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Oyster parasites *Bonamia ostreae* and *B. exitiosa* co-occur in Galicia (NW Spain): spatial distribution and infection dynamics

Andrea Ramilo[1], Mar González[1], María J. Carballal[1], Susana Darriba[2], Elvira Abollo[3], Antonio Villalba[1,*]

[1]Centro de Investigacións Mariñas (CIMA), Consellería do Medio Rural e do Mar, Xunta de Galicia, 36620 Vilanova de Arousa, Spain

[2]Instituto Tecnolóxico para o Control do Medio Mariño de Galicia (INTECMAR), Consellería do Medio Rural e do Mar, Xunta de Galicia, 36611 Vilagarcía de Arousa, Spain

[3]Centro Tecnológico del Mar — Fundación CETMAR, 36208 Vigo, Spain

ABSTRACT: Bonamiosis constrains the flat oyster industry worldwide. The protistan species *Bonamia ostreae* had been considered solely responsible for this disease in Europe, but the report of *B. exitiosa* infecting *Ostrea edulis* 5 yr ago in Galicia (NW Spain), and subsequently in other European countries, raised the question of the relevance of each species in bonamiosis. The spatial distribution of *B. exitiosa* and *B. ostreae* in Galicia was addressed by sampling 7 natural *O. edulis* beds and 3 culture raft areas, up to 3 times in the period 2009 to 2010. *B. ostreae* infected flat oysters in every natural bed and every raft culture area. True *B. exitiosa* infections (histological diagnosis) were detected in every raft culture area but only in 2 natural beds, i.e. in 4 rías. PCR-positive results for *B. exitiosa* were recorded in 4 out of 5 beds where true infections were not found, thus the occurrence of *B. exitiosa* in those 4 beds cannot be ruled out. Additionally, 4 cohorts of hatchery-produced oyster spat were transferred to a raft to analyse *Bonamia* spp. infection dynamics through oyster on-growing. The highest percentages of oysters PCR-positive for both *Bonamia* spp. were recorded in the first months of on-growing; other peaks of PCR-positive diagnosis were successively lower. Differences in the percentage of PCR-positive cases and in the prevalence of true infection between *B. exitiosa* and *B. ostreae* through on-growing were not significant. Our results support that *B. exitiosa* is adapted to infect *O. edulis* in the Galician marine ecosystem.

KEY WORDS: Bonamiosis · *Ostrea edulis* · Shellfish culture

INTRODUCTION

Flat oyster *Ostrea edulis* fishery was an important resource in Galicia (NW Spain) until the middle of the past century. Exhaustion of natural beds through overfishing led to increased oyster aquaculture based on on-growing of oyster spat imported from other European countries, mostly from France (Andreu 1968), which was spurred by raft mussel farming success in the region. Mass imports of spat favoured the introduction of pathogens such as *Bonamia ostreae*, the agent responsible for mass mortalities that almost collapsed the production of flat oysters in Europe (Pichot et al. 1980, Polanco et al. 1984, Hudson & Hill 1991, van Banning 1991, Culloty & Mulcahy 2001).

The genus *Bonamia* belongs to the phylum Haplosporidia (Perkins 1990, Carnegie & Cochennec-Laureau 2004) and includes 4 species: *B. ostreae* (Pichot et al. 1980), *B. exitiosa* (Hine et al. 2001),

*Corresponding author: villalba@cimacoron.org

B. roughleyi (Cochennec-Laureau et al. 2003) and *B. perspora* (Carnegie et al. 2006). *B. ostreae* and *B. exitiosa* have been included in the World Organisation for Animal Health (OIE) list of notifiable diseases (www.oie.int/en/animal-health-in-the-world/oie-listed-diseases-2013/). *B. ostreae* has been reported in various countries of the Northern Hemisphere, both in North America (Katkansky et al. 1969, Elston et al. 1986, Friedman & Perkins 1994, Marty et al. 2006) and Europe (Pichot et al. 1980, Polanco et al. 1984, Hudson & Hill 1991, van Banning 1991, Culloty & Mulcahy 2001). *B. ostreae* is believed to have been introduced to Europe through transfers of infected oysters from California (USA) to France and Spain (Elston et al. 1986, Cigarría & Elston 1997). Conversely, the known geographic distribution of *B. exitiosa* (and *Bonamia* sp. resembling *B. exitiosa*) was mostly limited to the Southern Hemisphere (Doonan et al. 1994, Campalans et al. 2000, Kroeck & Montes 2005, Corbeil et al. 2006), although *B. exitiosa* has also been detected on the Atlantic coast of the USA, in *Crassostrea virginica* and the exotic oyster *C. ariakensis* (Burreson et al. 2004, Dungan et al. 2012). According to this distribution, the European Animal Health regulation (Council of the European Union 2006) included *B. ostreae* in the list of non-exotic diseases requiring special surveillance and *B. exitiosa* in the list of exotic diseases. *B. exitiosa* was later detected on the Galician coast (Abollo et al. 2008) and subsequently in other European regions (Narcisi et al. 2010, Arzul et al. 2011, Carrasco et al. 2012). These reports highlight the need to re-examine the relevance of *B. ostreae* and *B. exitiosa* to bonamiosis in the European context.

Most prior epidemiological studies on bonamiosis in Europe were based on diagnosis with light microscopy (standard histology or tissue imprints). Discrimination between *Bonamia ostreae* and *B. exitiosa* based on these procedures is difficult and requires specific training (Abollo et al. 2008, Ramilo et al. 2013). Therefore, once the presence of *B. exitiosa* within flat oysters in some areas of Europe was discovered, the question of whether misidentifications may have occurred was raised. Infection dynamics of *B. ostreae* in flat oyster beds have been studied in Galicia (Montes et al. 1991, da Silva et al. 2005) as well as in other European countries (Tigé & Grizel 1984, McArdle et al. 1991, van Banning 1991, Culloty & Mulcahy 1996). Those studies agreed that older oysters are more susceptible to the disease than younger ones; in the oyster farming context, this means prevalence and mortality caused by the infection increase at late on-growing stages, close to market size. The application of molecular diagnostic procedures more sensitive than those based on light microscopy has enabled the detection of *B. ostreae* in younger stages (Lynch et al. 2005, Engelsma et al. 2010), even in oyster larvae. The infection dynamics of *B. exitiosa* have been studied in *Ostrea chilensis* from New Zealand (Hine 1991, Cranfield et al. 2005) and *O. puelchana* from Argentina (Kroeck et al. 2008); however, they have not yet been studied in *O. edulis*.

Here we report the results of a study aiming to evaluate the relevance of *Bonamia ostreae* and *B. exitiosa* in the bonamiosis of the flat oyster in Galicia. This goal was addressed by (1) determining the spatial distribution of *B. ostreae* and *B. exitiosa* in oysters along the Galician coast, involving the known natural flat-oyster beds and raft culture areas, and (2) characterising the infection dynamics of *B. ostreae* and *B. exitiosa* through oyster on-growing in an area where both parasites occur.

MATERIALS AND METHODS

Determining the spatial distribution of *Bonamia ostreae* and *B. exitiosa*

The 7 known natural oyster beds and the 3 raft culture areas of Galicia were involved in the study (Fig. 1). The natural oyster beds located in Ría de Pontevedra, Ría de Noia and Ría de Ferrol support a residual fishery, while those located in Ría de Ortigueira, Ría de Ares, Ría do Eo and Ría de Arousa are too scarce to support fishery. Oyster bed sampling was carried out in autumn of 2009 and spring and autumn of 2010, by randomly selecting between 28 and 55 (mostly 30) oysters from each bed. The intertidal beds (Ortigueira, Ferrol, Ares and Eo) were sampled on foot, detaching the oysters from the substrate by hand with a scraper, while the remaining beds (Noia, Arousa and Pontevedra) were sampled from a boat with an oyster dredge. Pontevedra and Ortigueira were sampled 3 times, Noia and Ferrol twice, and Ares, Eo and Arousa once. The culture raft areas were those named Redondela A in Ría de Vigo, and Grove A and Cambados D in Ría de Arousa; sampling of those culture areas involved randomly selecting 30 oysters from those present (beyond our control) in a commercial raft in each area in autumn of 2009 and again in spring of 2010. Introduction of oysters (including market sized oysters) with uncertain health status from various countries into the raft areas is common, and the duration of the period they

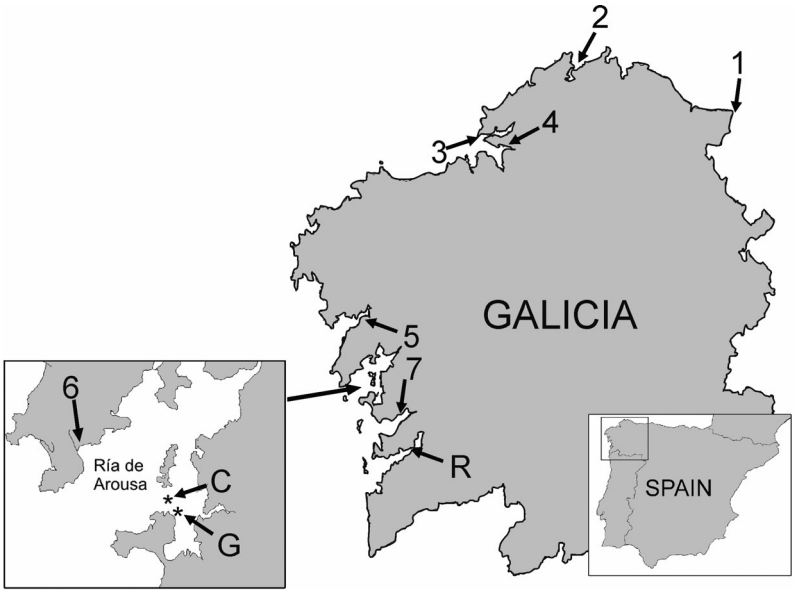

Fig 1. Galicia (NW Spain), showing the location of the natural beds and the culture raft areas included in the study as well as the locations (*) of the raft areas used in the oyster on-growing surveillance in Ría de Arousa. Oyster beds: (1) Ría do Eo, (2) Ría de Ortigueira, (3) Ría de Ferrol, (4) Ría de Ares, (5) Ría de Noia, (6) Ría de Arousa, (7) Ría de Pontevedra. Raft culture areas: (C) Camba-dos D, (G) Grove A, (R) Redondela A

stay in the rafts is highly variable, from a few weeks to a few months, all of which may significantly influence any prevalence trend. All oyster samples from beds and rafts were transported to the laboratory and processed for diagnosis by PCR and histology.

Characterising the infection dynamics of *Bonamia ostreae* and *B. exitiosa*

Four oyster spat cohorts produced in the hatchery facilities of CIMA were used in the study. Cohort A derived from oyster broodstock from Ría de Arousa, Cohort P from Ría de Pontevedra, and Cohorts O+A1 and O+A2 each derived from a different broodstock batch including oysters from Ría de Ortigueira and Ría de Arousa. The spawning events giving rise to Cohorts O+A1 and P occurred on 13 and 16 January 2009, respectively; the spawning giving rise to Cohort A occurred on 13 February 2009, and cohort O+A2 was a mix of larvae derived from a spawning on 16 February and another spawning on 31 March 2009. A total of 5900 individuals of Cohort A, 5800 of P, 6000 of O+A1 and 1450 of O+A2 were transferred to a culture raft located in the culture area named Cambados D (Ría de Arousa; Fig. 1) in summer 2009. Transference of Cohorts P and O+A1 was carried out on 23 June with oyster mean height (± SD) of 26.2 ± 3.50 mm and 21.7 ± 2.68 mm, respectively; Cohort A on 8 July, with 23.0 ± 4.65 mm; and Cohort O+A2 on 15 July 2009, with 19.1 ± 2.53 mm. The spat were set into lidded containers (250 per container) made of 1 cm mesh plastic net. The containers were set in standard perforated (2 cm mesh) circular plastic trays for oyster culture (4 containers per tray), the trays were piled in stacks, marked to identify the spat cohort inside and hung from the raft. The number of individuals per container was reduced as oysters grew; once oysters were large enough, the lidded containers were removed and the oysters set directly in the perforated circular trays. The on-growing period finished in June 2011. Sampling was performed monthly, from September 2009 onwards, and it involved taking 20 oysters per cohort to diagnose *B. exitiosa* and *B. ostreae* by species-specific PCR assays and histological analysis. Additional sampling was carried out quarterly, from September 2009 onwards, to evaluate growth and mortality of each cohort. Growth estimation was based on measuring height and whole weight of 30 oysters per cohort. Live and dead individuals within each of 4 to 6 lidded containers from different tray stacks were counted to estimate mortality rate of each cohort between sampling periods; the number of examined containers increased as stocking density decreased; once lidded containers were not needed, 6 oyster trays from different stacks were examined. Cumulative mortality was calculated from mortality rate estimations. After estimating mortality, all oysters from each cohort were pooled, and 30 individuals were randomly taken and their height and individual whole weight was measured to estimate growth.

Histology

A frontal section (ca. 5 mm thick) of the meat of each oyster, containing gills, visceral mass and mantle lobes, was fixed in Davidson's solution, dehydrated in an ethanol series and embedded in paraffin. Histological sections (5 µm thick) were stained with Harris' haematoxylin and eosin (Howard et al.

2004) and observed under a light microscope (1000× magnification) for the specific identification of *Bonamia exitiosa* and *B. ostreae* according to the distinguishing morphological criteria reported by Abollo et al. (2008). The procedure used to rank the intensity of *B. ostreae* and *B. exitiosa* infection for each oyster was adapted from Bachère et al. (1982) and Hine (1991) using the following scale: (0) null infection: no *Bonamia* spp. detected; (1) light infection: *Bonamia* spp. only observed after thorough searching and then only 1 to 2 parasites present in each infected haemocyte (rarely up to 4); few or no foci of haemocytic infiltration; (2) moderate infection: *Bonamia* spp. occurring in various foci of haemocytic infiltration, infected haemocytes enclosing few (1–4) parasites may coexist with haemocytes bearing up to 10 parasites; (3) heavy infection: *Bonamia* spp. widespread throughout host organs and abundant large areas of haemocytic infiltration. numerous parasites (>20) in each infected haemocyte.

DNA extraction and PCR diagnosis assays

Small pieces of gills and gonads from each oyster were preserved in 96% ethanol to perform molecular analysis. DNA extractions from both organs together (25–50 mg) were performed employing the commercial Wizard Genomic DNA Purification Kit (Promega), according to the manufacturer's protocol. DNA quality and quantity was checked in a spectrophotometer (Nanodrop® ND-1000, Nanodrop Technologies). DNA obtained was analysed by specific conventional PCR assays for *Bonamia ostreae* and *B. exitiosa*, using the primer pairs BOSTRE-F/BOSTRE-R and BEXIT-F/BEXIT-R, respectively (Ramilo et al. 2013). PCR assays were performed in a total volume of 25 µl containing 1 µl of genomic DNA (200 ng), PCR buffer at 1× concentration, 1.5 mM $MgCl_2$, 0.2 mM nucleotides (Roche Applied Science), 0.3 µM each specific primer for either *B. ostreae* (BOSTRE-F/-R) or *B. exitiosa* (BEXIT-F/-R) and 0.025 U μl^{-1} *Taq* DNA polymerase (Roche Applied Science). A positive control for *B. ostreae* or *B. exitiosa* (DNA from an oyster infected with either *B. ostreae* or *B. exitiosa*) and a negative control (no DNA) were used in every PCR assay. The PCR assays were carried out in a Tgradient thermocycler (Biometra), under the following reaction parameters: 94°C for 2 min, 35 cycles at a melting temperature of 94°C for 30 s, an annealing temperature of 55°C for *B. ostreae* or 58°C for *B. exitiosa* for 45 s, an extension temperature of 72°C for 1 min; followed by a final extension period of 72°C for 1 min. After PCR, 10 µl aliquots of amplified DNA were analysed by electrophoresis on 2% agarose gels, in 1% Tris acetate EDTA buffer, stained with ethidium bromide and scanned in a GelDoc XR documentation system (BioRad).

Statistical analysis

In order to determine whether the overall percentage of positive cases of *Bonamia ostreae* throughout the Galician natural beds differed from that of *B. exitiosa*, the mean value of the percentage of positive cases of each parasite after grouping all the samples was calculated for each natural bed; differences between the mean values of the percentage of positive cases of *B. ostreae* and of *B. exitiosa* were then analysed with a Mann-Whitney test. Differences in the raft culture areas were not analysed because the prevalence of both parasites is highly fluctuant depending on introductions of oysters from other countries, which was beyond our control during the study. The differences in the percentage of positive cases of *B. ostreae* and *B. exitiosa* between oyster cohorts through on-growing were analysed using a Friedman test in which the treatments were the cohorts and the blocks were the sampling dates (Conover 1999). In order to evaluate whether the overall percentage of positive cases of *B. ostreae* through on-growing differed from that of *B. exitiosa*, the mean value of the percentage of positive cases of each parasite after grouping all the cohorts was calculated for each monthly sample; differences between the mean percentage of positive cases of *B. ostreae* and *B. exitiosa* were then analysed with a Friedman test, in which the treatments were the parasite species and the blocks were the monthly samples. All statistical tests were performed with MINITAB 16 software; significance was established at $p \leq 0.05$.

RESULTS

Spatial distribution of *Bonamia exitiosa* and *B. ostreae* in *Ostrea edulis* along the Galician coast

Bonamiosis in natural beds. In total, 454 oysters were analysed. Results of diagnosis with histology showed cases of infection with *B. ostreae* in every natural bed, with a maximum prevalence of 83%, while infections with *B. exitiosa* were detected only in 2 beds, in Ría de Pontevedra and Ría de Ortigueira, with maximum prevalence of 4%. The preva-

lence of *B. ostreae* showed wide fluctuation in the beds sampled more than once. Cases of co-infection with both parasites were detected only in Ría de Pontevedra (Table 1). Differences between the prevalence of *B. ostreae* and that of *B. exitiosa* through the natural beds were significant ($p = 0.002$). PCR diagnosis provided more positive cases than histology for both parasites. Positives for *B. ostreae* were detected in every bed, in every sampling, while positives for *B. exitiosa* were not detected in 1 bed, that in the Ría do Eo, which was sampled just once. There were 2 beds, those in Ría de Ortigueira and Ría de Noia, in which PCR *B. exitiosa*-positives were detected in some samplings but not in every one. PCR-positive cases for both parasites in the same individual were detected in every bed except those in Ría de Ferrol and Ría do Eo (Table 1). The percentage of oysters with a PCR-positive diagnosis fluctuated between sampling times in the beds sampled more than once. Differences between the percentage of PCR *B. ostreae*-positive cases of and that of *B. exitiosa* through the natural beds were not statistically significant ($p > 0.05$).

Bonamiosis in raft culture areas. In total, 180 oysters were analysed. *Bonamia ostreae* and *B. exitiosa* were detected in every raft culture area in the autumn 2009 sampling, and cases of co-infection with both parasites were also detected, using histology and PCR diagnosis. However, in the spring 2010 sampling, neither *B. ostreae* nor *B. exitiosa* were detected in Redondela A and Cambados D, whereas PCR-positive cases for both parasites were found in samples from Grove A (Table 1). Again, PCR diagnosis provided more positive cases than histology for both parasites. The percentage of positive cases for *B. ostreae* and *B. exitiosa* was similar in 2 areas, Redondela A and Grove A, but in Cambados D, the percentage of positives for *B. exitiosa* was double that of *B. ostreae* with both diagnostic methods. The highest prevalence (histology) record of *B. ostreae* was 23% and that of *B. exitiosa* was 30%.

Infection dynamics through oyster on-growing

Growth and mortality. Fig. 2A shows the monthly mean values of whole weight of oysters of each cohort through the on-growing period. The growth pattern was similar among cohorts for 18 mo, up to the end of 2011. Subsequently, growth trends were different, especially in Cohort A, whose growth stopped from December 2010 and showed the lowest final mean weight. High mortality was detected in 3 cohorts (P, O+A1 and O+A2) in the first stage of on-growing (around 6 mo), reaching 70 to 90% cumulative mortality; afterwards, mortality was much lower (Fig. 2B). In the case of 1 of those cohorts, O+A2, on-growing started later, and thus the period of high mortality finished later. However, Cohort A showed lower mortality in that first stage of on-growing but it markedly increased in spring 2010. The final values of cumulative mortality were 88.1, 89.5, 96.8 and 86.15% for Cohorts P, A, O+A1 and O+A2, respectively. Mortality of juvenile oysters was not caused by bonamiosis (see 'Discussion').

Table 1. *Ostrea edulis*. Percentage of oysters in the samples with positive diagnosis for *Bonamia ostreae*, *B. exitiosa* and co-detection of both parasites in the same individual host, using histology and species-specific PCR assays, in 7 natural oyster beds and 3 raft culture areas, in 3 sampling events (autumn 2009, spring 2010 and autumn 2010). H+: histology positive (%); P+: PCR positive (%); n: sample size; nd: no data. The percentages in the *B. ostreae* and *B. exitiosa* columns include those individuals which were also co-infected with the other *Bonamia* sp., whereas the 'Co-infection' column includes only those individuals containing both *Bonamia* spp.

Location	*B. ostreae*									*B. exitiosa*									Co-infection								
	Autumn 2009			Spring 2010			Autumn 2010			Autumn 2009			Spring 2010			Autumn 2010			Autumn 2009			Spring 2010			Autumn 2010		
	H+	P+	n	H+	P+	n	H+	P+	n	H+	P+	n	H+	P+	n	H+	P+	n	H+	P+	n	H+	P+	n	H+	P+	n
Pontevedra	0	7	30	20	83	30	0	2	50	0	47	30	3	10	30	0	6	50	0	3	30	3	10	30	0	0	50
Ortigueira	3	63	30	13	57	30	21	26	47	3	30	30	0	0	30	4	4	47	0	23	30	0	0	30	0	0	47
Arousa	6	77	30		nd			nd		0	40	30		nd			nd		0	37	30		nd			nd	
Noia		nd			nd		4	10	50		nd			nd		0	0	50		nd			nd		0	0	50
Ferrol		nd		7	13	30	16	14	44		nd		0	10	30	0	7	44		nd		0	0	30	0	0	44
Ares		nd			nd		5	9	55		nd			nd		0	20	55		nd			nd		0	2	55
Eo		nd			nd		3	42	28		nd			nd		0	0	28		nd			nd		0	0	28
Redondela A	23	83	30	0	0	30		nd		20	83	30	0	0	30		nd		7	66	30	0	0	30		nd	
Grove A	7	53	30	0	7	30		nd		23	50	30	0	27	30		nd		3	23	30	0	3	30		nd	
Cambados D	13	43	30	0	0	30		nd		33	87	30	0	0	30		nd		7	43	30	0	0	30		nd	

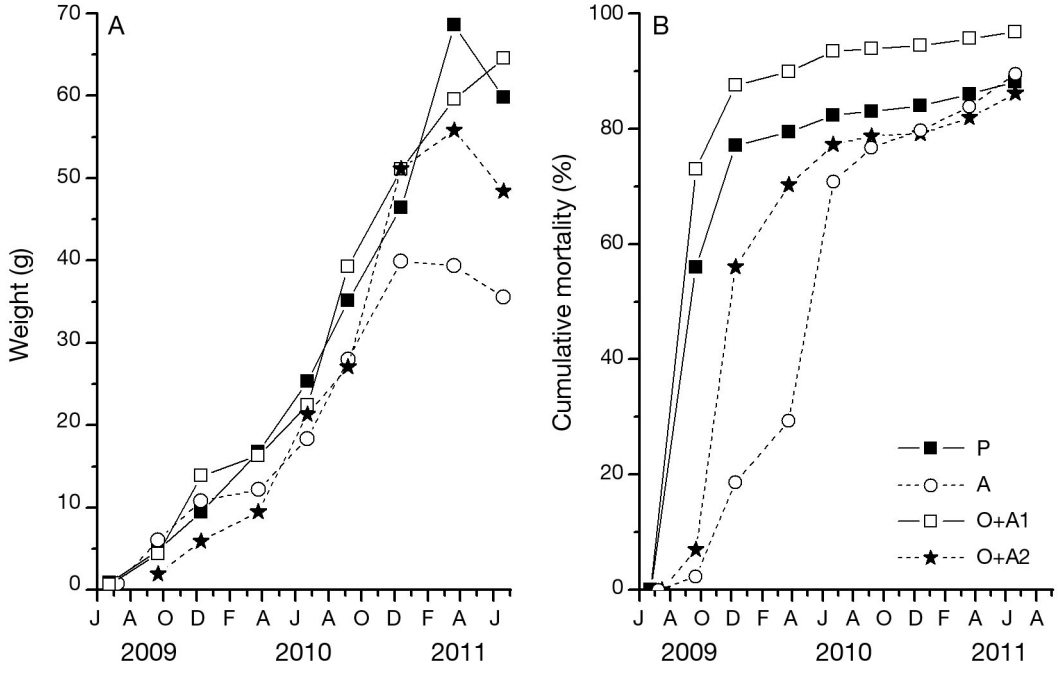

Fig. 2. *Ostrea edulis.* (A) Mean whole weight and (B) cumulative mortality of each cohort (represented by different symbols) through the on-growing period

First detection of infection. The earliest detection of *Bonamia exitiosa* infection with histology occurred in October 2009, in Cohort O+A2, 7 to 8 mo after spawning (Fig. 3, Table 2). In the case of infection with *B. ostreae*, the earliest detection with histology occurred in January 2010, in Cohort P, 12 mo after spawning. The weight and length of the smallest infected oyster at the earliest detection in each cohort is shown in Table 2. PCR-positive cases for both parasites were recorded in September 2009 (Fig. 3, Table 2).

Prevalence through on-growing. Fig. 3 shows the monthly records of the percentage of oysters with positive diagnosis for *Bonamia ostreae* and *B. exitiosa* by histology and PCR in each cohort through on-growing. No temporal pattern of infection prevalence common to all the cohorts was found. The highest prevalence (histology) was 20% for *B. ostreae* and 15% for *B. exitiosa*. The highest values of the percentage of oysters with PCR-positive diagnosis were recorded in the first months of on-growing (autumn to early winter 2009) for both parasites.

Table 2. *Ostrea edulis.* Sampling date and age of each cohort in which *Bonamia ostreae* and *B. exitiosa* were detected for the first time with histology and PCR. Weights and lengths are given for the smallest infected oyster found

Cohort	Date of deployment	*Bonamia* species	Histology				PCR			
			Sample date	Age (mo)	Weight (g)	Length (mm)	Sample date	Age (mo)	Weight (g)	Length (mm)
P	23 June 2009	*B. ostreae*	Jan 2010	12	42.47	66	Sep 2009	8	2.08	29
		B. exitiosa	Jun 2010	17	23.86	46	Sep 2009	8	3.71	26
A	8 July 2009	*B. ostreae*	Dec 2010	10	7.22	32	Sep 2009	7	0.72	17
		B. exitiosa	Jun 2010	16	2.61	29	Dec 2009	10	1.51	30
O+A1	23 June 2009	*B. ostreae*	Apr 2010	15	11.77	42	Sep 2009	8	15.11	68
		B. exitiosa	Aug 2010	19	16.37	48	Oct 2009	9	4.04	45
O+A2	15 July 2009	*B. ostreae*	Feb 2010	11–12[a]	28.47	54	Sep 2009	6–7[a]	2.57	31
		B. exitiosa	Oct 2009	7–8[a]	43.31	64	Sep 2009	6–7[a]	12.00	45

[a]Age is not precise because Cohort O+A2 larvae were spawned on 2 different dates

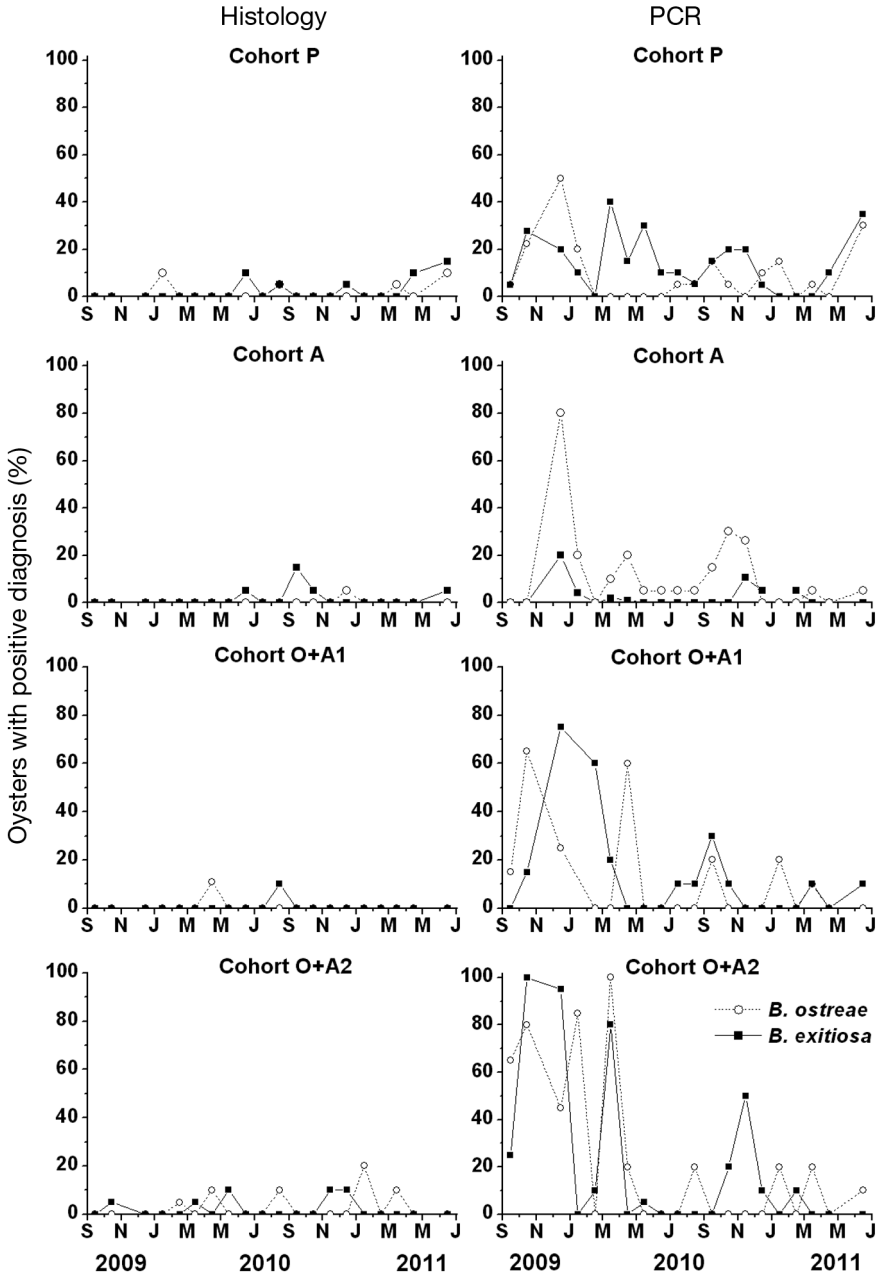

Fig. 3. *Ostrea edulis.* Percentage of oysters in the samples with positive diagnosis for *Bonamia ostreae* and *B. exitiosa* in each cohort by histology (left column) and species-specific PCR assays (right column)

However, in that period, just 1 case of true infection, corresponding to *B. ostreae*, was detected by histology, in January 2010 in Cohort P. After that period, other peaks of PCR-positive diagnosis were successively lower for both parasites. The highest percentages of PCR-positives corresponded to Cohort O+A2, in which up to 100% of oysters were positive for *B. exitiosa* in October 2009 and for *B. ostreae* in March 2010. The percentage of cases of infection with each parasite detected with histology was much lower than the percentage of PCR-positive cases. The mean percentages of positive cases of each parasite corresponding to each cohort through the on-growing period (after grouping all the samples) are shown in Table 3. Differences between percentages of positive cases for *B. ostreae* and *B. exitiosa* and between the 4 cohorts were not significant ($p > 0.05$), both by PCR and histology. Similarly, we found no significant differences ($p > 0.05$) in the percentage of positive cases through the on-growing period between *B. exitiosa* and *B. ostreae* after grouping all cohorts.

Table 3. *Ostrea edulis*. Mean percentage of oysters with positive diagnosis for *Bonamia ostreae* and *B. exitiosa* of each cohort after grouping all monthly samples, estimated with PCR and histology. Mean intensity is mean rank intensity (0 = no infection, 3 = heavy infection; see 'Materials and methods: Histology' for more details)

Cohort	Mean percentage positive				Mean intensity	
	Histology		PCR		Histology	
	B. ostreae	*B. exitiosa*	*B. ostreae*	*B. exitiosa*	*B. ostreae*	*B. exitiosa*
P	1.2	2.1	9.4	13.9	1.12	1.14
A	0.3	1.8	2.4	11.6	1.00	1.17
O+A1	0.9	0.6	10.8	12.5	1.00	1.00
O+A2	2.6	1.8	23.2	20.2	1.20	1.50
Average	1.2	1.6	11.45	14.55	1.08	1.20

Infection intensity. Intensity was light (rank 1) in most cases of infection with either *Bonamia ostreae* or *B. exitiosa*; some cases of moderate infection (rank 2) were also detected, but no heavy infection (rank 3) was recorded. Mean rank intensity of *B. exitiosa* after grouping all monthly samples and cohorts was slightly higher than that of *B. ostreae* (1.20 and 1.08, respectively). Cohort O+A2 showed the highest mean intensity for both parasites (Table 3).

DISCUSSION

The study of the spatial distribution of *Bonamia ostreae* and *B. exitiosa* infecting *Ostrea edulis* along the Galician coast revealed that the former parasite infected flat oysters in every natural bed and every raft culture area involved in the study. True *B. exitiosa* infections (positive histological diagnosis) were detected in every raft culture area but only in 2 natural beds (those sampled 3 times), i.e. 4 Galician rías. PCR-positive results for *B. exitiosa* were recorded in 4 of 5 beds where true infections were not found; thus the occurrence of *B. exitiosa* in those 4 beds cannot be ruled out. Infection with *B. ostreae* was significantly more prevalent than with *B. exitiosa* in Galician oyster beds considered as a whole, whereas the difference in the percentage of PCR-positive cases between both *Bonamia* species was not significant. These results could suggest that *B. ostreae* gave rise to true infection after contacting oysters in natural beds more easily than *B. exitiosa*. In raft culture areas, the prevalence of *B. exitiosa* was similar to that of *B. ostreae* in 2 culture areas but was higher in the third one. Nevertheless, the significance of the difference in raft culture areas is uncertain because the frequent introductions of oyster batches from multiple origins, with varying parasite loads, into the rafts results in highly fluctuant prevalence of *Bonamia* spp. in those areas. In fact, marked differences in the prevalence of both parasites were found between the 2 sampling events in the 3 areas. Higher oyster density in rafts than in beds could contribute to the higher prevalence of *B. exitiosa* in raft culture areas, because the main transmission route of this parasite is considered to be direct from oyster to oyster (Hine 1996, Cranfield et al. 2005). Furthermore, a good adaptation to environmental conditions of oysters in the natural beds is plausible, whereas oysters frequently do not adapt well when transferred to raft culture areas from distant regions with quite different environments. Culture often results in stress for molluscs (Filgueira et al. 2013). Thus, a lack of adaptation or increased stress of farmed oysters could favour clinical infections and the spread of *B. exitiosa*. Further sampling would be required to evaluate whether *B. exitiosa* is spreading through Galician beds. The widespread distribution of *B. ostreae* throughout the Galician coast has been reported previously (Polanco et al. 1984, Figueras 1991, Montes & Lama 1992).

Growth of the 4 cohorts of flat oyster was rather similar in the first 18 mo of on-growing. After that period, different growth trends were observed, especially in Cohort A, derived from Ría de Arousa broodstock. On-growing was marked by exceptionally high mortality in 3 cohorts in the first stage. High mortality of juvenile flat oysters is unusual in Galician farming areas. Since the first bonamiosis outbreaks in the early 1980s, high mortalities have usually occurred in the last stage of on-growing when the oysters are close to market age (Montes et al. 1989, 1991, da Silva et al. 2005). Nevertheless, juvenile flat oyster mortality, though lower than in our study, has been reported in the same farming area associated with a herpesvirus infection (da Silva et al. 2008). The high juvenile oyster mortality recorded in our study most likely resulted from a herpes-like viral infection that was highly prevalent in the samples during the high mortality period and has been reported elsewhere (Villalba et al. 2010). *Bonamia ostreae* and *B. exitiosa* were detected early in the on-growing process with PCR, but bonamiosis cannot be considered responsible for the juvenile oyster mortality because *Bonamia* infection, if any, had to be a very light, non-lethal infection: just 1 case of infection with *B. ostreae* and 1 case with *B. exitiosa* were detected with histology during the high mortality period.

Regarding bonamiosis dynamics through on-growing, most of the highest values of the percentage of PCR-positive oysters for *Bonamia ostreae* and *B. exitiosa* were recorded in the first months of on-growing; since then, other peaks of PCR-positive diagnosis were successively lower for both parasites. Many of the PCR-positive cases corresponded to histology-negative ones, which means that they were irrelevant from a clinical point of view. This early PCR-positive diagnosis in a high percentage of oysters would indicate that most oysters came into close contact with both parasites early. Even the hypothesis that oysters carried the parasites from the hatchery cannot be discarded because diagnosis was not performed at the transference from hatchery to the raft. However, proliferation of both parasites was somehow impeded, and true infections only occurred after several months. These results are consistent with the previously reported trend of higher prevalence in older than in younger oysters based on histological diagnosis (van Banning 1990, Culloty & Mulcahy 1996, da Silva et al. 2005). Why did the percentage of PCR-positive cases decrease after the first months? Did some oysters clear the parasites to undetectable levels? Unfortunately, the huge juvenile oyster mortality interfered with the study of bonamiosis dynamics, and that decrease could be due to death of weak oysters, those more susceptible to viral infection but perhaps also to bonamiosis, thus leaving the strongest, least susceptible individuals. This could explain the abnormally low prevalence of *B. ostreae* through the second year of on-growing (with a maximum of 20%), compared to previous reports of *B. ostreae* prevalence of >50% in Galicia (Figueras 1991, Montes et al. 1991) or even in the same raft culture area (Montes et al. 1989, da Silva et al. 2005). The prevalence of *B. exitiosa* estimated with histology did not exceed 15%. Interestingly, we found no significant differences in the percentage of positive cases (PCR and histology) through the on-growing period between *B. exitiosa* and *B. ostreae*. The intensity of infections was mostly low for both parasites, and no case of heavy infection was detected throughout the on-growing period, which was consistent with the unexpected low mortality recorded during the second year of on-growing and with the hypothetical selection of the least susceptible oysters at the juvenile stage due to herpes-like viral infection.

The sensitivity of PCR was higher than that of histology in detecting *Bonamia exitiosa* and *B. ostreae*. The low sensitivity of histology in diagnosing bonamiosis has been stated in previous studies (Diggles et al. 2003, Lynch et al. 2005, Balseiro et al. 2006, Corbeil et al. 2006, Marty et al. 2006, Engelsma et al. 2010, Ramilo et al. 2013). The earliest detection of true infection (histological diagnosis) with *B. ostreae* occurred 12 mo after spawning and 7 mo after transference to the raft. In a previous study on oyster spat on-grown in the same culture raft area, da Silva et al. (2005) did not detect *B. ostreae* by histology until 1 yr after the transference of the spat from the hatchery to the raft. The use of molecular diagnostics enables the detection of *Bonamia* spp. in earlier oyster stages than with histology, such as *B. ostreae* in 1.2 cm long spat (Lynch et al. 2005) and *B. ostreae* and *B. exitiosa* even in larvae (Arzul et al. 2011). There is no previous report on *B. exitiosa* infection dynamics in *Ostrea edulis*, but the highly prevalent infection with a herpes-like virus (Villalba et al. 2010) and the large juvenile oyster mortality in our study precludes us from considering the oyster age-related trend of the percentage of PCR-positive cases detected in our study as a general pattern.

The detection of true *Bonamia exitiosa* infections in 4 Galician rías plus the PCR-positive cases found in 3 other rías show that *B. exitiosa* is able to infect *Ostrea edulis* in the Galician marine ecosystem. Considering that *B. exitiosa* has also been detected in Italy (Narcisi et al. 2010), France (Arzul et al. 2011) and Catalonia (Carrasco et al. 2012), *B. exitiosa* should be transferred from the list of exotic diseases to the list of non-exotic ones within the European Animal Health regulation (Council of the European Union 2006).

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