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1 **Apparent interspecific transmission of *Aphanomyces astaci* from invasive signal to virile**
2 **crayfish in a sympatric wild population**

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11

12 **Running title:** Interspecific transmission of *Aphanomyces astaci* between invasive crayfish

13

14 **Abstract**

15 The crayfish plague pathogen (*Aphanomyces astaci*) causes mass mortalities of European
16 crayfish when transmitted from its original North American crayfish hosts. Little is known,
17 however, about interspecific transmission of the pathogen between different American
18 crayfish species, although evidence from trade of ornamental crayfish suggests this may
19 happen in captivity. We screened signal and virile crayfish for *A. astaci* at allopatric and
20 sympatric sites in a UK river. Whilst the pathogen was detected in signal crayfish from both
21 sites, infected virile crayfish were only found in sympatry. Genotyping of *A. astaci* from
22 virile crayfish suggested the presence of a strain related to one infecting British signal
23 crayfish. We conclude that virile crayfish likely contracted *A. astaci* interspecifically from
24 infected signal crayfish. Interspecific transmission of *A. astaci* strains differing in virulence

25 between American carrier species may influence the spread of this pathogen in open waters
26 with potential exacerbated effects on native European crayfish.

27

28 **Keywords:** *Pacifastacus leniusculus*; *Orconectes cf. virilis*; Wildlife disease; Transmission
29 pathways.

30

31 **1. Introduction**

32 The crayfish plague agent, *Aphanomyces astaci*, is arguably one of the most devastating
33 invasive parasites in European freshwaters (Lowe, 2004; DAISIE, 2009). Since its first
34 introduction in the mid-19th century (Alderman, 1996; Holdich, 2003), the pathogen has
35 spread throughout Europe, facilitated in recent decades by movements of invasive North
36 American (henceforth referred to as American) crayfish (Souty-Grosset *et al.*, 2006; Holdich
37 *et al.*, 2014; James *et al.*, 2014). Whilst American crayfish are often asymptomatic carriers of
38 *A. astaci* infection, the disease is usually lethal in European species (Unestam & Weiss, 1970;
39 Diéguez-Uribeondo *et al.*, 1997; Bohman, *et al.*, 2006; Kozubíková *et al.*, 2008). Once
40 introduced, *A. astaci* can spread rapidly, transmitted through zoospores that are released into
41 water (Oidtmann *et al.*, 2002) and can survive for at least 14 days (CEFAS, 2000). Spores are
42 mainly released during host moulting or death (Svoboda *et al.*, 2013), and within a cadaver *A.*
43 *astaci* can remain viable for several days (Oidtmann *et al.*, 2002). Therefore, the movement
44 of infected carcasses by predators could facilitate pathogen dispersal. If fish ingest infected
45 tissue, the pathogen can even survive passage through the gastro-intestinal tract, providing an
46 additional transmission pathway (Oidtmann *et al.*, 2002).

47 Whilst the transmission of *A. astaci* from non-native American to European crayfish
48 has been widely documented (e.g. Alderman *et al.*, 1990; Diéguez-Uribeondo, Temiño &
49 Múzquiz, 1997; Vennerström *et al.*, 1998; Bohman *et al.*, 2006), little is known about

50 interspecific pathogen transmission between these invasive carriers. Until now, four different
51 *A. astaci* genotype groups have been isolated in Europe; group A was obtained from infected
52 native European crayfish (*Astacus astacus* and *A. leptodactylus*) and groups B, D and E from
53 different American crayfish species (*Pacifastacus leniusculus*, *Procambarus clarkii* and
54 *Orconectes limosus*, respectively) (Svoboda *et al.*, 2017). The genotype groups infecting
55 additional *A. astaci* carriers known from European waters, calico (*Orconectes immunis*),
56 marbled (*Procambarus fallax* f. *virginalis*) and virile (*Orconectes* cf. *virilis*) crayfish
57 (Filipova *et al.*, 2013; Schrimpf *et al.*, 2013; Keller *et al.*, 2014; Tilmans *et al.*, 2014), are so
58 far unknown. Existing data suggest that *A. astaci* genotype groups are host-specific among
59 American crayfish (Grandjean *et al.*, 2014). There is no evidence of strains transmitting
60 between these crayfish in the wild, although it seems to occur in the aquarium trade (Mrugała
61 *et al.*, 2015).

62 Here, we investigate interspecific transmission of *A. astaci* upon contact of two
63 potential carrier species. Signal crayfish are widespread across the UK (James *et al.*, 2014)
64 and were initially stocked into the River Lea during the mid-1970s (Almeida *et al.*, 2014).
65 Conversely, virile crayfish are only found in the Lea catchment in the UK (James *et al.*,
66 2014), possibly unintentionally introduced there around 2004 (Ahern *et al.*, 2008). The two
67 species have been co-existing since at least 2011 (James *et al.*, 2015). Virile crayfish in this
68 river, as well as in a population from the Netherlands, have been reported to carry *A. astaci*
69 (Tilmans *et al.*, 2014), and it was suggested that these crayfish were already infected prior to
70 introduction. Here, we tested this hypothesis, the alternative hypothesis that virile crayfish
71 contracted *A. astaci* from co-existing signal crayfish, by evaluating the distribution of the
72 pathogen at allopatric and sympatric sites within the River Lea and an adjacent lake in
73 London, and by genotyping the pathogen from infected host specimens.

74

75

76 **2. Methods**

77 Invasive signal crayfish and virile crayfish were collected from the River Lea and an adjacent
78 lake in London, UK, during September 2014. Using baited traps employed over two
79 consecutive nights and checked daily, animals were caught from allopatric (Lat/Long:
80 51°45'14"N/ 000°00'16"E, 51°42'29"N/ 000°01'16"W for signal and virile crayfish
81 respectively, n = 30 for each species) and a sympatric site (Lat/Long: 51°42'24"N/
82 000°01'04"W, n = 9 signal and 30 virile crayfish) (Fig. 1). Upon capture, animals were
83 transported individually to Cardiff University (UK), humanely euthanized by freezing at -
84 80°C and stored in ca. 95% molecular grade ethanol before transport to Charles University in
85 Prague for further processing. For *A. astaci* screening, we harvested from each animal a
86 section of tail fan, soft abdominal cuticle, two limb joints, and any melanised cuticle (as in
87 Svoboda *et al.*, 2014). Tissue samples from each individual (40-50 mg) were ground together
88 in liquid nitrogen from which DNA was extracted using a DNeasy tissue kit (Qiagen) as per
89 manufacturer's guidelines.

90 All samples were screened for *A. astaci* presence using TaqMan MGB quantitative
91 PCR (qPCR) on the iQ5 BioRad thermal cycler according to Vrålstad *et al.*, (2009), slightly
92 modified to increase assay specificity (Strand *et al.*, 2011; Svoboda *et al.*, 2014). To check
93 for potential inhibition (as in Kozubíková *et al.*, 2011; Svoboda *et al.*, 2014) each DNA
94 isolate was analysed by qPCR at two concentrations (undiluted and 1:10 dilution). Negative
95 controls were included at each step of the protocol, and in all cases these remained negative.
96 Based on the estimated amount of *A. astaci* DNA in the isolates (expressed in PCR-forming
97 units, PFU), the extent of the infection was expressed in semi-quantitative agent levels (A0-
98 A7; according to Vrålstad *et al.*, 2009; Kozubíková *et al.*, 2011). Samples with agent levels of
99 A2 or higher were considered positive for *A. astaci*.

100 For *A. astaci* genotype group identification, we analysed *A. astaci*-positive samples
101 using nine *A. astaci*-specific microsatellite markers (Grandjean *et al.*, 2014). As amplification
102 success depends on the amount of pathogen DNA in the sample, genotyping was only
103 attempted for those with agent level A3 and higher (as in Grandjean *et al.*, 2014) and was
104 repeated three times for each sample. In case of an initial lack of amplification, DNA isolates
105 were concentrated on the Concentrator Plus 5305 (Eppendorf). The results of successful
106 genotyping were compared with the *A. astaci* reference strains described by Grandjean *et al.*,
107 2014 and an *A. astaci*-positive DNA isolate from signal crayfish in Lake Mochdre
108 (Newtown) Wales, UK (James *et al.*, 2017).

109

110 **3. Results**

111 Within allopatric sites on the River Lea, *Aphanomyces astaci* was detected in 83% (25 out of
112 30) signal crayfish but was not detected in any virile crayfish (n = 30). From the sympatric
113 site, 44% (4 out of 9) signal crayfish and 23% (7 out of 30) virile crayfish tested positive for
114 *A. astaci* infection. All *A. astaci*-positive samples yielded low levels of infection (A2-A3;
115 Vrålstad *et al.*, 2009). Of the *A. astaci* infected animals from the allopatric signal crayfish site
116 A3 level infections were detected in four animals (estimated PFUs: 51, 71, 106 and 111).
117 Within the sympatric site, A3 level infections were detected in three virile (estimated PFUs:
118 85, 167 and 1000) and two signal (estimated PFUs: 52 and 57) crayfish.

119 Due to low amount of *A. astaci* DNA, reliable amplification and scoring of the
120 microsatellites were only possible for two specimen of virile crayfish. Of the nine
121 microsatellite loci, amplification was achieved for seven loci for the first virile crayfish
122 specimen, and for six loci for the second one. For the first virile crayfish specimen the
123 multilocus genotype corresponded at five loci to the reference axenic culture of the genotype
124 group B (Table 1). Differences were observed at the Aast9 and Aast12 loci, where

125 homozygotes rather than heterozygotes were scored. Such variation at the Aast9 locus has
126 been also observed in the *A. astaci*-positive DNA isolate from signal crayfish in Wales, UK
127 (Table 1; James *et al.* 2017). For the second virile crayfish specimen the multilocus genotype
128 corresponded at five loci to the reference axenic culture of genotype group B (Table 1). A
129 difference was observed at the Aast9 locus, where a homozygote rather than a heterozygote
130 was scored. All six of the amplified loci for this second specimen matched the *A. astaci*
131 positive DNA isolate from signal crayfish in Wales (Table 1).

132

133 **4. Discussion**

134 Here we present two lines of evidence suggesting the interspecific transmission of
135 *Aphanomyces astaci* between two invasive American crayfish species from the UK. Firstly,
136 virile crayfish were infected at a site in the River Lea where they coexisted with signal
137 crayfish, but were not found to be infected at an allopatric site. In contrast, signal crayfish
138 were infected with *A. astaci* both in regions of sympatry and allopatry. Secondly, the *A.*
139 *astaci* multilocus genotype identified in virile crayfish was similar, although not identical, to
140 the reference strain of the genotype group B, isolated in Europe from infected signal crayfish
141 (Huang *et al.*, 1994; Grandjean *et al.*, 2014), and closely matched the multilocus genotype
142 detected in a UK signal crayfish population (Table 1). Therefore, the current study may
143 represent the first report of *A. astaci* being interspecifically transmitted within a wild, mixed
144 species population of American crayfish in their invasive range.

145 Whilst the multilocus genotypes obtained for the *A. astaci*-positive DNA isolates
146 from virile crayfish in the River Lea were not an identical match to the reference strain of the
147 group B from Europe, such within group variation has already been reported (Grandjean *et*
148 *al.*, 2014; Maguire *et al.* 2016; Mrugała *et al.*, 2016). This, combined with the fact that the *A.*
149 *astaci* genotype obtained from infected virile crayfish was not similar to any of the other four

150 genotype groups currently described (Grandjean *et al.*, 2014), make it a likely assumption
151 that the virile crayfish in this study harboured an *A. astaci* strain from the genotype group B
152 pathogen. Of all American crayfish species, this genotype group has only previously been
153 recorded from wild signal crayfish. In addition, the multilocus genotypes obtained from both
154 virile crayfish specimens closely matched that isolated from signal crayfish elsewhere in the
155 UK. Variation detected at the locus Aast 12 may have resulted inefficient amplification of
156 low concentration DNA template). Both virile crayfish specimens also shared a homozygote
157 pattern at locus Aast 9 in contrast to the heterozygote reported in the original reference
158 sequence (James *et al.*, 2017). Combined these data add to the evidence that virile crayfish
159 likely contracted *A. astaci* from infected signal crayfish within the UK.

160 Possessing a wide host range is one of the key factors in determining the success of an
161 introduced parasite (Kennedy 1994). It has been speculated that the interspecific transmission
162 of *A. astaci* genotypes between American crayfish species may be limited by host-pathogen
163 incompatibilities (Tilmans *et al.*, 2014). Our findings cast doubt on this hypothesis, and
164 highlight the need for greater investigation into the host range of different *A. astaci* genotype
165 groups. Given that *A. astaci* genotypes differ in their virulence (Makkonen *et al.*, 2012,
166 2014; Viljamaa-Dirks *et al.*, 2013) and climate requirements (Diéguez-Uribeondo *et al.*,
167 1995; Rezinciuc *et al.*, 2014) interspecific transmission may influence the spread of the
168 pathogen across Europe. This is likely to have implications for native crayfish conservation
169 given that *A. astaci* affords American crayfish species an even greater competitive advantage
170 over their susceptible European counterparts. Interspecific transmission may also result in
171 complex mixed genotype group infections with unknown consequences for both American
172 carrier crayfish and native European crayfish hosts. Ultimately, this may also influence
173 pathogen evolution.

174 This study provides the first indication of *A. astaci* being transmitted between
175 American carrier crayfish species in a wild population. We do, however, acknowledge the
176 limitations of our study in terms of incomplete genotyping and low sample size. Studies of *A.*
177 *astaci* interspecific transmission are particularly relevant given that many European countries
178 now harbour multiple non-native American crayfish species (Kouba *et al.*, 2014). With
179 microsatellite markers allowing the characterization of *A. astaci* genotypes from host tissue
180 samples, increased effort should be focused into genotyping the pathogen strains infecting
181 American carrier crayfish species. Characterising the strain(s) of *A. astaci* responsible for a
182 crayfish plague outbreak may also help identify the source of an epidemic and monitor the
183 spread of infection (see Kozubíková *et al.*, 2014, Vrålstad *et al.*, 2014).

184

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191

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332 **Figure 1.** Sample sites along the River Lea (UK) and an adjacent lake for invasive signal
333 (*Pacifastacus leniusculus*) and virile (*Orconectes cf. virilis*) crayfish. River flow direction is
334 indicated by the black arrow. Allopatric sites shown for signal (●) and virile crayfish (▲)
335 respectively, and the sympatric site (■). Note: the location of the allopatric virile crayfish site
336 is a lake adjacent to the river. More details about virile and signal crayfish distribution in this
337 region can be found in James *et al.*, (2016). Image courtesy of Maps data 2016 @Google.
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369 **Table 1.** Comparison of allele sizes of nine microsatellite loci from the original European
 370 reference strain of *Aphanomyces astaci* genotype group B (Grandjean *et al.*, 2014), a
 371 reference strain from *Pacifastacus leniusculus* from Wales, UK (James *et al.*, 2017) and *A.*
 372 *astaci*-positive samples of *Orconectes cf. virilis* examined in the current study.
 373

Locus	Original European reference strain VI03555 (<i>P. leniusculus</i>)	UK population (<i>P. leniusculus</i>)	UK population (<i>O. cf. virilis</i>) specimen 1	UK population (<i>O. cf. virilis</i>) specimen 2
Aast 2	142	142	142	142
Aast 4	87	87	87	n/a
Aast 6	148	148	n/a	148
Aast 7	215	215	215	n/a
Aast 9	164/182	164	164	164
Aast 10	132	132	n/a	132
Aast 12	226/240	226/240	240	226/240*
Aast 13	202	202	202	202
Aast 14	248	248	248	n/a

374 *both alleles were scored but respective peaks were observed in separate PCR runs. n/a – loci
 375 with no amplification, likely due to low concentration of *A. astaci* DNA in the isolate.