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1 **Co-occurrence and reproductive synchrony do not ensure hybridization**  
2 **between an alien tunicate and its interfertile native congener**

3

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21

22 **Abstract**

23           Biological invasions can promote secondary contacts between related species and thus  
24 provide excellent case studies for investigating the joint ecological and evolutionary  
25 trajectories of interfertile taxa. Here, we studied two tunicates living in sympatry, and  
26 sometimes in syntopy, in the English Channel, *Ciona intestinalis* species A (presumed native  
27 to the NW Pacific) and species B (native to the N Atlantic). In addition to monitoring their co-  
28 existence over time, we examined the level of interspecific gene flow, a process that may  
29 increase the invasiveness of the non-native species. The sampling scheme was repeated twice  
30 a year for three years (6 distinct generations) to determine the relative abundance of the two  
31 species in 11 localities along the coasts of the English Channel and the Iroise Sea (covering  
32 1600 km) in Brittany, France. We examined 23,000 individuals, including 5,315 specimens  
33 for which reproductive status was determined. Four species-diagnostic molecular markers  
34 traced interspecific gene flow on a random subset of 3,048 individuals. Regardless of the  
35 sampling date, the two species co-occurred in most of the study sites, with species A showing  
36 higher frequency in the autumn. The regional pattern of seasonal variation in relative  
37 abundance of the two congeners appears to correspond to different thermal growth optima.  
38 Abrupt variations in environmental parameters can act synergistically and may favor the non-  
39 native species locally. Despite syntopy, synchronous gamete production and successful *in*  
40 *vitro* interspecific crosses, only 4.3% individuals showed an admixed genome (i.e. footprint of  
41 present-day or past introgression events), most of them with a species A maternal lineage, of  
42 which only one was a putative first generation hybrid. Altogether, efficient barriers seem to  
43 prevent interspecific crosses between the two interfertile congeners in the wild: present-day  
44 hybridization may have less impact than competitive interactions on the fate of the two study  
45 species over their sympatric range.

46

## 47 **Introduction**

48           Biological introductions (i.e. transport of species away from their native range via  
49 human activities) are modifying species ranges at an unprecedented rate and on global scales  
50 (e.g. in the marine environment, Molnar et al. 2008). Among the various ecological and  
51 evolutionary consequences of species introduction, hybridization between native and non-  
52 native species can dramatically alter the evolutionary trajectories of both species (Allendorf et  
53 al. 2001). Hybridization *sensu lato* commonly refers to interspecific gene flow, including  
54 first-generation hybrids and repeated backcrossing of their offspring with parental species  
55 (Harrison 2012). Such interspecific gene flow between formerly allopatric taxa is not rare and  
56 can have diverse consequences (Allendorf et al. 2001), including an increased risk of  
57 extinction of the native species (Rhymer and Simberloff 1996; Fitzpatrick et al. 2010),  
58 increased invasiveness through, for instance, adaptive introgression mechanisms (Currat et al.  
59 2008; Schierenbeck and Ellstrand 2009) and the emergence of new species (Abbott 1992).  
60 Hybridization facilitated by human activities has been studied mainly in plant species (e.g.  
61 Schierenbeck and Ellstrand 2009; Guo 2014) and to a lesser extent in animals (e.g. Fitzpatrick  
62 et al. 2010; Steeves et al. 2010), with only a few cases reported in marine systems (but see, for  
63 instance, *Abudefduf vaigiensis* and *A. abdominalis*, Coleman et al. 2014). However, marine  
64 species live in a dispersive environment and there is a large number of cryptic, co-existing  
65 native and non-native marine species (e.g. in tunicates; Bock et al. 2012; Perez-Portela et al.  
66 2013). Furthermore, the increasing rate of marine biological invasions on the global scale  
67 facilitates numerous secondary contacts between allopatric taxa (Geller et al. 2010).

68           The recently described *Ciona intestinalis* species complex presents an interesting case  
69 to study. The nominal species *C. intestinalis* (Linnaeus, 1767) covers a complex of four  
70 cryptic species (Zhan et al. 2010). Two of them, *C. intestinalis* species A and *C. intestinalis*  
71 species B (hereafter referred to species A and species B, respectively) are now considered

72 pseudo-cryptic species since the advent of morphological criteria to distinguish them (Sato et  
73 al. 2012). In addition, very recently, Brunetti et al. (2015) showed that species A displays  
74 morphological features specific to the formerly described Japanese species *Ciona robusta*  
75 Hoshino and Tokioka 1967, later synonymized under *C. intestinalis*, whereas species B fits  
76 with the *C. intestinalis* description by Millar (1953). These new alpha-taxonomy discoveries  
77 have not yet been implemented in the World Register of Marine Species (e.g. *C. robusta* is  
78 still a non-accepted name) and we will use ‘species A’ and ‘species B’ in the following text.  
79 Despite a substantial time elapsed since their divergence (estimated at around 4 Mya; Nydam  
80 and Harrison 2011; Roux et al. 2013), the two species are not reproductively isolated: viable  
81 and fertile F1 hybrids are easily produced under laboratory conditions (Sato et al. 2014). As in  
82 many other tunicates (Shenkar and Swalla 2011), the distribution range of these two species  
83 has expanded in the last two centuries due to their accidental introduction via human activities  
84 (Zhan et al. 2010). In particular, species A, has been recently introduced (ca. 15-20 years ago;  
85 Nydam and Harrison 2011; J.D.D. Bishop, personal communication) in the native European  
86 range of species B. Although the two species are widely distributed around the world, the  
87 western English Channel and the South of Brittany in the Northeast Atlantic (hereafter  
88 referred to as WEC) constitute the only confirmed region in which both species have been  
89 reported in sympatry.

90         Like many other ascidians (Airoldi et al. 2015), the nominal species *C. intestinalis*  
91 often forms well-established populations in artificial habitats (e.g. marinas with floating  
92 docks, commercial harbors and aquaculture installations). In their area of sympatry — the  
93 WEC — species A and species B can also live in syntopy (i.e. in the same locality, Fig. 1).  
94 However, because of the cryptic nature of these two species, most published ecological  
95 studies do not distinguish between them (Procaccini et al. 2011). Furthermore, *C. intestinalis*  
96 spp. is a model organism for evolutionary developmental biology research as well as

97 phylogenetic studies. Nonetheless, ecological data are still lacking with regard to the recent  
98 discovery of the cryptic species. Due to its recent introduction in the WEC, species A  
99 experiences numerous, unprecedented biotic interactions and environmental changes that may  
100 affect its invasion dynamics (Blackburn et al. 2014). Consequently, there are many issues  
101 regarding the history, dynamics and fate of the recent introduction of species A in the native  
102 range of species B, including the stability of co-occurrence in species that share the same  
103 habitat and localities, the intensity of interspecific competitive interactions, and the  
104 occurrence of introgression or a hybrid swarm in sympatry.

105 To address these issues, we surveyed 11 marinas located along the coasts of Brittany  
106 for three years, and examine six distinct generations (i.e. the spring and autumn generations of  
107 the same year). We carried out genetic analyses to investigate 1) regional and local variation  
108 in the relative abundance of species A and species B; 2) their potential to mate; and 3) the rate  
109 and direction of interspecific crosses in the wild.

110

## 111 **Materials and Methods**

### 112 *Field survey: sampling, morphological species identification and determination of* 113 *reproductive status*

114 We selected 11 marinas (out of 27) with floating pontoons located along the 1600 km  
115 long coastline of Brittany, France. These marinas were representative of a range of  
116 characteristics (i.e. open to the sea or closed during low tide (by means of tidal or sill gates)),  
117 fully marine or under freshwater (estuarine) influence; see Table S1 for details of each  
118 locality). In spring 2012, *Ciona* spp. populations were sampled in 10 marinas over two weeks  
119 (Fig. 2a). The same sampling was repeated five times, with an additional site (no. 11 in Fig. 2)  
120 in autumn 2012, spring and autumn 2013, and without sites nos. 10 and 11 (see Fig. 2) in  
121 spring and autumn 2014. The six sampling dates correspond to at least six different adult

122 generations in European waters (e.g. Dybern 1965; Caputi et al. 2014). In each locality, ca.  
123 200-300 adult individuals (Table 1 for details) were randomly collected along two pontoons  
124 during SCUBA diving operations over ca. 50 m. Additionally, in most of the studied marinas  
125 (all except nos. 1, 2, 10 and 11, Fig. 2), seawater temperature was recorded using data loggers  
126 (TidbiT<sup>®</sup>v2) located at 1.5 m depth.

127         Following their collection, individuals were sedated with phenoxy-2-propanol  
128 (1:1000) for 20-30 min to facilitate species identification and excision of tissue samples.  
129 Species were identified using morphological criteria according to Sato et al. (2012). For each  
130 site, a random subset of 87 specimens ( $SD \pm 12.6$ ; sample size detailed in Table S2) was used  
131 to examine the reproductive status (modified from ProtocolJRA1-04.00,  
132 <http://www.assemblemarine.org/assets/ASSEMBLE-JRA1-Protocol-04.00mk.pdf>). Given that  
133 both species are simultaneous hermaphrodites, although slightly protandrous, three sequential  
134 stages of individual development were recorded: immature individuals, presence of sperm  
135 only, and presence of sperm and oocytes. A piece of branchial basket was preserved  
136 individually in 100% ethanol for further genetic studies.

137         All statistical analyses were performed using R vers. 3.1.1 (R Development Core  
138 Team 2005). To investigate temporal and spatial variations in the relative abundance of both  
139 species, a linear mixed-effects model with a binomial error distribution was computed using  
140 the *lme4* package (*glmer* function; Bates et al. 2014). Three sites (nos. 6, 10, 11, Fig. 2) with  
141 missing data or complete absence of species A were excluded. “Season” (spring and autumn  
142 generations) and “Year” (2012, 2013 and 2014) were considered as fixed factors. The factor  
143 “Sites” represented pseudo-replicates and was thus categorized as a random factor. In  
144 addition, pairwise comparisons among fixed factors were done. Comparisons of the  
145 proportion of individuals with sperm, or individuals producing sperm and oocytes, were tested  
146 using a Cochran-Mantel-Haenszel (CMH) chi squared test (Mantel 1963) using one

147 contingency table per site, at each sampling date. Only localities with more than 10  
148 individuals for each species were included in these analyses. For pairwise comparisons and  
149 CMH chi squared test, *P*-values were adjusted with a Bonferroni correction.

150

### 151 *Molecular identification of hybrids*

152 F1 hybrids and introgressed individuals (the two categories being referred to “admixed  
153 individuals” in the following text) cannot be discriminated morphologically (Sato et al. 2012).  
154 To identify them, species-diagnostic molecular markers were used on a random subset of  
155 individuals (average of 60 individuals  $\pm$  6.9 (SD) per location and sampling date; Table 2)  
156 collected in 2012 and 2013. DNA extraction was performed with Nucleospin® 96 Tissue Kit  
157 according to the manufacturer’s protocol (Macherey-Nagel, Germany).

158 Four species-diagnostic markers were used: one mitochondrial marker (mtCOI,  
159 cytochrome oxidase I) to identify the maternal lineage, and three nuclear loci, namely  
160 vAChTP (vesicular acetylcholine transporter), Cesa (cellulose synthase) and Hox5 (intronic  
161 nuclear regions of Hox5 gene) to identify the nuclear background. The Hox5 locus was  
162 amplified using the primers and PCR protocol described in Caputi et al. (2007). For this locus,  
163 species-diagnostic alleles differ in their length and allele size was scored on simple agarose  
164 gels (2%). For mtCOI, vAChTP and Cesa, amplifications (protocols detailed in Nydam and  
165 Harrison, 2007, 2010) were followed by enzymatic digestions as explained in Nydam and  
166 Harrison (2011).

167 For each site and sampling date, the proportion of admixed individuals (i.e. displaying  
168 at least one alternative species-diagnostic allele) was computed across the four loci. To test  
169 for stability of admixture across samples, a Pearson’s chi-squared test was used, and *P*-values  
170 were calculated using a Monte Carlo simulation with 5000 replicates. To further examine  
171 admixture patterns, a hybrid index per maternal lineage was computed for each sampling date

172 using the above-described random subset and additional selected individuals from the field  
173 samples to reach a minimum number of 20 individuals per species when possible. The hybrid  
174 index was calculated by adding, over the three nuclear loci, the number of alleles specific to  
175 species B. It varies from zero to six: zero if each of the three nuclear loci are homozygous for  
176 the species A allele and six if the three nuclear loci display only species B alleles.

177

### 178 ***Laboratory crosses***

179 Although the two species have been previously shown to be interfertile (e.g. Suzuki et  
180 al. 2005; Caputi et al. 2007; Sato et al. 2014), we carried out additional fertilization tests to  
181 ascertain that F1 hybrids could be easily produced with individuals from our study area. A  
182 series of bi-parental crosses (47 conspecific, 38 heterospecific and 23 self-crosses) were  
183 carried out using individuals sampled in autumn 2013 in the marinas of Aber Wrac'h (species  
184 B) and Moulin Blanc (species A and species B) (site nos. 6 and 7 in Fig. 2, respectively)  
185 following protocols available in Cirino et al. (2002) and Sato et al. (2014). Fertilization  
186 success was estimated by computing the proportion of eggs with cellular division after 1 h  
187 (i.e. fertilization rate). Following Sato et al. (2014), a linear mixed-effects model and pairwise  
188 comparisons were used to investigate differences in fertilization rate according to the type of  
189 cross (i.e. six categories: two heterospecific (with reverse parental roles for each species), two  
190 conspecific and two self-crosses) as a fixed factor. Because the same individuals were used in  
191 different crosses, a random effect of male genotype nested by female genotype was added.

192

## 193 **Results**

### 194 ***Significant variation in the relative abundance of the two species across generations***

195 In the first study generation (i.e. spring 2012), species A and species B co-occurred in  
196 8 localities out of the 10 surveyed, although species A was never abundant (Fig. 2). Detailed

197 values per site and sampling date are given in Table 1. Although abundance varied across  
198 localities, regionally 6.0% of the 2,316 individuals were morphologically identified as species  
199 A in spring 2012. This spatial pattern changed dramatically in autumn 2012 with 32.2% of the  
200 individuals identified as species A out of the 3,678 individuals collected in 11 marinas (Fig.  
201 2). Similar patterns were observed in 2013 and 2014 with an increase in the relative  
202 abundance of species A in autumn (Table 1, Fig. 2).

203 At the locality level, 7 of the 11 studied marinas showed temporal variation similar to  
204 the regional pattern, with some showing particularly strong contrasts between spring and  
205 autumn generations (e.g. Perros Guirec; no. 3 in Fig. 2). The remaining four localities showed  
206 distinct features. Moulin Blanc (no. 7, Fig. 2) showed a different pattern in 2013, with spring  
207 abundance of species A (35.8%) being much higher than in autumn (2.2%). In Roscoff (no. 5,  
208 Fig. 2), a regular decrease in species A was observed over the course of the survey, starting in  
209 spring 2012 with 8.8% of species A dropping to 0% during the last four sampling dates.  
210 Conversely, the presence of species A was first recorded in St Malo (no. 1, Fig. 2) during the  
211 last sampling date (autumn 2014; 1.1% of species A). Finally, species A was absent in one  
212 site (Aber Wrac'h, no. 6 in Fig. 2) on all sampling dates.

213 Altogether, a significant interaction was found between season and year (Table 3a)  
214 which reflects 1) an increase in the relative abundance of species A in autumn compared with  
215 spring of the same year and 2) a higher relative abundance of species A in 2012 compared  
216 with the following two years (Fig. 2).

217

### 218 ***Synchronous gamete production and successful fertilization between the two species***

219 The reproductive status of individuals at each sampling date for both study species is  
220 illustrated with ternary diagrams (Fig. 3): the distribution of the three categories varied among  
221 species and localities. When comparing species for the proportion of individuals with sperm

222 only, there were significant differences in spring and autumn 2013 ( $P<0.001$  and  $P<0.001$ ,  
223 respectively) and in autumn 2014 ( $P=0.032$ ). The proportion of individuals with both types of  
224 gametes showed a similar pattern with no significant difference except for two dates (in  
225 autumn 2012 and autumn 2014;  $P<0.001$ ). Altogether, regardless of the species, a high  
226 proportion of individuals produced gametes on all sampling dates. For example, in  
227 Trébeurden (no. 4, Fig. 2) in spring 2012, 100% of species A individuals and 84% of species  
228 B individuals showed both sperm and oocytes. Detailed values per site and sampling date are  
229 provided in Table S2.

230         There were significant differences in fertilization rates when comparing cross type  
231 (Fig. 4, Table 3b). In particular, selfing success was very poor. Fertilization rates of  
232 heterospecific crosses with oocytes from species B did not differ from those of the two  
233 conspecific crosses (ca. 80%, Fig. 4). Fertilization rates dropped to very low values (<6%)  
234 when species A was the female parent (Fig. 4).

235

### 236 *mtDNA-based species identification*

237         Altogether, 3,048 individuals collected during the four surveys carried out in 2012 and  
238 2013 were genotyped over four loci including one mitochondrial marker for identifying the  
239 maternal lineage. Only 33 of them (1.08%) showed discordance between morphological and  
240 mitochondrial identification, indicating a close association between maternal type and species  
241 assignment based on morphology. During the 2012 and 2013 field surveys, 90 individuals out  
242 of 15,463 (0.59%) were morphologically recorded as “unidentified” (Table 1); most (82%) of  
243 these showed a species B maternal lineage.

244

### 245 *A unique putative F1 hybrid and little genetic admixture in the wild*

246           Only 131 of the 3,048 individuals (4.3%) showed an admixed genome and all of them  
247 displayed very low hybrid index values (Fig. 5). In addition, only one (0.03%) individual was  
248 possibly a first-generation hybrid (i.e. heterozygote across the three nuclear loci). This  
249 putative F1 hybrid displayed a species B maternal lineage and was found in Perros Guirec  
250 (no. 3, Fig. 2) in autumn 2013.

251           Regardless of maternal type, at the regional level, the proportion of admixture varied  
252 from 0.8% (spring 2013) to 6.3% (autumn 2012). A slightly higher rate of admixture was  
253 found in the autumn generations compared with the spring generations (Table 1). The  
254 admixture rate varied slightly across localities (Table 1). In particular, in localities where the  
255 two species co-occurred, admixed individuals were frequently found. For instance during the  
256 first sampling date 1.6% in St Quay, Trébeurden and Moulin Blanc (nos. 2, 4 and 7, Fig. 2)  
257 and 9.7% in Perros Guirec and Roscoff (nos. 3 and 5, Fig. 2). In contrast, no admixed  
258 individuals were observed in the locality where species A was absent throughout the survey  
259 (Aber Wrac'h, no. 6 in Fig. 2). These variations are, however, explained by differences in  
260 admixture patterns between individuals with different maternal backgrounds (Table 2). Large  
261 differences in the admixture proportion were indeed observed between the two groups with  
262 82.4% of the 131 of individuals showing an admixed genome characterized by a species A  
263 maternal lineage. In addition, the admixture proportion of individuals with a species A  
264 maternal lineage was stable across localities and time (all localities and sampling date  
265 comparison,  $P=0.178$ ) and across localities at each sampling date separately ( $P>0.05$  except  
266 in autumn 2013:  $P=0.032$ ). In contrast, the proportion of admixed individuals with a species B  
267 maternal lineage was variable across localities and time (all localities and sampling date  
268 comparisons,  $P=0.002$ ) and across localities for each sampling date ( $P<0.05$  except in spring  
269 2013:  $P=0.129$ ). Altogether, these results show that most of the variation in admixture among

270 localities or sampling dates (Table 1) is due to variation in the relative abundance of species  
271 A, which showed the highest admixture rate.

272

## 273 **Discussion**

### 274 *The non-native tunicate Ciona intestinalis species A is well established*

275 Based on the morphological examination of 23,000 individuals covering six different  
276 generations, we documented the durable establishment of the non-native species (NNS) *C.*  
277 *intestinalis* species A along the coasts of Brittany, ca. 15-20 years after the first reports of its  
278 introduction in the WEC. The first detailed study on the co-occurrence of species A and  
279 species B in the English Channel (Nydam and Harrison 2011) revealed no samples of species  
280 A specimens during a follow-up survey (in 2009, two years after their first survey),  
281 interpreted as a possible decline of the NNS in the localities surveyed. This decline was not  
282 confirmed in our study. Our data, combined with those of Nydam and Harrison (2011),  
283 illustrate the importance of temporal monitoring over a substantial time window, particularly  
284 during the establishment stage, as well as during different seasons for short-lived species.

285 Despite the sustainable regional establishment of species A, there was local population  
286 instability: for instance, the Roscoff marina showed a steady decline leading to complete  
287 disappearance. Local population dynamics are likely to be unstable in this short-lived and  
288 recently introduced NNS, as observed in other introduced regions (e.g. Saldanha Bay in South  
289 Africa, Rius et al. 2011). Such major changes in site occupancy and local abundance in just a  
290 few years have already been documented in other NNS discovered in the English Channel  
291 (e.g. Bishop et al. 2014). Many environmental and biotic mechanisms influence the successful  
292 and durable establishment of NNS (Blackburn et al. 2014), including competitive interactions  
293 which may exacerbate invasion dynamics (Simberloff and Stiling 1996; Alpert 2006). This

294 mechanism may be particularly important here because the non-native species A co-occurs  
295 with its congener (Fig. 1).

296

297 *Seasonal variation in species abundance: the outcome of competition in a changing*  
298 *environment*

299 Regular field work, sampling and monitoring as well as panels experiments examining  
300 settlement dynamics of *Ciona* sp. (S. Bouchemousse, L. Lévêque, F. Viard, unpublished data)  
301 showed that spring and autumn generations are two separate generations, for the two study  
302 species. This is in agreement with the few data documenting the number of generations per  
303 year in the study area (Dybern 1965 in the North Atlantic for species B). By examining these  
304 two distinct generations of the same year, during three years, we documented a significant  
305 increase in the relative abundance of species A in autumn. Adults sampled in autumn likely  
306 correspond to juveniles that had settled from spring to early summer, when sea water  
307 temperatures increase from ca. 12°C in April to ca. 18°C in August-September in the study  
308 area (Figure S1). Conversely, adults sampled in spring likely correspond to juveniles that have  
309 settled in late summer / early fall, and which survive across the winter season (S.  
310 Bouchemousse, L. Lévêque, F. Viard, unpublished data). Seawater temperature greatly  
311 influences the development, growth and survival of *Ciona intestinalis* spp. (Dybern 1965;  
312 Marin et al. 1987), with species A and species B reported as warm-water and cold-water  
313 species, respectively (Procaccini et al. 2011; Caputi et al. 2014). We observed higher growth  
314 rates at 17°C, for species A than species B during laboratory experiments (data not shown),  
315 confirming previous findings (Petersen et al. 1995) based on the growth rate of the nominal  
316 species *C. intestinalis* in localities where only species A or species B are now reported (e.g. at  
317 15°C, growth rate varies between 2 and 3% in length per day for Japanese populations  
318 (species A) and between 0.7 and 1.1% per day for individuals from Scotland (species B)). In

319 the study area, temperatures above 15°C are only observed from June to October (Figure S1).  
320 It is also noteworthy that in the localities with the lowest maximum values of sea water  
321 temperatures (i.e. Aber Wrac'h and Roscoff, with a maximum always below 17°C) *C.*  
322 *intestinalis* species A has never been observed or declined up to disappearance. The observed  
323 seasonal variations may thus partly result from spatial competition among species A and B.  
324 The autumn generation of species A may have a competitive advantage, due to its faster  
325 growth rate during the warmer season.

326         Although temperature is likely the main environmental driver of the seasonal  
327 dynamics of the two congeners at a regional scale, other factors can drive local population  
328 dynamics. In particular, episodes of low salinity, due to rainfall and river inputs, can decrease  
329 survival of *C. intestinalis* (Lambert and Lambert 1998). Such perturbation events due to  
330 abrupt variations in environmental parameters have been recorded in our study area, as  
331 illustrated in Figure S2 for the marina of Moulin Blanc (no. 7 in Fig. 2), where additional  
332 monitoring (independent of our study; L. Lévêque, unpublished data) for *C. intestinalis* spp.  
333 abundance and salinity was carried out in 2013-2014. Salinity declined sharply and  
334 subsequently *Ciona* spp. died off massively during winter months from January to March  
335 2014. Synergistic effects between temperature and salinity may explain the very high  
336 percentage of species A observed in some localities such as Perros-Guirec (up to 90%; Fig.  
337 2): this marina is closed off from the sea at low tide and remains several days without  
338 seawater renewal during neap tides such that 1) salinity can rapidly decrease in winter (due to  
339 rainfall) and 2) temperature can increase on the surface in summer (Figure S1). After winter  
340 die-offs of both species, due both to low temperatures and low salinity episodes, increasing  
341 seawater temperature may favor a more rapid colonization of species A over species B, taking  
342 into account its faster growth rate, on pontoons from adjacent sources (e.g. pillars, sea-wall),  
343 giving locally and at some particular season, a competitive advantage to species A over

344 species B. Conversely, Roscoff is an open, fully marine marina in the coldest part of the  
345 Brittany coastline (i.e. 13°C annual average, Gallon et al. 2014). No massive mortality of  
346 *Ciona intestinalis* spp. has ever been observed and low temperatures may favor the  
347 development of species B, leading to competitive exclusion of species A.

348 Altogether, our study suggests that, with a few exceptions, environmental conditions in the  
349 study sites meet the niche requirements of the NNS species, though local variations in  
350 environmental parameters may favor one or the other of the two species, and thus may  
351 influence the outcome of competitive interactions among them. In particular, species A,  
352 thanks to its faster growth rate, may have a better adaptive potential than species B for  
353 (re)colonizing substrates during warmer seasons after disturbances due to variations in  
354 environmental factors, like a sharp decline in salinity after rains. Over larger temporal scales,  
355 the predicted increase of sea surface temperature (i.e. 0.35°C per decade; Gallon et al. 2014)  
356 and winter rainfalls (Ouzeau et al. 2014) in Brittany, due to climate change, could promote a  
357 substantial increase of the relative abundance of the NNS compared with its native congener  
358 in its introduced range.

359

### 360 ***Efficient barriers prevent hybridization in the wild***

361 Sexually mature, gamete-producing individuals occurred simultaneously in the two  
362 study species which are interfertile and live in syntopy in most of the study localities. Despite  
363 these features that are expected to favor hybridization, the admixture rate was very low  
364 (4.3%) and only one putative F1 hybrid was observed in this study on more than 3,000  
365 individuals, confirming previous studies (4.2% in localities of the WEC, Nydam and Harrison  
366 2011; 6.3% in one British locality, Sato et al. 2014). Ecological and/or genetic barriers thus  
367 seem to be at play and limit hybridization between the two species in the wild in Brittany. The  
368 success of interspecific fertilization in laboratory conditions (Fig. 4) seemingly suggests that

369 post-zygotic isolation mechanisms act in the wild through a reduction in hybrid fitness  
370 (Abbott et al. 2013). However, Sato et al. (2014) showed that F1 hybrids are viable and  
371 produce functional gametes, suggesting that hybrid depression, if any, is expressed at an  
372 earlier stage (e.g. larvae or juveniles). However, preliminary findings from a molecular study  
373 of juveniles of species A and species B in four marinas of the WEC (nos. 4, 5, 7 and 8; Fig. 2)  
374 showed rates of admixture similar to those observed in adults (S. Bouchemousse, L. Lévêque,  
375 F. Viard, unpublished data). Pre-zygotic isolation mechanisms can enhance assortative  
376 mating; however, given the close contact (see Fig. 1), habitat segregation and behavior cannot  
377 be efficient reproductive isolation mechanisms. Differences in the timing of reproductive  
378 development, as suggested by Sato et al. (2014), are also unlikely because we observed a high  
379 proportion of individuals producing gametes in both species and each sampling date. Gamete  
380 release occurs over several days or even weeks (Carver et al. 2003), invalidating any  
381 consideration of slight shifts in the timing of gamete release in light of the short survival time  
382 of the gametes in seawater (i.e. 16 h after release for sperm and 30 h for oocytes; Svane and  
383 Havenhand 1993). Assortative fertilization, facilitated by species-specific chemical attraction,  
384 can be a key mechanism for reproduction in broadcast-spawning marine invertebrates in  
385 marine systems (Palumbi 1994). This mechanism has been described already within species  
386 complexes, for instance in two tropical sea urchins of the *Echinometra* species complex  
387 (Geyer and Palumbi 2005) and in two mussels of the *Mytilus* species complex (Bierne et al.  
388 2002). Laboratory gamete choice experiments are needed to investigate this mechanism in the  
389 *C. intestinalis* species complex.

390

### 391 ***The source of admixture: historical or contemporary processes?***

392 In the laboratory, F1 hybrids are easily obtained in one direction only, i.e. with species  
393 B oocytes (Fig. 4). Asymmetrical interspecific fertilization success is common, including in

394 free-spawning marine invertebrates (e.g. Rawson et al. 2003; Geyer and Palumbi 2005). The  
395 direction of this asymmetry was however unexpected based on the observed admixture rates  
396 that we computed according to the maternal lineage (82.4% of the 131 admixed individuals  
397 showed a species A maternal lineage). There are two possible explanations of this discrepancy  
398 between laboratory and field data. First, the few offspring produced by the least productive  
399 heterospecific crosses (i.e. a species A female crossed with a species B male) may be  
400 particularly fertile and vigorous. Similar situations with successful hybrids produced by  
401 parental combinations with low fertilization success have already been described, even in  
402 marine systems (e.g. Blum et al. 2010). This process may foster adaptive introgression of the  
403 NNS (species A) and/or drive hybrids to replace parental species (e.g. Rosenfield et al. 2004;  
404 Schierenbeck and Ellstrand 2009). Second, conditions that promote successful crosses in the  
405 laboratory may not be the conditions found in the wild (Sato et al. 2014). Monitoring survival  
406 of hybrid offspring obtained from experimental crosses is one way to test these two  
407 hypotheses. It is also interesting to note that the direction of the asymmetry is variable across  
408 studies: the direction observed here is in agreement with what has been reported by Caputi et  
409 al. (2007) but not with Sato et al. (2014), although in both studies some of the crosses  
410 involved individuals from the North Atlantic. Factors promoting (preventing) the  
411 hybridization success are potentially numerous, and among them the origin and the genetic  
412 background, in particular the introgression profiles (see below), may be important factors to  
413 consider in future studies.

414         Alternatively, from a conceptually different viewpoint, observed admixture may  
415 reflect the footprint of a previous secondary contact between species A and species B (Roux  
416 et al. 2013). Roux et al. (2013) compared various speciation models (e.g. divergence models  
417 with or without interspecific gene flow) using Approximate Bayesian Computations based on  
418 full transcriptomes of species A and species B (10 individuals for each). The best model to

419 explain the data was a model of divergence with gene flow resulting from a secondary contact  
420 between the two species after their primary divergence (4 Mya ago). During this secondary  
421 contact, ca. 20% of loci presumably crossed the species barrier in both directions (Roux et al.  
422 2013). The authors then estimated the age of this secondary contact between 4,300 and 56,800  
423 years ago. Many secondary contacts have been documented in marine systems during this  
424 period which corresponds to the end of the Pleistocene (Maggs et al. 2008; Geller et al. 2010).  
425 The number of studies showing interspecific gene flow following these secondary contacts  
426 also recently increased (Abbott et al. 2013, see Becquet et al 2012 for an example). In this  
427 context, past hybridization is very plausible explanation of our results because it can account  
428 for 1) the discrepancy observed between laboratory and field experiments; 2) the temporal and  
429 spatial stability of the proportion of admixed individuals for species A; and 3) the complex  
430 evolutionary history of the two study cryptic species (Roux et al. 2013). Although historical  
431 processes may explain most of the admixture observed, contemporary interspecific gene flow  
432 remains possible based on our results in two localities: in St Malo, no admixed individuals  
433 were observed until autumn 2013 following the colonization of this locality by species A and  
434 in Roscoff, admixed individuals were observed in 2012, but none afterwards when species A  
435 disappeared. Finally, at Aber Wrac'h, the only locality where no species A specimens were  
436 ever observed, did not show any admixed specimens. Furthermore — and interestingly — in  
437 St Malo and Aber Wrac'h, all admixed individuals displayed a species B maternal lineage, as  
438 did the only putative F1 hybrid found in our study (in Perros Guirec).

439         Altogether, our results suggest that past secondary contacts influenced the  
440 introgression rates measured here and rare contemporary hybridizations in the wild account  
441 mainly for the admixed individuals with a species B maternal background, in agreement with  
442 laboratory assays. The status of the presumed species diagnostic markers used so far (Caputi  
443 et al. 2007; Nydam and Harrison 2011; Sato et al. 2014; this study) need to be re-evaluated

444 because the three loci behave differently: *CesA* and *vAChTP* highlight admixture in species B  
445 maternal backgrounds whereas *Hox5* revealed admixture with species A maternal  
446 backgrounds (see Table S3). Although not discernable in our laboratory crosses (or in the  
447 literature, Caputi et al. 2007), we suggest that *Hox5* crossed the species barrier a long time  
448 ago, in contrast to the two other loci (similar to the *Glu* locus which was mistakenly  
449 considered as a diagnostic marker of *Mytilus* species, Borsa et al. 2012). High-throughput  
450 genotyping based on SNPs are currently in progress to better investigate the fate of the  
451 introgression hot-spots defined by Roux et al. (2013) and the true extent of contemporary  
452 hybridization in wild *Ciona* populations.

453

454 In conclusion, our study highlights the importance of temporal monitoring adapted to  
455 the life cycle and the generation time of short-lived NNS. This type of monitoring is critical  
456 for early-warning and accurate assessments of the durable establishment of introduced  
457 species. Our results also illustrate how difficult it is to predict the outcome of human-  
458 mediated introductions, even in supposedly well-known model species: we expected to find a  
459 large proportion of hybrids and introgression events between species A and species B, but  
460 only rare events (if any) were uncovered despite extensive sampling. Our results pave the way  
461 for future research on the reproductive isolation mechanisms acting in the wild between these  
462 model organisms, as well as on the processes that can sustain long-term co-occurrence of two  
463 functionally similar and congeneric species living in syntopy.

464

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477           **References**

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607

Table 1. Sample size ( $N_{\text{tot}}$ ), number of individuals unidentified based on morphological criteria ( $N_{\text{uni}}$ ), and percentage of *Ciona intestinalis* species A specimens (based on morphology;  $\%_{\text{spA}}$ ), for each study site and sampling date. The overall introgression rate (i.e. proportion of individuals showing an admixed multilocus genotype whatever their maternal lineage;  $\%_{\text{mix}}$ ) is indicated for the four surveys for which there were molecular data (i.e. all except in 2014).

| Site               | Spring 2012<br>26 April – 10 May |                  |                   |                   | Autumn 2012<br>26 Oct. – 6 Nov. |                  |                   |                   | Spring 2013<br>15 – 25 April |                  |                   |                   | Autumn 2013<br>14 – 24 Oct. |                  |                   |                   | Spring 2014<br>15 – 22 May |                   | Autumn 2014<br>24 – 31 Oct. |                   |
|--------------------|----------------------------------|------------------|-------------------|-------------------|---------------------------------|------------------|-------------------|-------------------|------------------------------|------------------|-------------------|-------------------|-----------------------------|------------------|-------------------|-------------------|----------------------------|-------------------|-----------------------------|-------------------|
|                    | $N_{\text{tot}}$                 | $N_{\text{uni}}$ | $\%_{\text{spA}}$ | $\%_{\text{mix}}$ | $N_{\text{tot}}$                | $N_{\text{uni}}$ | $\%_{\text{spA}}$ | $\%_{\text{mix}}$ | $N_{\text{tot}}$             | $N_{\text{uni}}$ | $\%_{\text{spA}}$ | $\%_{\text{mix}}$ | $N_{\text{tot}}$            | $N_{\text{uni}}$ | $\%_{\text{spA}}$ | $\%_{\text{mix}}$ | $N_{\text{tot}}$           | $\%_{\text{spA}}$ | $N_{\text{tot}}$            | $\%_{\text{spA}}$ |
| 1- St Malo         | 311                              | 1                | 0.0               | 0.0               | 375                             | 1                | 0.0               | 0.0               | 736                          | 1                | 0.0               | 0.0               | 490                         | 1                | 0.0               | 1.6               | 399                        | 0.0               | 823                         | 1.10              |
| 2- St Quay         | 178                              | 14               | 5.6               | 1.6               | 335                             | 0                | 33.1              | 3.3               | 269                          | 2                | 3.0               | 0.0               | 341                         | 1                | 12.6              | 0.0               | 157                        | 0.6               | 763                         | 14.2              |
| 3- Perros Guirec   | 297                              | 6                | 13.5              | 9.7               | 305                             | 0                | 90.2              | 10.9              | 223                          | 3                | 8.5               | 0.0               | 531                         | 8                | 67.0              | 3.2               | 217                        | 14.3              | 495                         | 96.0              |
| 4- Trébeurden      | 228                              | 4                | 6.6               | 1.6               | 262                             | 0                | 61.1              | 12.7              | 436                          | 0                | 6.7               | 0.0               | 474                         | 4                | 43.2              | 8.1               | 281                        | 6.4               | 809                         | 16.4              |
| 5- Roscoff         | 160                              | 8                | 8.8               | 9.7               | 203                             | 0                | 2.5               | 0.0               | 329                          | 5                | 0.0               | 0.0               | 626                         | 0                | 0.0               | 0.0               | 311                        | 0.0               | 508                         | 0.0               |
| 6- Aber Wrac'h     | 171                              | 3                | 0.0               | 0.0               | 236                             | 2                | 0.0               | 0.0               | 290                          | 1                | 0.0               | 0.0               | 523                         | 0                | 0.0               | 0.0               | 36                         | 0.0               | 315                         | 0.0               |
| 7- Moulin Blanc    | 168                              | 1                | 18.5              | 1.6               | 234                             | 1                | 42.7              | 13.8              | 120                          | 1                | 35.8              | 7.8               | 543                         | 1                | 2.2               | 1.7               | 99                         | 0.3               | 424                         | 3.3               |
| 8- Château         | 205                              | 0                | 3.4               | 0.0               | 721                             | 6                | 19.1              | 8.3               | 300                          | 0                | 0.0               | 2.2               | 778                         | 0                | 6.0               | 7.9               | 295                        | 0.0               | 906                         | 8.0               |
| 9- Camaret-sur-Mer | 260                              | 4                | 6.5               | 0.0               | 306                             | 1                | 30.4              | 10.0              | 214                          | 2                | 5.1               | 0.0               | 543                         | 4                | 20.3              | 9.4               | 236                        | 4.7               | 463                         | 38.2              |
| 10- Concarneau     | 338                              | 1                | 1.5               | 0.0               | 474                             | 1                | 60.1              | 9.7               | 264                          | 0                | 0.0               | 0.0               | 712                         | 0                | 27.2              | 3.2               | -                          | -                 | -                           | -                 |
| 11- Quiberon       | -                                | -                | -                 | -                 | 227                             | 1                | 8.4               | 0.0               | 367                          | 1                | 0.5               | 0.0               | 360                         | 0                | 3.6               | 3.2               | -                          | -                 | -                           | -                 |
| Mean               | 232                              | 4.2              | 6.4               | 2.4               | 334                             | 1.2              | 33.9              | 6.2               | 323                          | 1.5              | 5.9               | 0.9               | 538                         | 1.7              | 17.9              | 3.5               | 226                        | 2.9               | 612                         | 19.7              |
| Standard deviation | 66                               | 4.3              | 5.9               | 3.9               | 150                             | 1.7              | 29.6              | 5.6               | 160                          | 1.5              | 10.5              | 2.4               | 131                         | 2.6              | 21.7              | 3.5               | 113                        | 4.9               | 213                         | 31.1              |
| <b>Total</b>       | <b>2316</b>                      | <b>42</b>        | <b>6.0</b>        | <b>2.0</b>        | <b>3678</b>                     | <b>13</b>        | <b>32.2</b>       | <b>6.3</b>        | <b>3548</b>                  | <b>16</b>        | <b>3.1</b>        | <b>0.8</b>        | <b>5921</b>                 | <b>19</b>        | <b>16.6</b>       | <b>3.5</b>        | <b>2031</b>                | <b>3.1</b>        | <b>5506</b>                 | <b>22.0</b>       |

Table 2. Proportion of admixed individuals, i.e. individuals showing a mixture of alleles diagnostic for *Ciona intestinalis* species A and *C. intestinalis* species B, for each maternal lineage separately ( $\%_{\text{mix spA}}$  and  $\%_{\text{mix spB}}$  for individuals diagnosed with species A mitochondria and a species B mitochondria, respectively), for each site and sampling date studied in 2012 and 2013.  $N_{\text{spA}}$  and  $N_{\text{spB}}$  are the number of specimens for which admixture was examined.

| Site               | Spring 2012      |                       |                  |                       | Autumn 2012      |                       |                  |                       | Spring 2013      |                       |                  |                       | Autumn 2013      |                       |                  |                       |
|--------------------|------------------|-----------------------|------------------|-----------------------|------------------|-----------------------|------------------|-----------------------|------------------|-----------------------|------------------|-----------------------|------------------|-----------------------|------------------|-----------------------|
|                    | $N_{\text{spA}}$ | $\%_{\text{mix spA}}$ | $N_{\text{spB}}$ | $\%_{\text{mix spB}}$ | $N_{\text{spA}}$ | $\%_{\text{mix spA}}$ | $N_{\text{spB}}$ | $\%_{\text{mix spB}}$ | $N_{\text{spA}}$ | $\%_{\text{mix spA}}$ | $N_{\text{spB}}$ | $\%_{\text{mix spB}}$ | $N_{\text{spA}}$ | $\%_{\text{mix spA}}$ | $N_{\text{spB}}$ | $\%_{\text{mix spB}}$ |
| 1- St Malo         | 0                | -                     | 56               | 0.0                   | 0                | -                     | 62               | 0.0                   | 0                | -                     | 60               | 0.0                   | 0                | -                     | 64               | 1.6                   |
| 2- St Quay         | 5                | 20.0                  | 58               | 0.0                   | 28               | 7.1                   | 33               | 0.0                   | 2                | 0.0                   | 55               | 0.0                   | 19               | 0.0                   | 44               | 0.0                   |
| 3- Perros Guirec   | 18               | 33.3                  | 44               | 0.0                   | 56               | 12.5                  | 8                | 0.0                   | 4                | 0.0                   | 44               | 0.0                   | 50               | 4.0                   | 12               | 0.0                   |
| 4- Trébeurden      | 4                | 25.0                  | 59               | 0.0                   | 40               | 15.0                  | 23               | 8.7                   | 5                | 0.0                   | 54               | 0.0                   | 29               | 10.3                  | 33               | 6.1                   |
| 5- Roscoff         | 5                | 40.0                  | 26               | 3.8                   | 1                | 0.0                   | 57               | 0.0                   | 0                | -                     | 77               | 0.0                   | 0                | -                     | 60               | 0.0                   |
| 6- Aber Wrac'h     | 0                | -                     | 62               | 0.0                   | 0                | -                     | 58               | 0.0                   | 0                | -                     | 63               | 0.0                   | 0                | -                     | 62               | 0.0                   |
| 7- Moulin Blanc    | 11               | 9.1                   | 51               | 0.0                   | 33               | 18.2                  | 25               | 8.0                   | 19               | 21.1                  | 32               | 0.0                   | 3                | 33.3                  | 56               | 0.0                   |
| 8- Château         | 0                | -                     | 64               | 0.0                   | 23               | 21.7                  | 37               | 0.0                   | 0                | -                     | 46               | 2.2                   | 6                | 16.7                  | 57               | 7.0                   |
| 9- Camaret-sur-Mer | 8                | 0.0                   | 56               | 0.0                   | 28               | 17.9                  | 32               | 3.1                   | 3                | 0.0                   | 46               | 0.0                   | 17               | 29.4                  | 47               | 2.1                   |
| 10- Concarneau     | 1                | 0.0                   | 63               | 0.0                   | 48               | 12.5                  | 14               | 0.0                   | 0                | -                     | 60               | 0.0                   | 20               | 5.0                   | 42               | 2.4                   |
| 11- Quiberon       | -                | -                     | -                | -                     | 5                | 0.0                   | 59               | 0.0                   | 0                | -                     | 55               | 0.0                   | 5                | 40.0                  | 56               | 0.0                   |
| Mean               | 5.2              | 18.2                  | 53.9             | 0.4                   | 23.8             | 11.7                  | 37.1             | 1.8                   | 3.0              | 4.2                   | 53.8             | 0.2                   | 13.6             | 17.3                  | 48.5             | 1.7                   |
| Standard deviation | 5.8              | 15.8                  | 11.5             | 1.2                   | 20.0             | 7.8                   | 19.2             | 3.4                   | 5.6              | 9.4                   | 11.8             | 0.7                   | 15.6             | 15.1                  | 15.4             | 2.6                   |

Table 3. Results of linear mixed models testing a) the effects of season and sampling year on the relative abundance of *Ciona intestinalis* species A and *C. intestinalis* species B in syntopic localities and b) the effect of cross type on the fertilization rate. Degrees of freedom (d.f.), residual d.f., residual deviation and *P*-value (from Chi-squared test) are given. Significant *P*-values are shown in italics.

| Variable                      | Model         | d.f. | Residual d.f. | Residual deviation | <i>P</i> -value   |
|-------------------------------|---------------|------|---------------|--------------------|-------------------|
| a) Relative species abundance | Null model    |      | 46            | 2968.1             |                   |
|                               | Year          | 2    | 44            | 2804.9             | <i>&lt;0.01</i>   |
|                               | Season        | 1    | 45            | 1667.9             | <i>&lt;0.001</i>  |
|                               | Year x Season | 5    | 41            | 1319.2             | <i>&lt; 0.001</i> |
| b) Fertilization rate         | Null model    |      | 105           | 720.85             |                   |
|                               | Cross type    | 5    | 100           | 203.44             | <i>&lt;0.001</i>  |

## Figure Legends

Figure 1. Artificial plastic substrates are heavily colonized in the study area, including by *Ciona intestinalis* species A (white form) and *C. intestinalis* species B (with yellow rim around siphons) as shown in this picture, which also illustrates the syntopy between the two study species. Photo credit: Wilfried Thomas, Station Biologique of Roscoff.

Figure 2. Relative abundance of *Ciona intestinalis* species A (in black) and species B (in gray) in the 11 study sites (1: St Malo, 2: St Quay, 3: Perros Guirec, 4: Trébeurden, 5: Roscoff, 6: Aber Wrac'h, 7: Moulin Blanc, 8: Château, 9: Camaret-sur-Mer, 10: Concarneau, 11: Quiberon) from spring 2012 to autumn 2014. Maps with the same letter indicate non-significant differences between sampling dates (pairwise comparisons,  $P > 0.05$ ).

Figure 3. Ternary diagrams representing the proportion of the three recorded reproductive stages for *Ciona intestinalis* species A (black circles) and for species B (gray circles), for each sampling date. Numbers in the circles refer to site numbers (see Table 1).

Figure 4. Fertilization rate for conspecific crosses (i.e. using female (Fe) and male (Ma) gametes of the same species, gray bars), heterospecific crosses (i.e. using female and male gametes from different species, white bars) and self-crosses (i.e. using gametes from the same individual, black bars). Values with the same letter are not significantly different (pairwise comparisons,  $P > 0.05$ ).

Figure 5. Hybrid index computed for each sampling date for individuals with a *Ciona intestinalis* species A (black bars) and *C. intestinalis* species B (gray bars) maternal lineage.

Maternal lineage was ascertained with a diagnostic mitochondrial marker (see text).  $N_{spA}$  and  $N_{spB}$  are the number of specimens examined. The individual (sampled in autumn 2013) indicated by an arrow has a hybrid index of 3 and is the only putative F1 hybrid found of the 3,048 study individuals.

Figure 1.



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Figure 2.

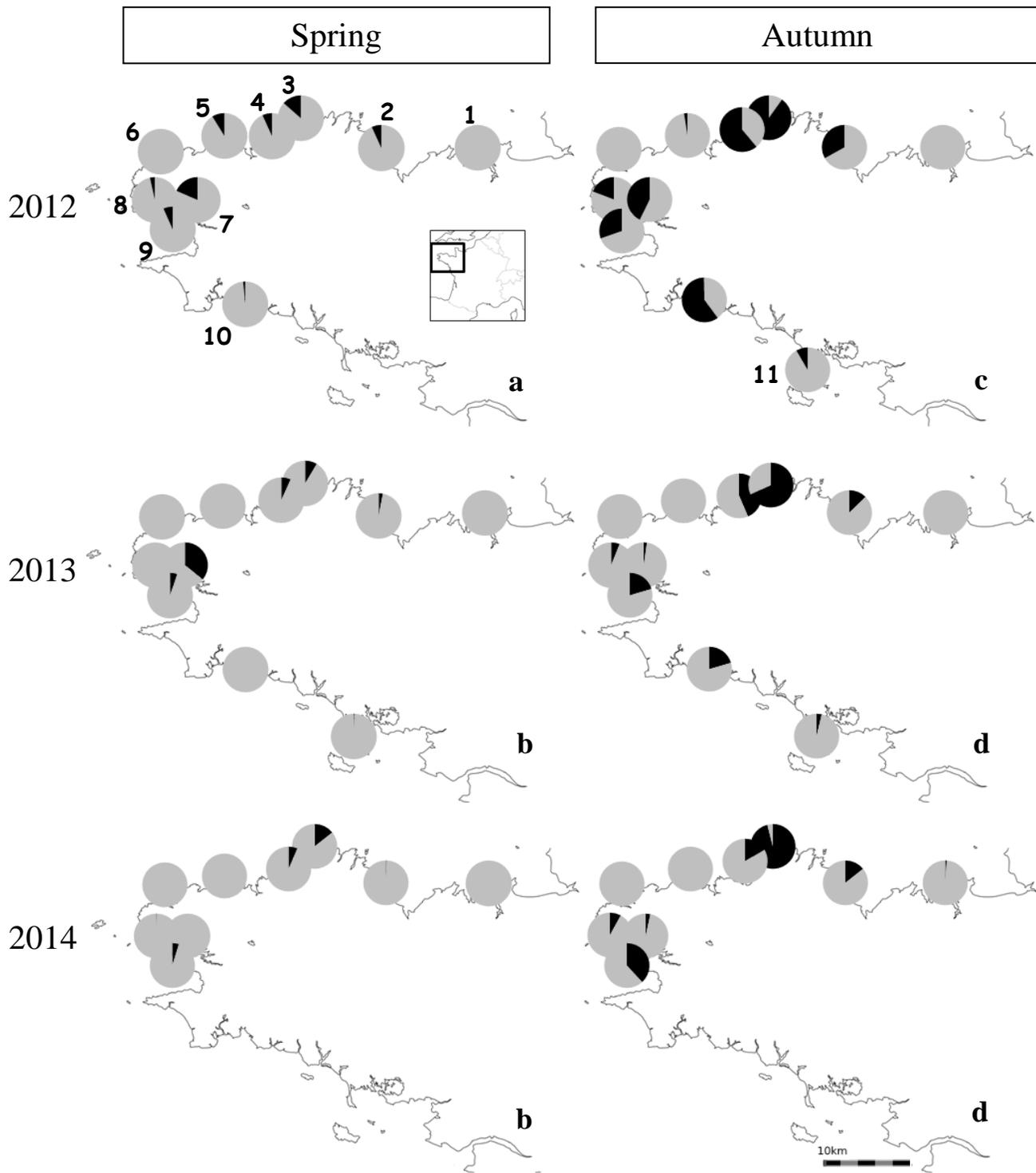


Figure 3.

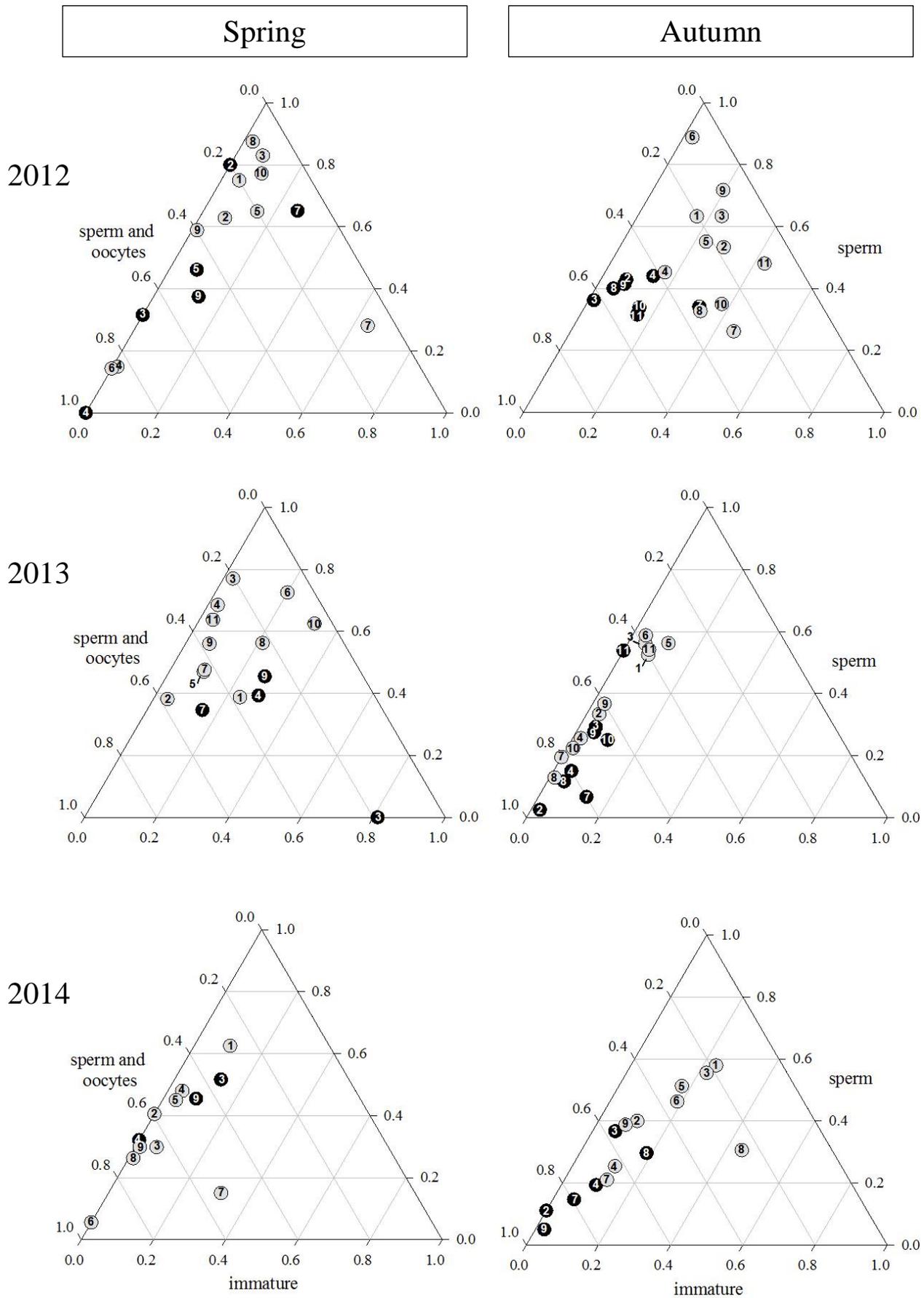


Figure 4.

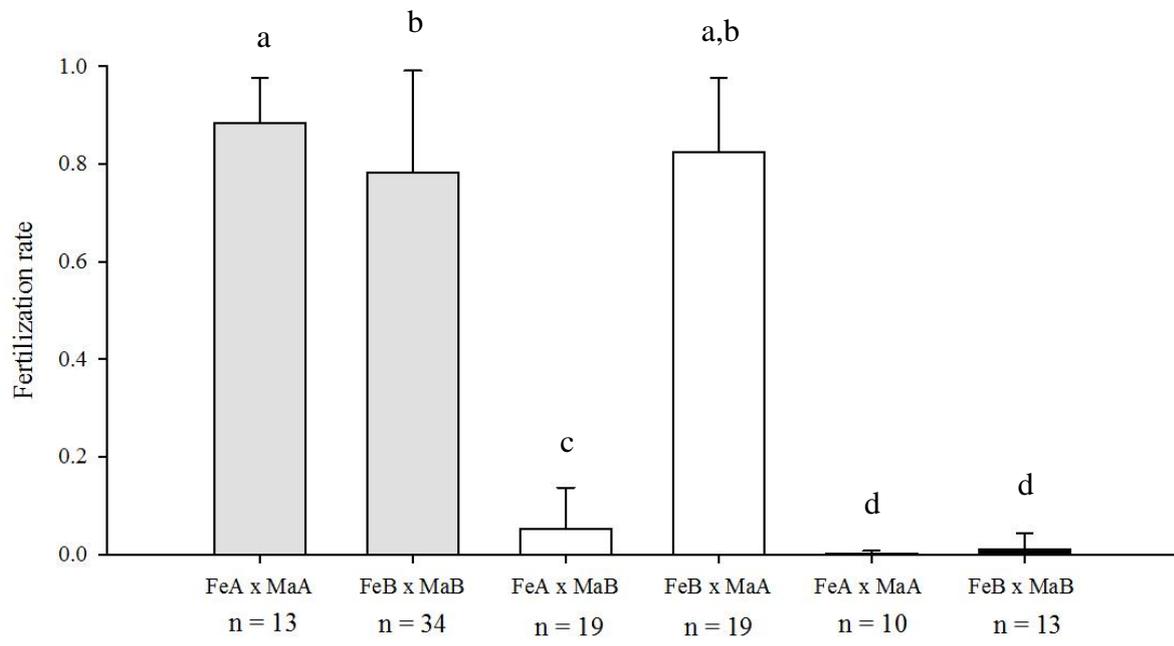
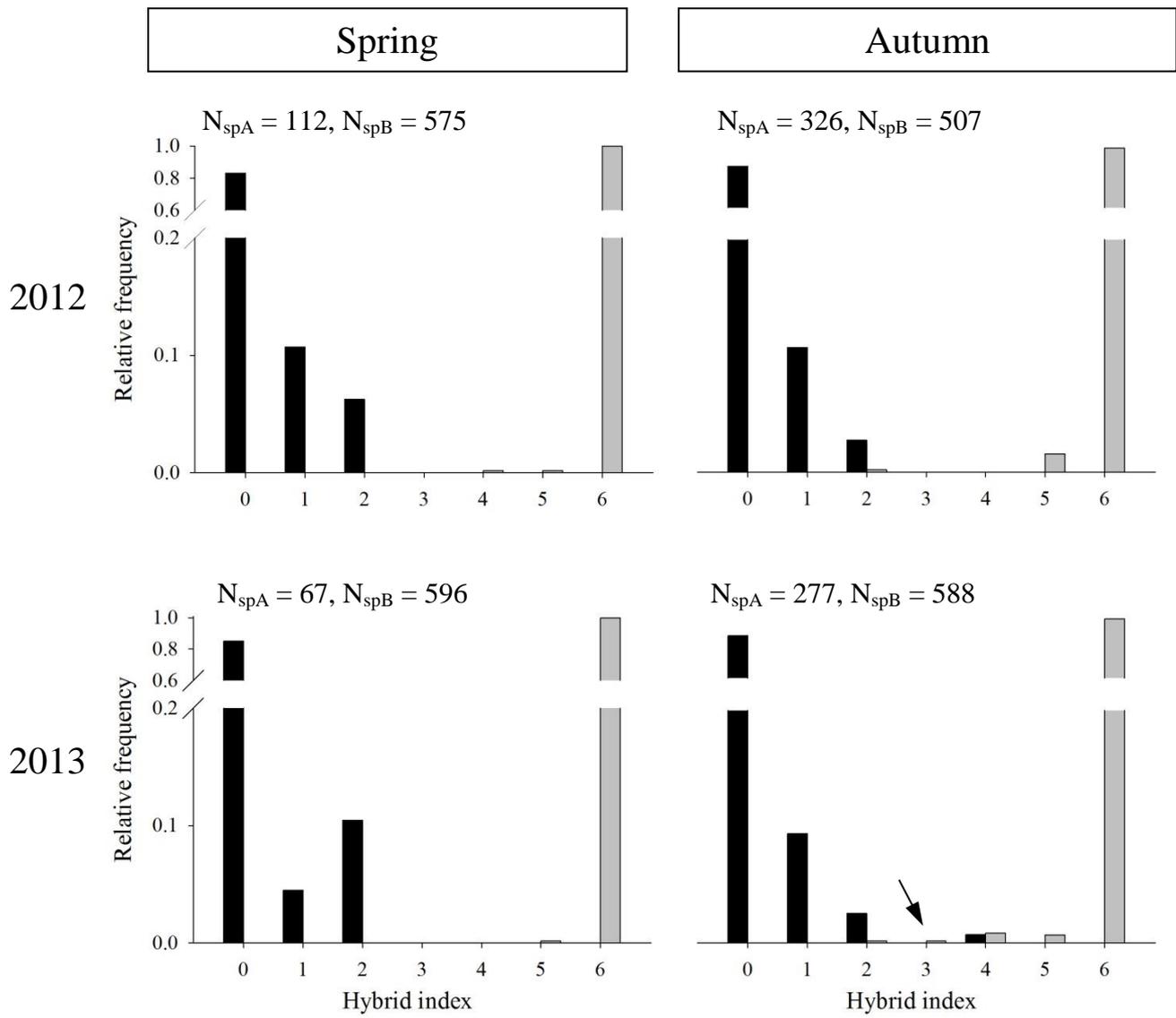
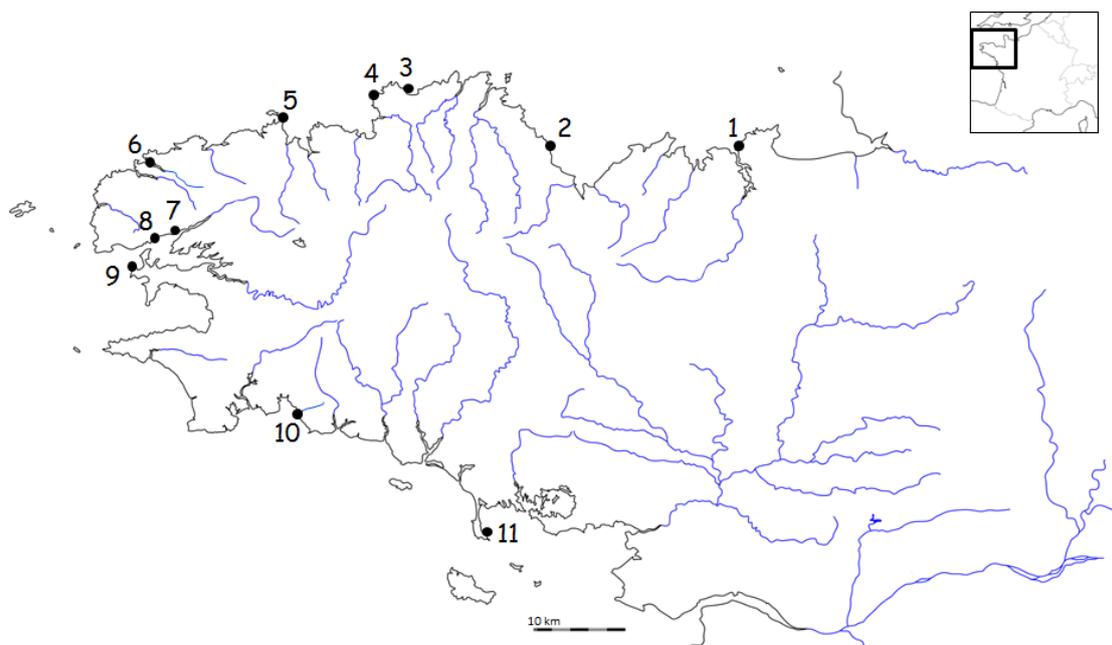


Figure 5.



## Supplementary Information for online appendix

Table S1. Geographical coordinates and characteristics of each site, i.e. open or closed marinas (by means of tidal or sill gates) at low tide, freshwater (estuarine) or marine influence.



| Site               | Coordinates          | Open/ Closed | Estuarine/Marine |
|--------------------|----------------------|--------------|------------------|
| 1- St Malo         | 48.638617; -2.026411 | Open         | Marine           |
| 2- St Quay         | 48.646136; -2.820925 | Open         | Marine           |
| 3- Perros Guirec   | 48.804877; -3.441954 | Closed       | Marine           |
| 4- Trébeurden      | 48.769510; -3.585811 | Closed       | Marine           |
| 5- Roscoff         | 48.717500; -3.965401 | Open         | Marine           |
| 6- Aber Wrac'h     | 48.598626; -4.562438 | Open         | Estuarine        |
| 7- Moulin Blanc    | 48.390365; -4.432140 | Open         | Estuarine        |
| 8- Château         | 48.379489; -4.489469 | Open         | Estuarine        |
| 9- Camaret-sur-Mer | 48.279674; -4.596113 | Open         | Marine           |
| 10- Concarneau     | 47.870150; -3.914604 | Open         | Estuarine        |
| 11- Quiberon       | 47.488223; -3.103269 | Open         | Marine           |

Table S2. Detailed sampling size used for analyzing the reproductive status and proportions of the observed reproductive stages for *C. intestinalis* species A ( $N_{spA}$ ) and species B ( $N_{spB}$ ) at each study site and sampling date. Three categories were recorded: 1) immature individuals (imm), 2) individuals with sperm only (sp) and 3) individuals with both sperm and oocytes (sp+ooc).

A) Spring 2012

| Site               | Species A  |            |             |             | Species B  |             |             |             |
|--------------------|------------|------------|-------------|-------------|------------|-------------|-------------|-------------|
|                    | $N_{spA}$  | %imm       | %sp         | %sp+ooc     | $N_{spB}$  | %imm        | %sp         | %sp+ooc     |
| 1- St Malo         | 0          | -          | -           | -           | 80         | 5.0         | 75.0        | 20.0        |
| 2- St Quay         | 10         | 0.0        | 80.0        | 20.0        | 70         | 7.1         | 62.9        | 30.0        |
| 3- Perros Guirec   | 38         | 0.0        | 31.6        | 68.4        | 53         | 7.5         | 83.0        | 9.4         |
| 4- Trébeurden      | 15         | 0.0        | 0.0         | 100.0       | 74         | 1.4         | 14.9        | 83.8        |
| 5- Roscoff         | 13         | 7.7        | 46.2        | 46.2        | 40         | 15.0        | 65.0        | 20.0        |
| 6- Aber Wrac'h     | 0          | -          | -           | -           | 77         | 0.0         | 14.3        | 85.7        |
| 7- Moulin Blanc    | 23         | 26.1       | 65.2        | 8.7         | 64         | 64.1        | 28.1        | 7.8         |
| 8- Château         | 7          | 0.0        | 71.4        | 28.6        | 80         | 2.5         | 87.5        | 10.0        |
| 9- Camaret-sur-Mer | 16         | 12.5       | 37.5        | 50.0        | 73         | 1.4         | 58.9        | 39.7        |
| 10- Concarneau     | 5          | 0.0        | 20.0        | 80.0        | 79         | 10.1        | 77.2        | 12.7        |
| 11- Quiberon       | -          | -          | -           | -           | -          | -           | -           | -           |
| Mean               | 12.7       | 5.8        | 44.0        | 50.2        | 69         | 11.4        | 56.7        | 31.9        |
| Standard deviation | 11.5       | 9.5        | 27.3        | 31.2        | 13.2       | 19.1        | 27.6        | 29.6        |
| <b>Total</b>       | <b>127</b> | <b>7.1</b> | <b>41.7</b> | <b>51.2</b> | <b>690</b> | <b>10.4</b> | <b>56.2</b> | <b>33.3</b> |

B) Autumn 2012

| Site               | Species A  |             |             |             | Species B  |             |             |             |
|--------------------|------------|-------------|-------------|-------------|------------|-------------|-------------|-------------|
|                    | $N_{spA}$  | %imm        | %sp         | %sp+ooc     | $N_{spB}$  | %imm        | %sp         | %sp+ooc     |
| 1- St Malo         | 0          | -           | -           | -           | 79         | 16.5        | 63.3        | 20.3        |
| 2- St Quay         | 42         | 7.1         | 42.9        | 50.0        | 45         | 28.9        | 53.3        | 17.8        |
| 3- Perros Guirec   | 69         | 1.4         | 36.2        | 62.3        | 30         | 23.3        | 63.3        | 13.3        |
| 4- Trébeurden      | 50         | 14.0        | 44.0        | 42.0        | 42         | 16.7        | 45.2        | 38.1        |
| 5- Roscoff         | 5          | 20.0        | 60.0        | 20.0        | 78         | 23.1        | 55.1        | 21.8        |
| 6- Aber Wrac'h     | 0          | -           | -           | -           | 80         | 2.5         | 88.8        | 8.8         |
| 7- Moulin Blanc    | 41         | 31.7        | 34.1        | 34.1        | 42         | 45.2        | 26.2        | 28.6        |
| 8- Château         | 40         | 5.0         | 40.0        | 55.0        | 55         | 32.7        | 32.7        | 34.5        |
| 9- Camaret-sur-Mer | 41         | 7.3         | 41.5        | 51.2        | 46         | 19.6        | 71.7        | 8.7         |
| 10- Concarneau     | 59         | 15.3        | 33.9        | 50.8        | 40         | 37.5        | 35.0        | 27.5        |
| 11- Quiberon       | 19         | 15.8        | 31.6        | 52.6        | 77         | 42.9        | 48.1        | 9.1         |
| Mean               | 33.3       | 13.1        | 40.5        | 46.5        | 55.8       | 26.3        | 53.0        | 20.8        |
| Standard deviation | 23.8       | 9.2         | 8.5         | 12.7        | 18.9       | 12.8        | 18.4        | 10.4        |
| <b>Total</b>       | <b>366</b> | <b>11.5</b> | <b>38.5</b> | <b>50.0</b> | <b>614</b> | <b>25.1</b> | <b>55.2</b> | <b>19.7</b> |

## C) Spring 2013

| Site               | Species A        |             |             |             | Species B        |             |             |             |
|--------------------|------------------|-------------|-------------|-------------|------------------|-------------|-------------|-------------|
|                    | N <sub>spA</sub> | %imm        | %sp         | %sp+ooc     | N <sub>spB</sub> | %imm        | %sp         | %sp+ooc     |
| 1- St Malo         | 0                | -           | -           | -           | 80               | 23.8        | 38.8        | 37.5        |
| 2- St Quay         | 6                | 33.3        | 16.7        | 50.0        | 76               | 3.9         | 38.2        | 57.9        |
| 3- Perros Guirec   | 16               | 81.3        | 0.0         | 18.8        | 74               | 2.7         | 77.0        | 20.3        |
| 4- Trébeurden      | 28               | 28.6        | 39.3        | 32.1        | 73               | 2.7         | 68.5        | 28.8        |
| 5- Roscoff         | 0                | -           | -           | -           | 115              | 9.6         | 47.0        | 43.5        |
| 6- Aber Wrac'h     | 0                | -           | -           | -           | 80               | 20.0        | 72.5        | 7.5         |
| 7- Moulin Blanc    | 26               | 15.4        | 34.6        | 50.0        | 42               | 9.5         | 47.6        | 42.9        |
| 8- Château         | 0                | -           | -           | -           | 80               | 21.3        | 56.3        | 22.5        |
| 9- Camaret-sur-Mer | 11               | 27.3        | 45.5        | 27.3        | 75               | 6.7         | 56.0        | 37.3        |
| 10- Concarneau     | 0                | -           | -           | -           | 80               | 32.5        | 62.5        | 5.0         |
| 11- Quiberon       | 2                | 0.0         | 0.0         | 100.0       | 80               | 3.8         | 63.8        | 32.5        |
| Mean               | 8.1              | 31.0        | 22.7        | 46.4        | 77.7             | 12.4        | 57.1        | 30.5        |
| Standard deviation | 10.8             | 27.4        | 20.0        | 29.1        | 16.5             | 10.3        | 13.1        | 15.9        |
| <b>Total</b>       | <b>89</b>        | <b>33.7</b> | <b>29.2</b> | <b>37.1</b> | <b>855</b>       | <b>12.6</b> | <b>57.0</b> | <b>30.4</b> |

## D) Autumn 2013

| Site               | Species A        |            |             |             | Species B        |            |             |             |
|--------------------|------------------|------------|-------------|-------------|------------------|------------|-------------|-------------|
|                    | N <sub>spA</sub> | %imm       | %sp         | %sp+ooc     | N <sub>spB</sub> | %imm       | %sp         | %sp+ooc     |
| 1- St Malo         | 0                | -          | -           | -           | 80               | 7.5        | 52.5        | 40.0        |
| 2- St Quay         | 40               | 2.5        | 2.5         | 95.0        | 57               | 3.5        | 33.3        | 63.2        |
| 3- Perros Guirec   | 65               | 4.6        | 29.2        | 66.2        | 41               | 4.9        | 56.1        | 39.0        |
| 4- Trébeurden      | 40               | 5.0        | 15.0        | 80.0        | 43               | 2.3        | 25.6        | 72.1        |
| 5- Roscoff         | 0                | -          | -           | -           | 80               | 11.3       | 56.3        | 32.5        |
| 6- Aber Wrac'h     | 0                | -          | -           | -           | 80               | 3.8        | 58.8        | 37.5        |
| 7- Moulin Blanc    | 15               | 13.3       | 6.7         | 80.0        | 77               | 0.0        | 19.5        | 80.5        |
| 8- Château         | 43               | 4.7        | 11.6        | 83.7        | 70               | 1.4        | 12.9        | 85.7        |
| 9- Camaret-sur-Mer | 40               | 5.0        | 27.5        | 67.5        | 60               | 3.3        | 36.7        | 60.0        |
| 10- Concarneau     | 40               | 10.0       | 25.0        | 65.0        | 58               | 1.7        | 22.4        | 75.9        |
| 11- Quiberon       | 13               | 0.0        | 53.8        | 46.2        | 72               | 6.9        | 54.2        | 38.9        |
| Mean               | 26.9             | 5.0        | 19.5        | 75.5        | 65.3             | 4.2        | 38.9        | 56.8        |
| Standard deviation | 22.1             | 4.3        | 16.4        | 15.9        | 14.5             | 3.2        | 17.2        | 19.8        |
| <b>Total</b>       | <b>296</b>       | <b>5.0</b> | <b>19.0</b> | <b>76.0</b> | <b>718</b>       | <b>4.5</b> | <b>39.7</b> | <b>55.8</b> |

## E) Spring 2014

| Site               | Species A        |            |             |             | Species B        |            |             |             |
|--------------------|------------------|------------|-------------|-------------|------------------|------------|-------------|-------------|
|                    | N <sub>spA</sub> | %imm       | %sp         | %sp+ooc     | N <sub>spB</sub> | %imm       | %sp         | %sp+ooc     |
| 1- St Malo         | 0                | -          | -           | -           | 80               | 10.0       | 62.5        | 27.5        |
| 2- St Quay         | 4                | 0.0        | 25.0        | 75.0        | 79               | 0.0        | 40.5        | 59.5        |
| 3- Perros Guirec   | 31               | 12.9       | 51.6        | 35.5        | 67               | 6.0        | 29.9        | 64.2        |
| 4- Trébeurden      | 28               | 0.0        | 32.1        | 67.9        | 75               | 4.0        | 48.0        | 48.0        |
| 5- Roscoff         | 0                | -          | -           | -           | 80               | 3.8        | 45.0        | 51.3        |
| 6- Aber Wrac'h     | 0                | -          | -           | -           | 36               | 0.0        | 5.6         | 94.4        |
| 7- Moulin Blanc    | 0                | -          | -           | -           | 80               | 31.3       | 15.0        | 53.8        |
| 8- Château         | 1                | 0.0        | 0.0         | 100.0       | 80               | 1.3        | 26.3        | 72.5        |
| 9- Camaret-sur-Mer | 11               | 9.1        | 45.5        | 45.5        | 77               | 1.3        | 29.9        | 68.8        |
| 10- Concarneau     | -                | -          | -           | -           | -                | -          | -           | -           |
| 11- Quiberon       | -                | -          | -           | -           | -                | -          | -           | -           |
| Mean               | 8.3              | 4.4        | 30.8        | 64.8        | 72.7             | 6.4        | 33.6        | 60.0        |
| Standard deviation | 12.5             | 6.2        | 20.2        | 25.4        | 14.4             | 9.9        | 17.4        | 18.6        |
| <b>Total</b>       | <b>75</b>        | <b>6.7</b> | <b>41.3</b> | <b>52.0</b> | <b>654</b>       | <b>6.9</b> | <b>35.5</b> | <b>57.6</b> |

## F) Autumn 2014

| Site               | Species A        |            |             |             | Species B        |             |             |             |
|--------------------|------------------|------------|-------------|-------------|------------------|-------------|-------------|-------------|
|                    | N <sub>spA</sub> | %imm       | %sp         | %sp+ooc     | N <sub>spB</sub> | %imm        | %sp         | %sp+ooc     |
| 1- St Malo         | 9                | 11.1       | 55.6        | 33.3        | 76               | 23.7        | 57.9        | 18.4        |
| 2- St Quay         | 27               | 0.0        | 11.1        | 88.9        | 65               | 10.8        | 40.0        | 49.2        |
| 3- Perros Guirec   | 79               | 6.3        | 36.7        | 57.0        | 27               | 22.2        | 55.6        | 22.2        |
| 4- Trébeurden      | 31               | 9.7        | 19.4        | 71.0        | 59               | 11.9        | 25.4        | 62.7        |
| 5- Roscoff         | 0                | -          | -           | -           | 80               | 17.5        | 51.3        | 31.3        |
| 6- Aber Wrac'h     | 0                | -          | -           | -           | 80               | 18.8        | 46.3        | 35.0        |
| 7- Moulin Blanc    | 34               | 5.9        | 14.7        | 79.4        | 76               | 11.8        | 21.1        | 67.1        |
| 8- Château         | 27               | 18.5       | 29.6        | 51.9        | 72               | 44.4        | 30.6        | 25.0        |
| 9- Camaret-sur-Mer | 40               | 2.5        | 5.0         | 92.5        | 49               | 8.2         | 38.8        | 53.1        |
| 10- Concarneau     | -                | -          | -           | -           | -                | -           | -           | -           |
| 11- Quiberon       | -                | -          | -           | -           | -                | -           | -           | -           |
| Mean               | 27.4             | 7.4        | 25.3        | 67.3        | 64.9             | 18.8        | 40.8        | 40.4        |
| Standard deviation | 24.3             | 6.0        | 19.0        | 22.2        | 17.6             | 11.0        | 13.2        | 18.1        |
| <b>Total</b>       | <b>247</b>       | <b>6.9</b> | <b>25.7</b> | <b>67.4</b> | <b>584</b>       | <b>19.2</b> | <b>40.2</b> | <b>40.6</b> |

Table S3. Proportions of admixture per locus, sampling date and maternal lineage (i.e. identified with one species-diagnostic mitochondrial marker) for the 131 individuals that showed an admixed multilocus genotype with the three nuclear loci scored (i.e. CesA, Hox5 and vAChTP).

| <b>Generation</b> | <b>Maternal Lineage (mtDNA)</b> | <b>N<sub>ind</sub></b> | <b>%CesA</b> | <b>%Hox5</b> | <b>%vAChTP</b> |
|-------------------|---------------------------------|------------------------|--------------|--------------|----------------|
| Spring 2012       | sp A                            | 19                     | 5.2          | 89.5         | 5.2            |
|                   | sp B                            | 2                      | 100.0        | 0.0          | 0.0            |
| Autumn 2012       | sp A                            | 44                     | 2.3          | 97.7         | 0.0            |
|                   | sp B                            | 9                      | 88.8         | 11.1         | 11.1           |
| Spring 2013       | sp A                            | 10                     | 0.0          | 100.0        | 0.0            |
|                   | sp B                            | 1                      | 100.0        | 0.0          | 0.0            |
| Autumn 2013       | sp A                            | 35                     | 17.1         | 82.9         | 5.7            |
|                   | sp B                            | 11                     | 100.0        | 27.3         | 9.1            |

Figure S1. Monthly mean of seawater temperature (°C) recorded for this study using data loggers (TidbiT<sup>®</sup>v2) located at 1.5 m depth in most of the studied marinas (all except nos. 1, 2, 10, 11, in Fig. 2 in the main text).

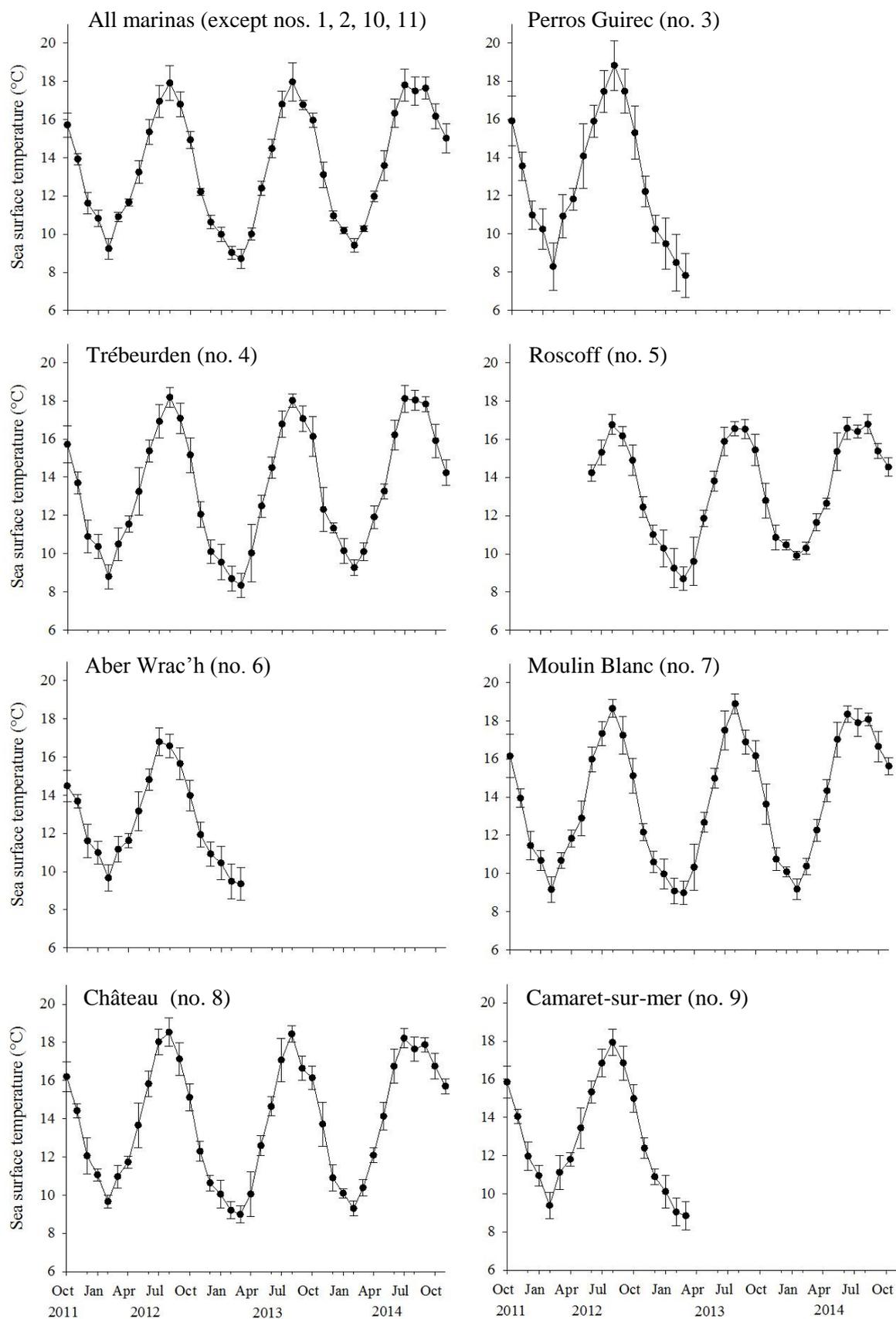


Figure S2. Salinity values and abundance of *C. intestinalis* spp. recorded in Moulin Blanc (no. 7 in Fig. 2 in the main text) between November 2012 and December 2014. Each month, salinity was recorded at two depths (0.25 m and 1.5 m) using a Hach Lange HQ40d multimeter equipped with a CDC 40115 conductivity probe. Every three months, the abundance of *C. intestinalis* spp. was estimated by SCUBA divers using a visual census method along ca. 30 m under one pontoon, according to a semi-quantitative scale (0 = absent, 1 = 0-20%, 2 = 20-40%, 3 = 40-60%, 4 = 60-80% and 5 = 80-100% of coverage).

