

1 **Title**

2 Ephemeral detection of *Bonamia exitiosa* (Haplosporida) in adult and larval European flat
3 oysters *Ostrea edulis* in the Solent, United Kingdom

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35 **Abstract**

36 The haplosporidian parasite *Bonamia exitiosa* was detected using PCR in four adult and
37 six larval brood samples of the European flat oyster *Ostrea edulis* from the Solent, UK. This
38 represents the second reported detection of this parasite along the south coast of England.

39 Adult oysters were collected and preserved from seabed populations or restoration
40 broodstock cages between 2015 - 2018. The larvae within brooding adults sampled during
41 2017 and 2018 were also preserved. Molecular analysis of all samples was performed in
42 2019. The DNA of *B. exitiosa* was confirmed to be present within the gill tissue of one oyster
43 within the Portsmouth wild fishery seabed population (n = 48), sampled in November 2015;
44 the congeneric parasite *Bonamia ostreae* was not detected in this individual. This is the
45 earliest record of *B. exitiosa* in the Solent. Concurrent presence of both *B. ostreae* and *B.*
46 *exitiosa*, determined by DNA presence, was confirmed in the gill and heart tissue of three
47 mature individuals from broodstock cages sampled in October 2017 (n = 99), two from a
48 location on the River Hamble and one from the Camber Dock in Portsmouth Harbour. *B.*
49 *exitiosa* was not detected in the November 2018 broodstock populations. A total of six larval
50 broods were positive for *B. exitiosa*, with five also positive for *B. ostreae*. None of the
51 brooding adults were positive for *B. exitiosa* suggesting that horizontal transmission from the
52 surrounding environment to the brooding larvae is occurring. Further sampling of broodstock
53 populations conducted by the Fish Health Inspectorate at the Centre for Environment,
54 Fisheries and Aquaculture Science in June 2019 did not detect infection of *O. edulis* by *B.*
55 *exitiosa*. These findings together suggest that the pathogen has not currently established in the
56 area.

57

58 **Key words**

59 Oyster; *Ostrea edulis*; *Bonamia exitiosa*; *Bonamia ostreae*; Molecular diagnosis;
60 haplosporida

61

62 **1. Introduction**

63 As efforts to restore the European flat oyster *Ostrea edulis* gain momentum across
64 Europe, disease prevalence and resistance within populations will play a pivotal role in their

65 success. Of particular concern is the impact of European Commission (EC) notifiable
66 protozoan parasites within the genus *Bonamia* (Haplosporidia; Sprague 1979), especially *B.*
67 *ostreae*. The disease bonamiosis, caused by members of the genus of intrahaemocytic
68 protozoan parasites *Bonamia*, including *Bonamia ostreae*, has severely impacted *O. edulis*
69 populations. The microcells (2 - 5 µm diameter) of *B. ostreae* enter into the haemocytes of
70 the oysters by host-specified phagocytosis (Chagot et al., 1992) and become systemic,
71 overwhelming and eventually killing the infected individual. The distribution, spread and
72 mass mortality events caused by *B. ostreae*, since its introduction to Europe in the 1970s and
73 '80s (MacKenzie et al., 1997), are well documented (Figueras, 1991; Cigarria et al., 1995,
74 Laing et al., 2005; Culloty and Mulcahy, 2007) with its impact as a non-native species
75 driving disease emergence highlighted by Peeler et al. (2011).

76 Another member of the genus, *B. exitiosa*, first detected in the southern hemisphere in
77 association with the host *Ostrea chilensis* (Dinamani et al., 1987; Cranfield et al., 1991; Hine
78 et al., 2001) has subsequently been detected in *O. edulis* across continental Europe. The first
79 detection occurred in 2006 (Galician coast, Spain (Abollo et al., 2008)), shortly followed by
80 another in 2007 (Adriatic Sea, Italy (Narcisi et al., 2010)). The species has subsequently been
81 detected in France (Mediterranean Sea (Arzul et al., 2010)), the Spanish Mediterranean coast
82 (Carrasco et al., 2012), Britain (Cornwall (Longshaw et al., 2013)) and Portugal (Algarve
83 (Batista et al., 2016)). The first UK positive population in which *B. exitiosa* was detected,
84 was in the River Fal (Cornwall) (Longshaw et al., 2013), 28 years after the first diagnosis of
85 *B. ostreae* in the UK, also in the River Fal (Bucke and Feist, 1985; Hudson and Hill 1991).
86 To date there have been no reported mass mortality events in Europe where *B. exitiosa* has
87 been considered the aetiological agent and a small number of infected individuals were
88 detected within the sampled populations.

89 *Bonamia exitiosa* was first described infecting *Ostrea chilensis* in New Zealand (Hine
90 et al., 2001), after a mass mortality event devastated an ‘immunologically naïve’ oyster
91 population between 1986 and 1992 (Doonan et al., 1994; Cranfield et al., 2005).
92 Retrospective analysis demonstrated that tissue samples infected with *B. exitiosa* date back to
93 1964, supporting the assumption that the species is endemic to New Zealand (at least) and
94 that a relatively stable host/parasite relationship exists (Hine and Jones, 1994; Hine, 1996).

95 Unlike *B. ostreae*, where aspects of the life cycle remain unanswered (Culloty and
96 Mulcahy, 2007), the life cycle of *B. exitiosa* is relatively well documented and is key to our
97 understanding of dispersal mechanisms across a wide geographic range (Cranfield et al.,
98 2005; Hill et al., 2014). The intrahaemocytic *B. exitiosa* spreads through the dispersal of
99 infective particles released from the gonads, kidneys, gills and gut tissue of the diseased or
100 dying oyster host (Hine, 1991a, 1991b). Once ingested by oysters in close proximity these
101 new hosts become infected when the parasite enters the blood via the gut (Hine and Jones,
102 1994). There is currently no literature available describing the occurrence of vertical
103 transmission from parent to larval brood, or of horizontal transmission in any of the host
104 oyster species.

105 *B. exitiosa* is currently known to infect wild and aquaculture stocks of multiple oyster
106 species from around the globe including *Ostrea chilensis*, *O. edulis*, *O. angasi*, *O. puelchana*,
107 *O. stentina*, and *Saccostrea glomerata*, with at least occasional infection of *O. lurida*,
108 *Crassostrea virginica* and *C. ariakensis* noted as well. The parasite has been associated with
109 mass mortality events for some of these oyster species (Burreson et al., 2004; Corbeil et al.,
110 2006; Hill et al., 2010; Kroeck, 2010; Carnegie et al., 2014; Hill et al., 2014; Engelsma et al.,
111 2014).

112 We used molecular technology to document the presence of *B. exitiosa* and *B. ostreae*
113 in *O. edulis* populations in the Solent, UK over multiple years.

114

115 **2. Material and Methods**

116 **2.1. Oyster provenance**

117 Oyster samples were collected within the Solent (the stretch of water separating
118 Southern England from the Isle of Wight) between 2015 - 2018 for on-going monitoring
119 conducted as part of the Solent Oyster Restoration Project
120 (www.bluemarinefoundation.com/project/solent/). These samples were stored in 98% ethanol
121 and were held at 4°C at the Institute of Marine Sciences (University of Portsmouth,
122 Portsmouth, UK) until retrospective screening was conducted in 2019 for pathogen presence.
123 In November 2015, oysters were collected from the seabed using a commissioned dredge
124 fisher in the area managed by the Southern and Sussex Inshore Fisheries and Conservation
125 Authorities, as described in Helmer et al. (2019). A sub-sample of these from Chichester
126 Harbour (n = 48) and Portsmouth Harbour (n = 48) (locations H+S and E and T, respectively,
127 Fig. 1) were sampled immediately and stored for later molecular analysis of pathogen DNA
128 presence. The remaining oysters sourced from the fishery were translocated into restoration
129 broodstock cages suspended from existing floating structures in Portsmouth Harbour - BA
130 (individuals from H+S) and Langstone Harbour - UP (individuals from E and T) in December
131 2015 (Fig. 1). Additional oysters (BA n = 42, UP n = 16) were sampled from these cages in
132 July 2016 and stored as above.

133 Oysters sampled in October 2017 (n = 99) and November 2018 (n = 70) were originally
134 purchased from the catch of the 2016 dredge fishery in Langstone Harbour (Locations L and
135 S, Fig. 1). The 2016 seabed oysters were translocated into broodstock cages at two marina
136 locations in the River Hamble in November 2016 (PH and HP, Fig. 1), and then distributed to

137 four additional locations across the Solent in March 2017 (SW, BA, UP and SP, Fig. 1, in
138 addition to PH and HP). Oyster samples were taken and preserved from all marina locations
139 during October 2017 and November 2018. Oysters collected in 2017 and 2018 were
140 monitored for the presence of larvae within the pallial cavity and the white, grey or black
141 “sick” larval stage was also recorded (Fig. 2). Brooding adults, and their larvae, were
142 sampled and preserved for later molecular analysis. Adult gill and heart tissues were stored
143 separately from the larval brood; a 250- μ l aliquot of each brood was preserved in ethanol. A
144 total of 35 broods were analysed, 31 from 2017 and 4 from 2018, with 21 of these having
145 been sampled from brooding adults that were also screened for *B. exitiosa*.

146 The Fish Health Inspectorate (FHI) of England and Wales was contacted immediately
147 upon PCR detection of *B. exitiosa*. Upon suspicion of presence of this exotic pathogen, the
148 FHI carried out statutory sampling of *O. edulis* populations to test for the presence of *B.*
149 *exitiosa*: 129 oysters were sampled from Port Hamble Marina (PH, Fig. 1), along with 150
150 from the Camber Dock, Portsmouth Harbour (BA Fig. 1) in March 2019, and an additional 26
151 oysters were sampled from the University of Portsmouth research platform in Langstone
152 Channel (UP, Fig. 1) in June 2019. Tissue ‘steaks’ were dissected from each oyster and fixed
153 for histopathology and molecular analyses and were processed for both methods as described
154 in Longshaw et al. (2013).

155

156 **2.2. Genomic DNA extraction and PCR amplification**

157 A 5-mm section of gill tissue and the whole heart from each of the adult 2017 brooding,
158 2017 broodstock and 2018 broodstock samples were removed and stored in 98% ethanol
159 before maceration with a sterile scalpel or pellet pestle. A 5-mm section of gill tissue was
160 analysed from the 2015 seabed and 2016 broodstock samples, and was also removed and
161 stored in 98% ethanol prior to maceration. The larval broods, rinsed with 0.2 μ m filtered

162 seawater and 98% ethanol prior to storage in 98% ethanol, required no mechanical
163 breakdown for the extraction process. All DNA extractions were performed using DNeasy[®]
164 Blood & Tissue kits (QIAGEN[™]) following the manufacturer's tissue protocol.
165 Quantification of DNA was conducted using a NanoDrop[®] 1000 Spectrophotometer
166 (NanoDrop[®], Thermo Fisher Scientific Inc., Wilmington, USA).

167 The *Ostrea edulis* species-specific primer pair Oe fw_1 + Oe rev_4 (5'-ATG-GGA-
168 CGA-TTT-GAT-AGA-GC-3' and 5'-CCC-AAA-TAA-CGG-GAA-AAG-TGC-TAA-CCA-
169 CCA-GAA-TGA-3', respectively) (Gercken and Schmidt, 2014) was used to amplify the
170 cytochrome c oxidase subunit I (COI) gene from *O. edulis* as a positive control for oyster
171 species confirmation. Due to the potential for concurrent infection of both *B. ostreae* and *B.*
172 *exitiosa* and the specificity of OIE recommended primer pairs for *B. ostreae* over *B. exitiosa*
173 (Helmer et al. unpublished results), the species-specific primer pairs BOSTRE-F + BOSTRE-
174 R (5'-TTA-CGT-CCC-TGC-CCT-TTG-TA-3' and 5'-TCG-CGG-TTG-AAT-TTT-ATC-GT
175 -3', respectively) (Ramilo et al., 2013) and BEXIT-F + BEXIT-R (5'-GCG-CGT-TCT-TAG-
176 AAG-CTT-TG-3' and 5'-AAG-ATT-GAT-GTC-GGC-ATG-TCT-3', respectively) (Ramilo
177 et al., 2013) were used to amplify the 18S-ITS1 rRNA gene region present from *B. ostreae*
178 and *B. exitiosa*, respectively. The OIE recommended 18S primer pair BO + BOAS (5'-CAT-
179 TTA-ATT-GGT-CGG-GCC-GC-3' and 5'-CTG-ATC-GTC-TTC-GAT-CCC-CC-3',
180 respectively) (Cochennec et al., 2000) was also used to amplify *B. ostreae* DNA. Polymerase
181 chain reaction (PCR) amplifications consisted of 12.5 μ l 2 x DreamTaq[™] PCR Master Mix
182 (Thermo Fisher Scientific Inc.) or 12.5 μ l 2 x DreamTaq Green PCR Master Mix (Thermo
183 Fisher Scientific Inc.), 0.2 μ M forward and reverse primers (Invitrogen, Thermo Fisher
184 Scientific Inc.) and 20 - 200 ng genomic DNA made up to a final volume of 25 μ l with
185 molecular biology grade water. A negative control, with molecular biology grade water in
186 place of template DNA, was run alongside each reaction. No negative controls amplified

187 during the course of the current study. No positive control was available at the outset. *B.*
188 *exitiosa*-positive PCR products generated using the BEXIT primer pair during earlier
189 sampling in the current study, later confirmed by sequencing of the 18S-ITS1 gene region,
190 were used as positive controls in the latter PCR analysis.

191 PCRs were run in a G-STORM 482 Thermal Cycler (Gene Technologies Ltd., Essex,
192 England) under the respective conditions described by Cochenec et al. (2000), Ramilo et al.
193 (2013), Gercken and Schmidt (2014). PCR products were separated on 1% (Oe) or 2%
194 (BOSTRE, BEXIT and BO + BOAS) 1x TAE (40 mM Tris, 20 mM acetic acid, 1 mM
195 EDTA) agarose gels stained with 4 ul ethidium bromide. Electrophoresis ran at 100 V for 1 h.
196 A 1-kb GeneRuler™ DNA ladder (Thermo Fisher Scientific Inc.) or 100-bp DNA ladder
197 (New England Biolabs® or PCR Biosystems Ltd) and PCR products were visualized by
198 ultraviolet (UV) transillumination (VWR Gel Documentation Smart Version).

199 PCR products of all *B. exitiosa*-positive amplifications using the BEXIT-F + BEXIT-R
200 primer pair and reference samples and strong bands from Oe fw_1 + Oe rev_4, BOSTRE-F +
201 BOSTRE-R and BO + BOAS primer pairs were purified using a QIAquick® PCR Purification
202 Kit (Qiagen) following the manufacturer's protocol. Amplicons were sequenced by Sanger
203 sequencing (Source BioScience, Nottingham, England) using the respective primer pairs used
204 for PCR, and the electropherograms analysed by eye in MEGA X (Pennsylvania State
205 University, USA). Where possible, contigs were assembled using CAP3 sequence assembly
206 program (Huang & Madan, 1999). The resulting contig sequences were BLASTn®-searched
207 against the nr/nt database of the National Center for Biotechnology Information web server.
208 Sequences were deposited into GenBank (Accession numbers MT184259 - MT184268).

209

210 **3. Results**

211 Of the 96 oysters sampled from the 2015 Seabed populations, one individual (1.04%),
212 from the Portsmouth fishery area (H+S Fig. 1), was positive for *B. exitiosa* based on the
213 amplification of the expected 246 bp using the BEXIT-F + BEXIT-R primer pair. The
214 sequence of the PCR-amplification product showed 99.59% identity to a *B. exitiosa* sequence
215 from Tunisia (JF831718.1). *B. ostreae* was not detected in this individual using the BOSTRE-
216 F + BOSTRE-R primer pair. *B. ostreae* DNA was detected in 34.4% and 49% of the oysters
217 using the BO + BOAS and BOSTRE primer pairs, respectively.

218 Of the broodstock oysters sampled in 2017, three (3.03%) screened positive for *B.*
219 *exitiosa* DNA. Of those, two were located in the same marina on the River Hamble (PH, Fig.
220 1) and the other in Portsmouth Harbour (BA, Fig. 1). The sequence of the PCR-amplification
221 products from the River Hamble showed 100% identity to a *B. exitiosa* sequence from North
222 Carolina (JF831588.1), whilst the sample from Portsmouth Harbour showed 100% identity to
223 a *B. exitiosa* isolate sequence from Australia (JF831683.1). Both oysters from the River
224 Hamble and the individual from Portsmouth Harbour were also positive for *B. ostreae* DNA,
225 with a 208-bp amplicon from the BOSTRE-F + BOSTRE-R primer pair. *B. ostreae* DNA was
226 detected in 85.7% and 98% of the oysters using the BO + BOAS and BOSTRE primer pairs,
227 respectively. No oysters sampled from the 2016 or 2018 broodstock cages tested positive for
228 *B. exitiosa*. However, in 2016, 34.5% and 46.6% of oysters tested PCR-positive for *B.*
229 *ostreae* using the BO + BOAS and BOSTRE primer pairs, respectively. In 2018, the same
230 primer pairs resulted in 54.3 % and 81.4 % of oysters also testing PCR-positive for *B.*
231 *ostreae*.

232 Of the 10 larval broods analysed without the respective adult collected for analysis,
233 none were PCR-positive for *B. exitiosa*. A total of 21 brooding adults and their larval broods
234 from 2017 were analysed; none of the brooding adult oysters tested positive for *B. exitiosa*
235 (Fig. 3). The larval brood from one PCR-negative adult oyster in Chichester Harbour tested

236 positive using PCR and showed 100% identity to a *B. exitiosa* sequence from North Carolina
 237 (JF831588.1). Another four broods were PCR-positive with the BEXIT primers, but the F and
 238 R sequences did not form a contiguous sequence due to either low sequence quality or lack of
 239 consensus. The latter could be due to multiple parasites occurring within the brood. Further
 240 work is required to clarify the validity of these results. Of the four broods collected from
 241 Chichester Harbour in 2018, one provided a sequence contig that showed 100% identity to a
 242 *B. exitiosa* sequence from North Carolina (JF831588.1). The positive results obtained for all
 243 adult and larval samples are summarised in Table 1 and the sample groupings from all years
 244 in Table 2. In 2017, 64.5% and 77.4% of the larvae tested PCR-positive for *B. ostreae* using
 245 the BO + BOAS and BOSTRE primer pairs, respectively. This was also the case for 50% and
 246 75% of larval samples in 2018, using the same respective primer pairs. Sequences with a
 247 similarity to *B. exitiosa* greater than 98% were submitted to GenBank (Accession numbers
 248 MT184259 - MT184268).

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254 Table 1. Details of samples that tested positive by PCR screening for *Bonamia exitiosa* with
 255 highest sequence identity from GenBank BLASTn search. Samples with no contiguous
 256 sequence that provide tentative results are grouped with respective borderlines and grey
 257 scaled, F or R denotes the forward or reverse primer sequence used.

| Study sample information | | | GenBank search results | | | | |
|--------------------------|--------------|----------|------------------------|------------|-------------------|--------------|-------------------|
| Location in Figure 1 | Sample group | Sequence | Top GenBank match | Identity % | Geographic region | Host species | GenBank accession |
| <hr/> | | | | | | | |

| | | | | | | | |
|-------------|-----------------|--------|--------------------|--------|-----------------|-----------------------------|----------|
| H+S | 2015 Seabed | Contig | <i>B. exitiosa</i> | 99.59 | Tunisia | <i>Ostrea stentina</i> | JF831718 |
| Port Hamble | 2017 Broodstock | Contig | <i>B. exitiosa</i> | 99.18 | North Carolina | <i>Ostrea stentina</i> | JF831588 |
| Port Hamble | 2017 Broodstock | Contig | <i>B. exitiosa</i> | 100.00 | North Carolina | <i>Ostrea stentina</i> | JF831588 |
| Portsmouth | 2017 Broodstock | Contig | <i>B. exitiosa</i> | 100.00 | Australia | <i>Saccostrea glomerata</i> | JF831683 |
| Chichester | 2017 Larvae | Contig | <i>B. exitiosa</i> | 100.00 | North Carolina | <i>Ostrea stentina</i> | JF831588 |
| Chichester | 2018 Larvae | Contig | <i>B. exitiosa</i> | 100.00 | North Carolina | <i>Ostrea stentina</i> | JF831588 |
| Langstone | 2017 Larvae | F | <i>B. exitiosa</i> | 98.59 | Argentina | <i>Ostrea stentina</i> | JF831559 |
| Langstone | 2017 Larvae | R | <i>B. exitiosa</i> | 100.00 | Tunisia | <i>Ostrea stentina</i> | JF831718 |
| Chichester | 2017 Larvae | F | <i>B. exitiosa</i> | 97.77 | New Zealand | <i>Ostrea chilensis</i> | KY680634 |
| Chichester | 2017 Larvae | R | N/A | N/A | N/A | N/A | N/A |
| Chichester | 2017 Larvae | F | N/A | N/A | N/A | N/A | N/A |
| Chichester | 2017 Larvae | R | <i>B. exitiosa</i> | 100.00 | Australia (NSW) | <i>Saccostrea glomerata</i> | JX977122 |
| Chichester | 2017 Larvae | F | <i>B. exitiosa</i> | 93.70 | California | <i>Ostrea conchaphila</i> | JF831733 |
| Chichester | 2017 Larvae | R | <i>B. exitiosa</i> | 98.40 | Tunisia | <i>Ostrea stentina</i> | JF831718 |

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263 Table 2. Summary of sample populations, sample type, number of oysters from each location
264 and population sampled. Bold numbers in parentheses indicate the number of PCR-positive
265 *Bonamia exitiosa* samples from the respective sample set obtained using high quality
266 consensus sequence reads. Numbers not in bold indicate those samples where identification
267 requires further analysis.

Number of oysters per location

| River Itchen | River Hamble | Portsmouth Harbour | Langstone Harbour | Chichester Harbour | Total |
|--------------|--------------|--------------------|-------------------|--------------------|-------|
|--------------|--------------|--------------------|-------------------|--------------------|-------|

| Sampling year | Sample type | SW | PH | HP | H+S | BA | UP | E / T | SP | |
|--|--------------|----|--------|----|--------|--------|-------|-------|---------|----|
| 2015 Seabed populations | Gill | | | | 48 (1) | | | 48 | | 96 |
| 2016 Broodstock cages | Gill | | | | | 42 | 16 | | | 58 |
| 2017 Broodstock cages | Gill + Heart | 17 | 17 (2) | 17 | | 17 (1) | 17 | | 14 | 99 |
| 2017 Brooding individuals (within cages) | Gill + Heart | 1 | 3 | 3 | | 2 | 5 | | 8 | 22 |
| 2017 Larvae | Larvae | 2 | 4 | 4 | | 4 | 8 (1) | | 9 (1,3) | 31 |
| 2018 Broodstock cages | Gill + Heart | 10 | 12 | 12 | | 12 | 12 | | 12 | 70 |
| 2018 Larvae | Larvae | | | | | | | | 4 (1) | 4 |

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269 The 305 samples collected by the Fish Health Inspectorate (FHI) of England and Wales
270 and analysed by the Statutory Diagnostic Team at the Centre for Environment, Fisheries and
271 Aquaculture Sciences (CEFAS) were all PCR-negative for *B. exitiosa* using the lineage-
272 specific BEXIT primers. *B. exitiosa* was not observed in any histology screens of the animals.

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277 4. Discussion

278 The current study describes the second detection of *B. exitiosa* in *O. edulis* in the UK,
279 with concurrent detection of *B. ostreae*, as previously reported by Abollo et al. (2018),
280 Ramilo et al. (2014) and Lane et al. (2016). It should be noted that not all the samples in the
281 present study were analysed by histology or heart smears, therefore only the DNA of *B.*

282 *exitiosa* and *B. ostreae* was detected for a proportion of the population in this study. Infection
283 of native oysters by *B. exitiosa* was not confirmed by microscopic examination or histology
284 because no diseased oysters were observed; therefore, the possibility the pathogen was
285 dormant or not viable cannot be ruled out (Burreson, 2008). The detection of *B. exitiosa* was
286 ephemeral in nature and limited to a small portion of the populations monitored and no
287 disease symptoms or mortality was attributed to *B. exitiosa*. Mortality experienced within the
288 monitored populations is more likely attributed to a combination of post-spawning mortality
289 (Helmer, unpublished data), environmental stressors, such as temperature and salinity, and
290 the high prevalence of *B. ostreae* observed. As *B. ostreae* is well established in the area
291 (Laing et al., 2014) it is unsurprising that such high proportions of the oysters sampled tested
292 PCR-positive for this pathogen.

293 Despite the lack of histological analysis to indicate infection intensity by *B. exitiosa* in
294 this case, and the lack of DNA detection during statutory disease assessments of oysters
295 sampled from two proximal sites in 2016, 2018 and 2019, the distribution and potential
296 impacts of *B. exitiosa* across Europe requires further investigation. In addition, a detailed
297 investigation into the phylogeny and origin of the strains for members of the *Bonamia* genus
298 in *O. edulis* populations is strongly recommended, as the complete status across Europe is
299 currently unknown, even though the presence of *B. ostreae* is relatively well documented.

300 The only other characterised *Bonamia* species is *B. perspora* (Carnegie et al., 2006). It
301 is believed that *B. perspora* is host specialist and currently maintains a well-defined and
302 restricted geographical range infecting *Ostrea stentina* in North Carolina (Carnegie et al.,
303 2006; Hill et al., 2014), thus unlikely to be present in *O. edulis* within Europe. Infections of
304 *B. perspora* were not observed in populations of *O. stentina* in Argentina, Tunisia and New
305 Zealand (Hill et al., 2014) but its presence in *O. stentina* in areas across Europe remains
306 untested. *Bonamia roughleyi* was first described as a distinct species (Cochennec-Laureau et

307 al., 2003), but Carnegie et al. (2014) questioned its identity, arguing that there is a lack of
308 genetic distinction between *B. exitiosa* and *B. roughleyi*.

309 To date there have been no reported mass mortalities of *O. edulis* within Europe where
310 *B. exitiosa* has been identified as the aetiological agent; all accounts have reported *B. ostreae*
311 to be the responsible pathogen. All reported detections of *B. exitiosa* in *O. edulis* have been in
312 a small proportion of the tested populations, with Abollo et al. (2008) reporting the highest
313 prevalence of 40.2% with 16.5% co-infection with *B. ostreae*. Batista et al. (2016) reported
314 positives in 83.3% of samples but the small sample size was small (n = 24). In many cases
315 co-infection with *B. ostreae* was reported; we found only one adult and one larval brood
316 infected with *B. exitiosa* but not *B. ostreae*.

317 The ability of *O. edulis* to tolerate co-infection with *B. exitiosa* and *B. ostreae* may be
318 due to the similarity of the two pathogen species but also their difference in lethality, with the
319 18-week 50% lethal dose of *B. ostreae* in *O. edulis* (Hervio et al., 1995) being 40% lower
320 than that of *B. exitiosa* in *O. chilensis*, the former determined to be $\sim 1.1 \times 10^5$ infective
321 particles (Diggles and Hine, 2002). This indication that *B. ostreae* is far more virulent than *B.*
322 *exitiosa* suggests that any resistance, tolerance or resilience to *B. ostreae* within European
323 populations of *O. edulis*, developed in the 30-40 years since its introduction (1970s - 80s)
324 (MacKenzie et al., 1997; Culloty and Mulchay, 2007; Lynch et al., 2014), may provide a
325 level of resistance, tolerance or resilience to *B. exitiosa* that impedes its rapid proliferation.
326 Another possibility is that interspecific competition between the two pathogens is occurring,
327 with *B. ostreae* excluding or outcompeting *B. exitiosa*. Such interactions are yet to be
328 investigated in these species.

329 The detection of *B. exitiosa* has implications for management of infected populations as
330 this pathogen is included, along with *B. ostreae*, within the list of notifiable species by the

331 World Organisation for Animal Health (OIE) (<http://www.oie.int/animal-health-in-the->
332 [world/oie-listed-diseases-2019/](http://www.oie.int/animal-health-in-the-world/oie-listed-diseases-2019/), last accessed 26 March 2019) and the EC Council Directive
333 2006/88/EC ([https://eur-](https://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2006:328:0014:0056:en:PDF%20)
334 [lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2006:328:0014:0056:en:PDF%20](https://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2006:328:0014:0056:en:PDF%20), last
335 accessed 18 May 2019) with this document legally ensuring that the Fish Health Inspectorate
336 of England and Wales, the responsible entity for fish and shellfish health, regularly monitor
337 for *B. ostreae* and *B. exitiosa*.

338 The increase in geographical distribution of *B. exitiosa* (Hill et al., 2014) is likely to be
339 attributed to its dispersal potential. Survival of infective particles in seawater has been shown
340 to be 50% after 48 h at 18°C (Diggles and Hine, 2002) and detection of *B. exitiosa* in *O.*
341 *edulis* larvae (Arzul et al., 2011) suggests that if the pathogen is viable in, or incidentally
342 attached to the larvae, dispersal or spread could be accelerated. The detection of *B. exitiosa* in
343 larval broods of PCR-negative adults indicates the occurrence of horizontal transmission by
344 release of the pathogens from dead or dying oysters (Hine 1991a, b; Audemard et al., 2014)
345 or other vector taxa. Evidence of horizontal transmission has been described for *B. ostreae* in
346 *O. edulis* (Arzul et al., 2011; Flannery et al., 2016), with Lynch et al. (2010) also detecting *B.*
347 *ostreae* DNA in the pallial fluid. The capacity of *O. edulis* larvae to feed within the pallial
348 cavity during the brooding period (Hine and Jones, 1994; Helm et al., 2006) and detection of
349 *B. ostreae* in the epithelia surrounding the visceral cavity of infected larvae (Arzul et al.,
350 2011), further highlights the opportunity for transmission of *B. exitiosa* in this manner. This
351 mounting evidence of larval infection highlights one of many potential transmission
352 pathways of *Bonamia* infection to naïve oyster populations, with larvae having been shown to
353 travel up to 12 km from the source location (Wilson, 1987).

354 Restoration efforts, whereby oysters are transported into areas that have been left fallow
355 for prolonged periods, should continue to include monitoring for both *B. ostreae* and *B.*

356 *exitiosa*. van Banning (1998) showed that *B. ostreae* can persist in the environment in the
357 absence of *O. edulis* and infect newly introduced naïve oysters, with Lynch et al. (2007)
358 suggesting that this could be due to the potential of multiple macroinvertebrate species to act
359 as carriers. All eight potential carrier species observed in that study, including *Actinia equine*,
360 *Carcinus maenas* and *Asciidiella aspersa*, are present and abundant within the Solent and
361 across much of Europe. The presence of *B. ostreae* in the 2007 study was not limited to
362 benthic species - grouped zooplankton species also yielded positive results adding to the
363 potential for vectoring. For example, the copepod *Paracartia grani* acts as an intermediate
364 host for another serious oyster parasite, *Marteilia refringens* (Audemard et al., 2002). The
365 ability of *B. exitiosa* to utilise intermediate hosts is currently unknown and also requires
366 further research.

367 Evidence that the Pacific oyster *Crassostrea gigas* may be a host for both *B. ostreae*
368 and *B. exitiosa* (Lynch et al., 2010; Flack, unpublished results) is of particular concern as the
369 species' distribution and abundance across Europe has increased in recent years (Anglès
370 d'Auriac et al. 2017). This potentially provides "stepping-stones" for disease transfer
371 between remaining populations of *O. edulis* that are currently fragmented. Further clarity of
372 the disease vector role played by *C. gigas* is required for areas across Europe where it is
373 present in aquaculture or wild populations. Confirmation of *C. gigas* as a disease vector may
374 require the active management and removal of significant populations in order to aid
375 prevention of disease transmission within flat oyster populations. Alternatively, Pacific
376 oysters could be paratenic or dead-end hosts acting as sinks for the pathogens, indefinitely or
377 until prevalence reaches a threshold. Similarly, the role of disease transmission by the
378 invasive and highly abundant American slipper limpet *Crepidula fornicata* (Helmer et al.,
379 2019) is unclear and needs to be determined, with the potential of additional supporting
380 information to justify their removal on a large scale.

381 Incidents of *B. exitiosa* infecting *O. edulis* where *B. ostreae* is not present have been
382 observed previously (Batista et al., 2016). The first European detection of *B. exitiosa* on the
383 Galician coast in 2006, followed by infections along the French Atlantic coast in 2008 and
384 then the River Fal in 2010, suggests that a combination of anthropogenic oyster movements,
385 larval dispersal and infective particle transmission enables the relatively rapid transmission of
386 *B. exitiosa* north-eastward. The ability of *Bonamia* species to parasitize a range of hosts,
387 alongside infective particle dispersal and larval infection, is likely to have contributed to its
388 dispersal on a global scale, including New Zealand, Australia and Argentina in the southern
389 hemisphere, and Atlantic coastlines (US and Europe), Pacific coastlines (US) Mediterranean
390 Sea and English Channel in the northern hemisphere.

391 It is uncertain if the presence of *B. exitiosa* poses a threat to progress made with the
392 selective breeding for resistance to *B. ostreae* in the European flat oyster (Hervio et al., 1995;
393 Culloty et al., 2004; Lynch et al., 2014). Mortality events of *O. edulis* should continue to be
394 monitored rigorously, as the species is unlikely to fare well with the introduction of this
395 additional non-native protozoan if it reaches a significant prevalence within a population. The
396 impact of such an event can be seen from the mass mortalities in Europe induced by the
397 initial introduction of *B. ostreae* from the west coast of the USA (Elston et al., 1986;
398 MacKenzie et al., 1997). It is therefore recommended that monitoring for, and restricted
399 movement of, oysters infected with *B. exitiosa* be incorporated into section 4 (Respect
400 *Bonamia*-free areas) of the Berlin Oyster Recommendations (Pogoda et al., 2019). These
401 recommendations were compiled through a collaborative assessment of the current European
402 restoration efforts with the aim of developing and sharing best restoration practices for the
403 species, a prime example of information sharing.

404 For *O. edulis* restoration efforts to be successful, as they have been for other oyster
405 restoration projects in disease-stricken sites around the world, (Proestou et al., 2016),

406 deploying large quantities of oysters in high-density populations will be required to recreate a
407 fraction of historical population densities. This may incur significant mortality due to disease,
408 but assuming the stocks used are genetically robust and diverse it also provides an
409 opportunity for natural resistance to develop over time.

410

411 **5. Conclusion**

412 The low levels of detection of *B. exitiosa*, along with no increase in detection in high-
413 density oyster populations over subsequent years, suggests the parasite has either failed to
414 establish in the Solent or may have established at low enzootic levels. However, the current
415 study highlights the risk of emerging and known pathogens to oyster restoration and
416 aquaculture in Europe and further emphasises the requirement for continued control of oyster
417 translocation. Biosecurity controls are the only method currently available to prevent or
418 postpone the spread of *Bonamia* parasites, but as can be observed by the continued spread of
419 pathogens, and as reported here, these control measures are not always successful. Therefore,
420 it is clear that further research is required fully understand the mechanism of transmission,
421 the vector species and environmental pathways through which *Bonamia* pathogens enter
422 previously disease-free sites in order to successfully manage bonamiosis.

423 ***Ethical approval***

424

425 All applicable international, national and/or institutional guidelines for the care and use of
426 animals were followed. This article does not contain any studies with human participant
427 performed by any of the authors.

428

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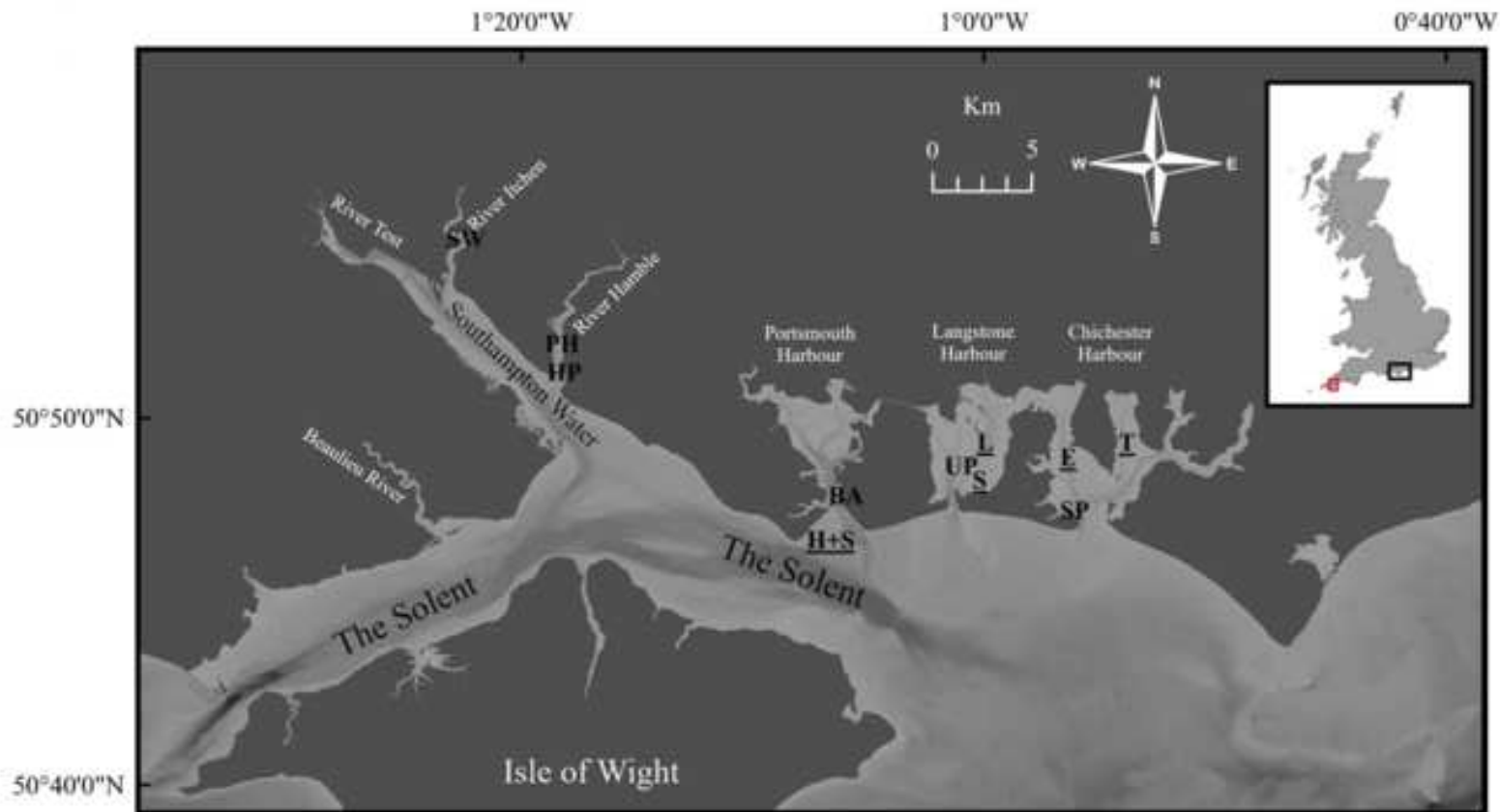


Fig. 1. The wider Solent (black box), showing the original source locations for the 2015 wild fishery populations (H+S, E, and T), 2016 broodstock cage populations (BA sourced from H+S, UP sourced from E and T) and 2017/18 broodstock cage populations (SW, PH, HP, BA, UP and SP, all sourced from L and S). Red box indicating the location of the River Fal where the first detection of *Bonamia exitiosa* in the UK occurred (Longshaw et al., 2013). Modified from Helmer et al. (2019). Map created using ArcMap software. (<http://desktop.arcgis.com/en/arcmap/>).

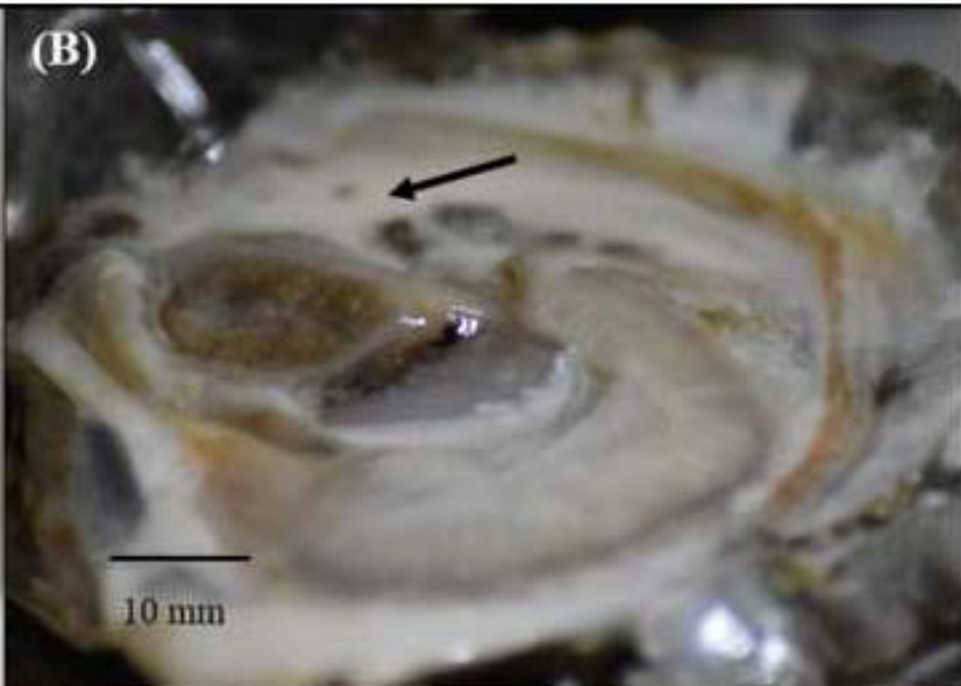
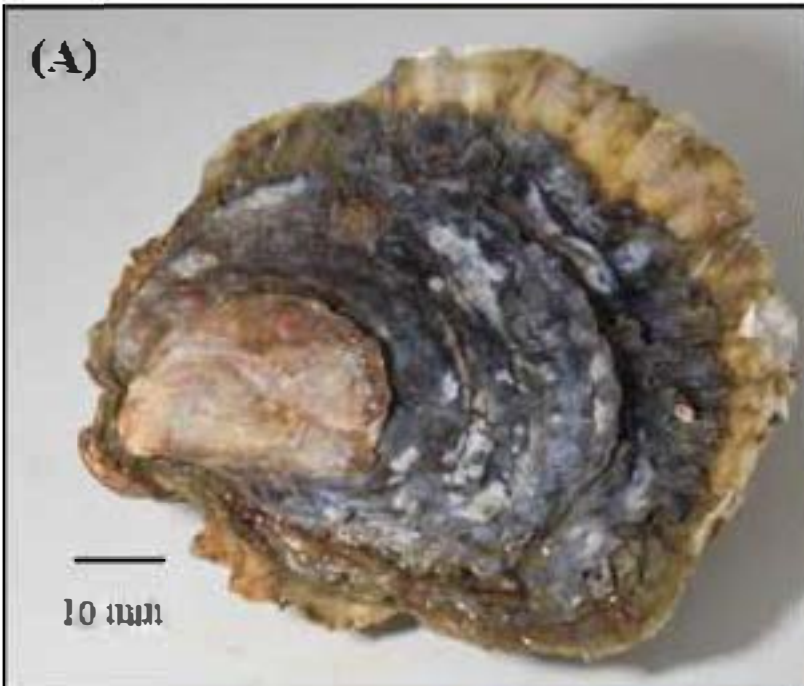


Fig. 2. (A) Brooding oyster prior to shucking; brooding oysters containing larvae referred to as (B) white 'sick?', (C) grey 'sick?' and (D) black 'sick?' stages of development. Larvae within the pallial cavity in and around the gill and mantle structures, indicated by arrows.

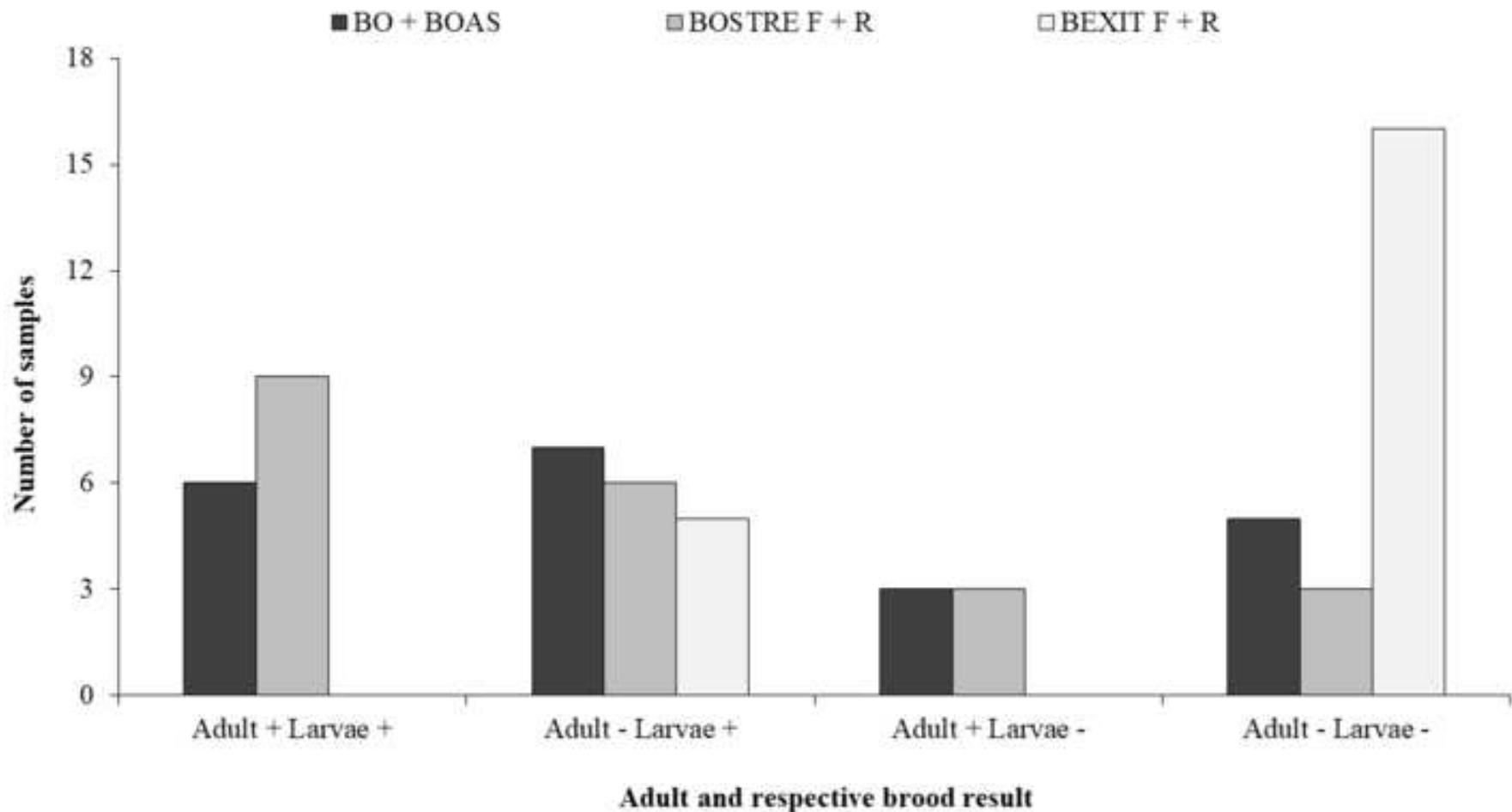


Fig. 3. The direct comparison of the positive or negative amplifications of *Bonamia ostreae* (BO + BOAS and BOSTRE-F + BOSTRE-R) and *Bonamia exitiosa* (BEXIT-F + BEXIT-R) DNA from individual brooding oysters and their respective larval brood collected during the 2017 spawning season. All samples provided positive amplifications for *Ostrea edulis* using the Oe primer pair.