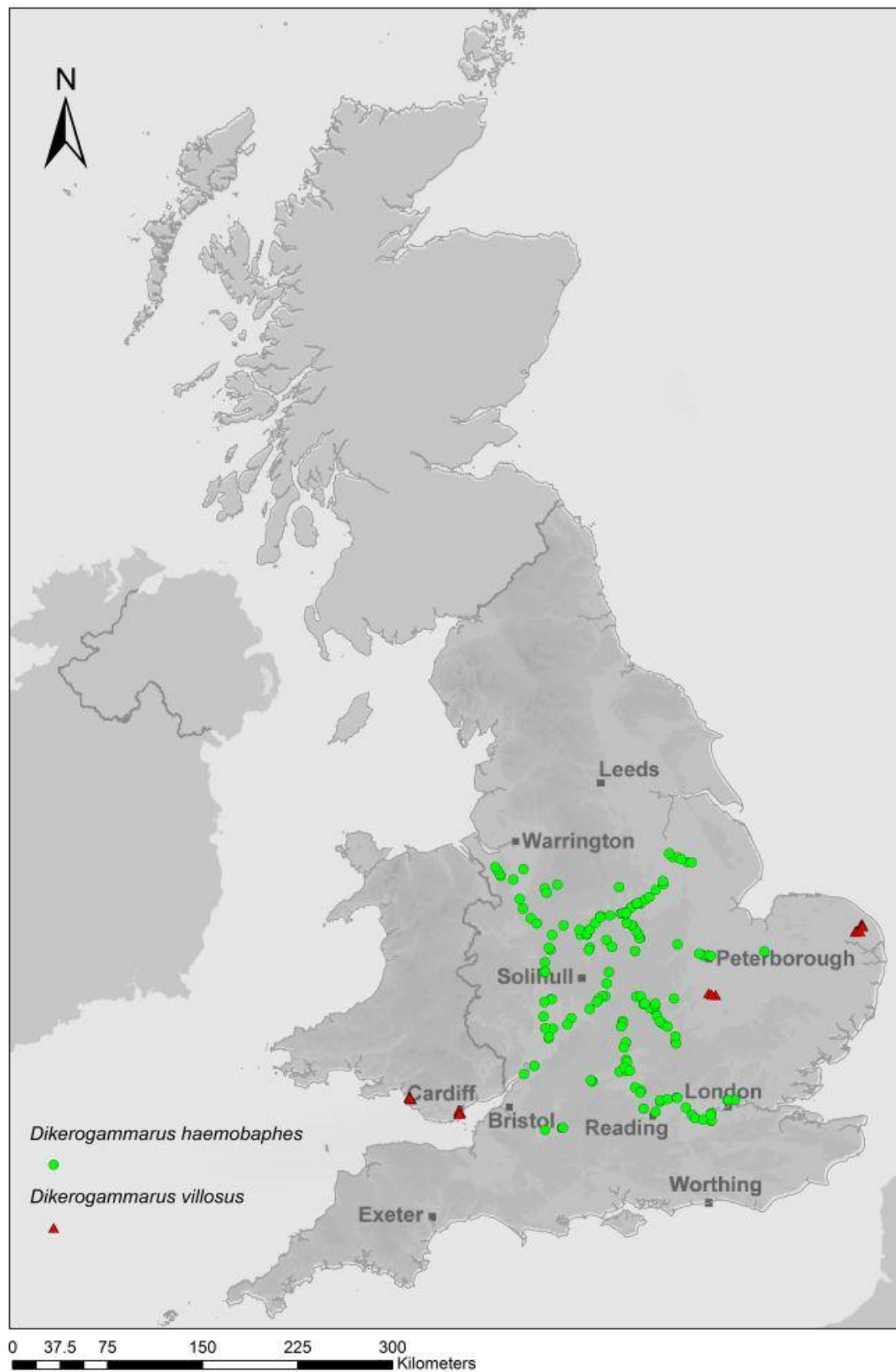
Figure 2(on next page)

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).
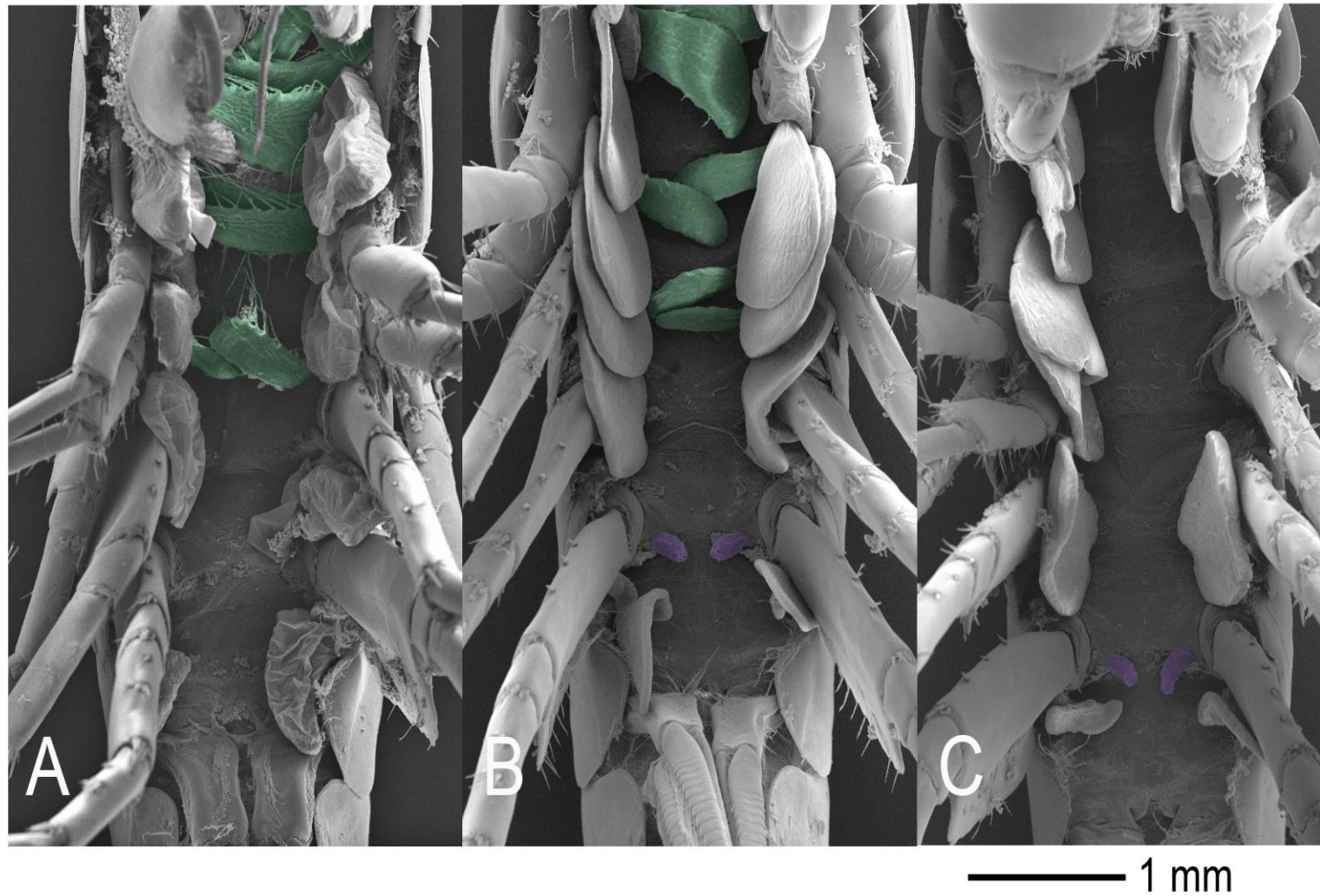
Figure 3(on next page)

Figure 3

Fig. 3 Frequency of sexual phenotypes and prevalence of *D. berillonum* infection. a) Sexual phenotypes found in two *D. haemobaphes* populations and *G. pulex*. b) Infection of *D. berillonum* 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).
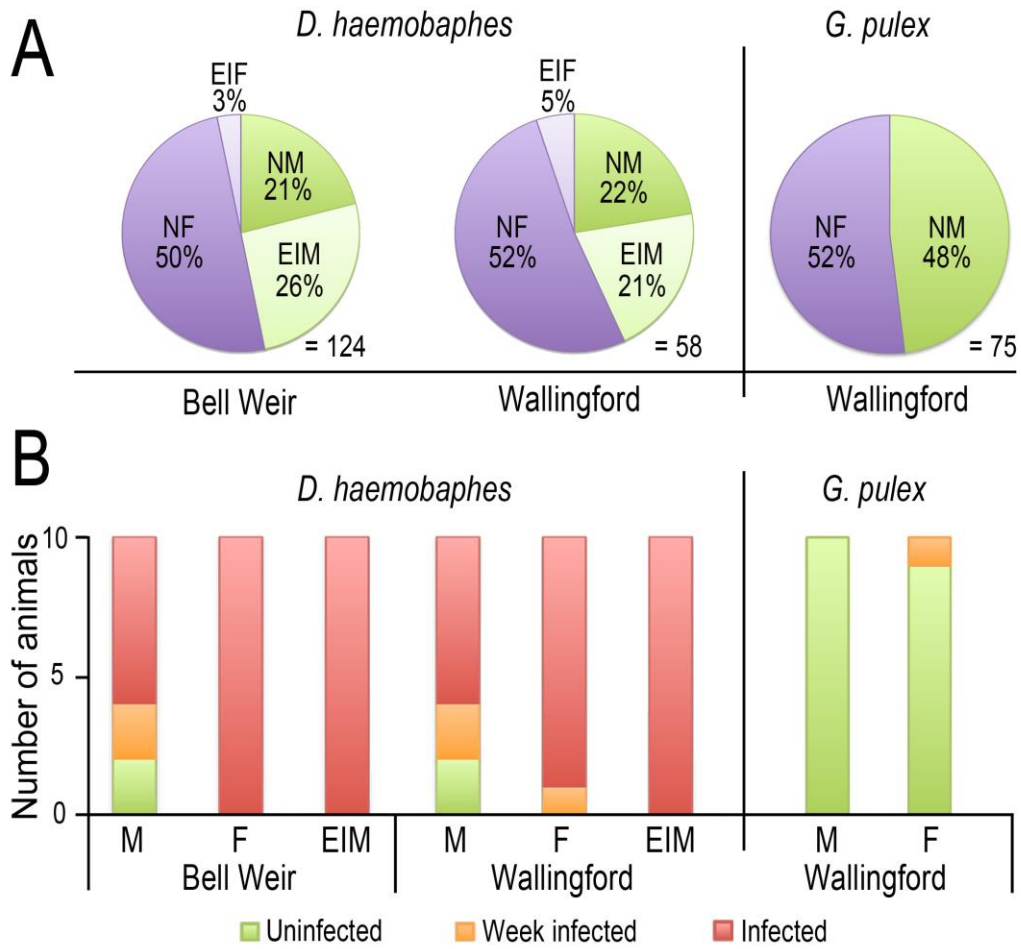
Table 1(on next page)

Table 1

Target	Primer	Sequence	Reference
Microsporidea 16S	V1f	5′-CACCAGGTTGATTCTGCCTGAC-3′	Weiss *et al.*, 1994
	1342AC	5′-ACGGGCGGTGTGTACAAGGTACAG-3′	Yang *et al.*, 2011
Acanthocephala 18S	537F	5′-GCCGCGGTAATTCCAGCTC-3′	Near, Garey & Nadler, 1998
	1133R	5′-CTGGTGTGCCCCTCCGTC-3′	
	1073F	5′-CGGGGGGAGTATGGTTGC-3′	
	18SR	5′-TGATCCTTCTGCAGGTTCACCTAC-3′	
	18SF	5′-AGATTAAGCCATGCATGCGTAAG-3′	
	549R	5′-GAATTACCGCGGCTGCTGG-3′	
Nematode/acanthocephala/apicomplexa	Nem18SlongF	5′-CAGGGCAAGTCTGGTGCCAGCAGC-3′	Wood *et al.*, 2013
	Nem18SlongR	5′-GACTTTCGTTCTTGATTAATGAA-3′	
Paramyxea	Para18SF3	5′-CTACGGCGATGGCAGGTC-3′	Short *et al.*, 2012b
	Para18SR3	5′-GGGCGGTGTGTACAAAGG-3′	
Wolbachia	WSPEC-F	5′-CATACCTATTCGAAGGGATAG-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
D. haemobaphes	Nematoda	11/60	472bp	537F 1133R	KM486061	*Hysterothylacium deardorffoverstreetorum*	JF718550	100%
	Microsporidia	51/60	1148bp	V1f 1342AC	KM486059	*Dictyocoela berillonum*	KF830272	99.9%
G. pulex	Acanthocephala	3/20	547bp	537F 1133R	KM486063	*Echinorhynchus gadi Echinorhynchus truttae*	AY830156	98%
	Microsporidia	10/20	1135bp	V1f 1342AC	KM486060	*Pleistrophora hippoglossoideos Pleistrophora typicalis Pleistrophora mulleri*	EF119339	99.6%
	Apicomplexa	10/20	402bp	537F 1133R	KM486064	*Mattesia geminate*	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).