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RESEARCH ARTICLE

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Growth, mortality and disease susceptibility of oyster *Ostrea edulis* families obtained from brood stocks of different geographical origins, through on-growing in the Ría de Arousa (Galicia, NW Spain)

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Abstract Infection by *Bonamia ostreae* has caused extensive mortalities of oysters *Ostrea edulis* through European and United States coasts for at least 25 years. The development of a bonamiosis-resistant strain seems a promising strategy to fight against the disease. As a first step, evaluation of variability in productive traits and disease susceptibility of European populations was performed to identify favourable oyster populations with which to start selective breeding in Galicia (NW Spain). Oysters taken from Greece, Ireland, Ortigueira (Galicia) and Coroso (Galicia) were used as brood stock, and 19 seed families were produced (4–5 families from each origin). The oyster families were used to assess variability through on-growing in an area of the Ría de Arousa heavily affected by bonamiosis. Results showed significant differences in growth, mortality and susceptibility to bonamiosis and other diseases, both between origins and between families under origins. Bonamiosis was associated with mortality in the late stage of oyster on-growing. Indications of natural selection of bonamiosis less-susceptible oysters due to the long exposure of the Ortigueira population to bonamiosis were found. Other symbionts and pathological conditions were detected, of which herpes-like viral infections and disseminated neoplasia could also cause mortality. An index of the overall incidence of pathological conditions (OIPC) was estimated for each family. A significant correlation between the OIPC and the cumulative mortality of the families was noted. On average, oysters from autochthonous origins showed better performance. The results obtained with the best-performing families suggest that the profitability of oyster farming in Galicia would improve, even under bonamiosis pressure, by using appropriate oyster spat.

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Introduction

Galician oyster (*Ostrea edulis*) beds have supported a traditional, profitable and intensive fishery for centuries, which led, at the middle of the past century, to the exhaustion of most Galician oyster populations and the decline of the oyster industry (Andreu 1968). Then, oyster farming was developed as an alternative to the traditional fishery, but the scarcity of a local supply of oyster spat made it necessary to import large amounts of oysters from different countries, mainly from France. In the meantime, two consecutive epizootic outbreaks caused *O. edulis* mass mortalities in France due to the protozoans *Marteilia refringens* (Grizel et al. 1974) and *Bonamia ostreae* (Pichot et al. 1980). *B. ostreae*, which had probably been introduced into France with oyster spat from California (Elston et al. 1987), spread throughout other European countries with oyster transfers: England (Alderman 1981), Holland (van Banning 1991), Ireland (Culloty and Mulcahy 2001), and Galicia (Polanco et al. 1984). Thus, bonamiosis became the most important constraint for the oyster (*O. edulis*) industry in these areas because it causes mass mortalities in the late stage of oyster on-growing. *B. ostreae* infects the host haemocytes, which are the effector cells of the host immune system; haemocytes fail to kill the parasite, and the latter divides inside the former leading to haemocyte rupture and evoking a heavy haemocytic infiltration of tissues (Comps 1983). Molecular taxonomy supported allocating this species to the phylum Haplosporidia (Carnegie et al. 2000; Cochennec et al. 2000).

Different strategies have been tried to minimise the effect of this disease or to eradicate it and to recover the

natural oyster beds. In Holland, attempts to eradicate the disease, by thoroughly removing oysters from beds and banning oyster deployment in affected areas, failed (van Banning 1988, 1991). In France, a plan combining zootechnical prophylaxis and eradication measures was performed (Grizel et al. 1986) but its effectiveness was limited and, eventually, the oyster industry of the country that supported the highest world production of *O. edulis* replaced this species by the non-susceptible *Crassostrea gigas*. In Galicia, the oyster industry was forced to shift to a survival strategy involving the introduction of submarket- or even market-sized oysters from other countries, which are on-grown for a short period (less than 1 year), and harvested before bonamiosis-caused mortality decimates the oyster batch. This strategy is close to storing rather than farming oysters, and involves a risky dependence on availability of submarket-sized oysters. When spat are introduced in Galicia for on-growing, mass mortality occurs earlier than, or just when the minimum market size is reached (Montes et al. 2003), and thus only oysters of the lowest market class can be sold. *B. ostreae* deserves special attention in the European Union legislation (Anonymous 1991, 1994), as well as in the "Office International des Epizooties" (Anonymous 2002), as part of an international fighting strategy to avoid introduction of the parasite in non-affected areas. One strategy that has been performed with encouraging results with other diseases of oysters, involves developing resistant strains by selective breeding programmes. This was the case in the diseases caused by *Haplosporidium nelsoni* (Ford and Haskin 1988; Ragone-Calvo et al. 2003) and *Perkinsus marinus* (Ragone-Calvo et al. 2003), and the "Juvenile Oyster Disease" (Davis and Barber 1999) in the oyster *C. virginica*, the "summer mortality" in *C. gigas* (Beattie et al. 1988), and those caused by *M. sydneyi* and *Mykrocytos roughleyi* in *Saccostrea commercialis* (Nell et al. 2000; Nell and Hand 2003). Those results, together with the report of an *O. edulis* population from California exhibiting a degree of resistance to *B. ostreae* (Elston et al. 1987), encouraged the performance of selective breeding programmes for bonamiosis resistance in France (Martin et al. 1993; Baud et al. 1997; Naciri-Graven et al. 1998, 1999) and Ireland (Culloty et al. 2001), which have yielded higher survival and lower *B. ostreae* prevalence. Nevertheless, evidence of inbreeding in the selected lines of the French programme has been noticed (Launey et al. 2001). Therefore, the Centro de Investigacións Mariñas (CIMA) has decided to develop a selective breeding programme to produce oysters, *O. edulis*, with increased resistance to bonamiosis. Evaluation of the variability of productive traits, disease susceptibility, physiological features and immune capability through oyster populations was thought to be a useful initial stage, because particular populations with certain traits could be chosen for the programme. One study on variability at allozyme loci had suggested the existence of two separated, ancient Atlantic and Mediterranean *O. edulis* stocks (Saavedra et al. 1995). Genetic differentiation due to isolation has been further confirmed by microsatellite polymorphism (Launey et al. 2002). According to the available genetic information in 2000, four oyster populations were chosen for variability assessment, including extreme and intermediate locations in the geographic range: Greece (eastern Mediterranean), Ireland (northern Atlantic), and two populations from Galicia, Spain. Oysters from those locations were used as brood stocks and 19 full- or half-sib families were produced (4–5 families from each origin). The oyster families were used to assess variability through cultivation up to market size in an area heavily affected by bonamiosis. All the oyster families shared common environments and handling since birth to ensure that differences that eventually could be detected between them were not due to environmental factors. This paper reports the inter- (between origins) and intra-population (between families) variability in productive (growth and survival) traits and disease susceptibility (in a broad sense) through oyster on-growing in Galicia.

Materials and methods

Production of oyster families

Four oyster populations were selected as brood stock for the experiments: one in the North of Ireland (IR), where bonamiosis has never been detected (Culloty and Mulcahy 2001), one in Greece (GR) that has not been affected (Anonymous 2002), one in Ría de Ortigueira (OR) (Galicia, NW Spain), where the disease has occurred since the early 1980s (Polanco et al. 1984), and one in Coroso (CO) (Ría de Arousa, Galicia, NW Spain) where the disease was detected later and the prevalence is lower (R.F. Conchas, personal communication). Unfortunately, there is no oyster bed that has not been affected by bonamiosis in Galicia. Thus, in December 2000, adult oysters from each origin were transported to the hatchery facilities of CIMA, and distributed in 5 trays with 15–20 individuals each, thus resulting in 20 trays (5 trays per origin). Oysters were conditioned for 2–3 months in an open seawater circuit. Seawater temperature was raised progressively up to 20 ± 1°C, and supplemented daily with a mixed algal diet of *Tetraselmis suecica*, *Chaetoceros calcitrans*, *Isochrysis galbana* and *Skeletonema costatum*, to favour gametogenesis out of the normal season. Controlled pair mating is difficult with *O. edulis* because it is a hermaphrodite species; the ripe ovocytes are not expelled out of the shell but they are kept in the pallial cavity instead, where fertilisation with spermatozoa from other oyster/s eventually occurs, the oysters brooding the resulting larvae in the pallial cavity, and then discharging D-shaped larvae. An 80-μm-mesh sieve was placed at the outlet of each tray and every time a batch of larvae was detected in it, the number of larvae was counted. When this number was higher than 1.5 million, the complete batch was rejected because more than one mother could have been

involved. Therefore, the larvae in each collected batch probably derived from a single mother, thus being either a half- or full-sib family. Every larval family was separately reared in 100-l tanks filled with 0.2 μm-filtered seawater at 20 ± 1°C, at a final concentration of 3–5 larvae ml^{-1}, according to the procedure described by Román (1992). Once larvae reached the setting size, they were transferred to tanks provided with setting substrate (razor-clam shell powder). After ca. 22 days, oyster spat were set inside PVC cylinders with a mesh bottom and an up-welling system to ensure continuous water flow. All the cylinders were placed in the same tank. All the spat in a cylinder belonged to the same family. Larvae and spat were fed with a mixed algal diet of *T. suecica*, *Chaetoceros calcitrans*, *I. galbana*, and *Monochrysis lutheri*. Once spat overpassed 1 cm height, they were transferred to a raft for on-growing. A total of 19 families were produced, 5 from each origin, except for IR from which only 4 families were obtained.

Experimental design for oyster raft on-growing

In September 2001, approximately 4,000 individuals of each family were transferred to a raft located near Cambados (Ría de Arousa, Galicia, NW Spain), in an oyster-culture area heavily affected by bonamiosis since the 1980s (Montes et al. 1989). Oyster spat was distributed into lidded containers (250 ind. per container) made of 1-cm-mesh plastic net. Each container was marked to identify the spat family inside. The containers were set in standard perforated (2 cm mesh) circular plastic trays for oyster culture (four containers per tray). The trays were piled in stacks of ten trays (eight stacks in total) and hung from a raft. Each stack of trays hosted two containers of each spat family, which were randomly placed in the stack, in a randomised complete block design. Thus, each family was represented, twice, in every stack of trays (block). Two stacks of trays were randomly selected every month for sampling in order to estimate the growth and mortality, and to diagnose pathological conditions. One of the two spat containers from each family in each stack was used to estimate mortality by counting dead and live individuals, whereas the other spat container of each family was used to estimate growth by measuring size and weight of ten randomly selected individuals. Five individuals from the latter ten were randomly chosen for disease diagnosis (see below). The number of individuals per container was reduced as oysters grew, and thus the number of trays increased. The use of spat containers ceased in June 2002 and oysters were directly set in the trays to favour water renewal through oysters. As a consequence, the small oysters that were not retained in the 2-cm-mesh trays were set apart and no longer considered in the study. Size sorting and setting ''tails'' apart is a common practice in oyster farming. Because no significant difference either in growth or mortality had been found between stacks of trays (blocks), oyster rearrangement also involved setting oysters of the same family in the same stack of trays. Consequently, sampling design was also modified into a hierarchical design, with families nested under origins. One stack of trays of each family was marked for sampling. The upper tray in the stack was used for monthly mortality estimates. The oyster-culture trays have two internal cross-dividers splitting the trays into four equal portions. Two replicates (two halves of the upper tray) were considered for monthly mortality estimations by counting dead and live oysters in each half. The height of the shell of dead individuals was measured. Six oysters were randomly taken monthly from the other trays in the stack for disease diagnosis (see below). Two samples of ten oysters each were randomly taken quarterly from the trays in the stack to estimate growth by measuring their height and whole weight. Oyster density in the trays was adjusted quarterly, being the same for every family, from 200 ind. per tray in June 2002 to a final density of 60 ind. per tray. Sampling finished in September 2003, after 2 years of raft culture. The families IR1, IR2 and IR4 reached 100% cumulative mortality before the end of the experiment, and thus only 14, 15 and 18 monthly samples could be taken, respectively. Seawater temperature and salinity in the raft area at 0–5 m depth ranged from 9 to 19°C and 25.5 to 35.6 PSU, respectively (data supplied by the Programme of Monitoring of Phytoplankton and Oceanographic Conditions of the ''Centro de Control do Medio Mariño'').

Analysis of pathological conditions

The occurrence of pathological conditions was evaluated by examining histological sections (HS). Furthermore, when the oysters were large enough to allow haemolymph withdrawal (from September 2002), bonamiosis was diagnosed by examining haemolymph cell monolayers (HCM), which has proved to be more sensitive than examining HS (da Silva and Villalba 2004). Sampled oysters were processed by first withdrawing haemolymph to perform haemolymph cell monolayers, and then excising a section of meat to produce histological sections stained with Harris' hematoxylin and eosin, as described by da Silva and Villalba (2004). Additional histological sections were made from some oysters to perform Feulgen picromethyl blue staining (Howard and Smith 1983). The monthly prevalence of *B. ostreae*, symbionts and other pathological conditions was calculated as the percentage of affected oysters in each monthly sample. The estimation of *B. ostreae* infection intensity involved examination of HS and ranking each oyster using the scale proposed by da Silva and Villalba (2004): null, light, moderate and heavy infection. The oysters in which infection was detected in HCM but was not in HS were allocated to the light infection class.

An index of the overall incidence of pathological conditions (OIPC) was obtained for each family as follows: the mean prevalence of each symbiont and

pathological condition in each family for the whole study period was calculated. Then, families were ranked for each symbiont and pathological condition, from the lowest to the highest mean prevalence. Families IR1, IR2 and IR4 were excluded from the ranking because they reached 100% cumulative mortality before the end of the study, and thus samples of the last months could not be taken from these families. The OIPC of each family was calculated as its mean rank. Similarly, the OIPC corresponding to each origin was calculated.

Statistical analysis

The mean values of whole weight and shell height and the percentage of cumulative mortality were analysed by a three-factor ANOVA (stack of trays as a block, origin as main factor, and family nested under origins, Sept 01–Jun 02) or by a two-factor nested ANOVA (origin as main factor and family nested under origins, Jul 02–Sept 03) with replicates (GLM ANOVA; MINITAB 13.1 software). The percentages of cumulative mortality data were arcsin-transformed to meet homogeneity requirements before ANOVAs. The differences in prevalence of *B. ostreae* between families were analysed using a Friedman test, in which the treatments were the families and the blocks were the months, followed by paired comparisons (Conover 1999). The differences in prevalence of *B. ostreae* between origins were analysed by calculating a mean prevalence for each origin every month and then using a Friedman test as previously. The differences in infection intensity between origins were analysed by *G*-tests of independence with contingency tables (Sokal and Rohlf 1981). Families IR1, IR2 and IR4 were excluded from the comparison of prevalence and intensity of infection by *B. ostreae* because they reached 100% cumulative mortality before the end of the study, and thus samples of the last months (when the prevalence and intensity of bonamiosis were higher) could not be taken from these families. The differences in OIPC between origins and between families were analysed by the Kruskal-Wallis test followed by paired comparisons using the Fisher's LSD procedure (MINITAB 13.1 software). Correlation between cumulative mortality and OIPC was estimated using the Spearman's rank correlation coefficient (r_S) (MINITAB 13.1 software).

Results

Growth and mortality

Figure 1 shows the monthly mean values of whole weight of oysters from different origins and families through the on-growing period. There were significant differences between families in both height and weight when spat was transferred from the nursery to the raft, but the differences between origins were not significant. Growth was very slow up to size sorting in June 2002. Never-

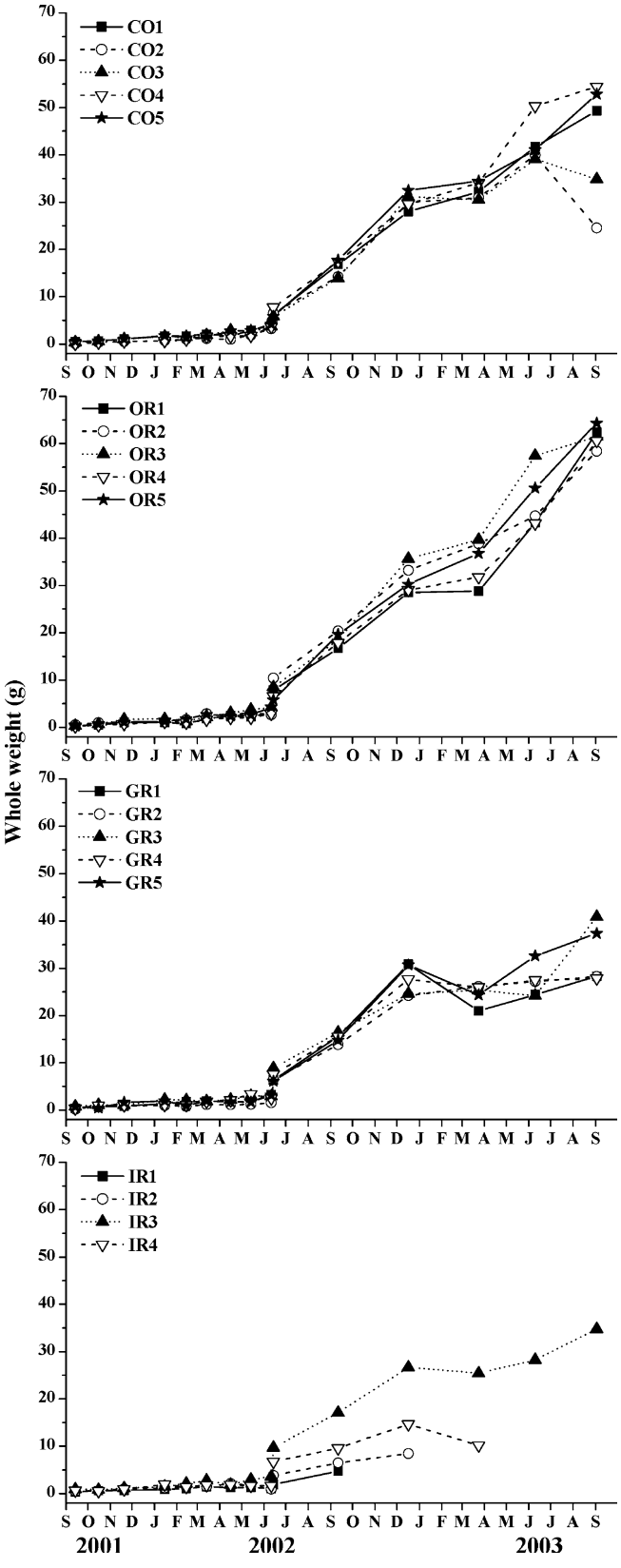

Fig. 1 Monthly variation of the mean whole weight of oysters of each family from every origin through on-growing. Two estimates were made in June 2002, immediately before and after size sorting (removing "tails"). Families of each origin are identified by numbers (CO Coroso; OR Ortigueira; GR Greece; IR Ireland)

theless, significant differences in both height and weight were detected between origins ($F = 6.02$, $P = 0.007$ and $F = 5.63$, $P = 0.009$, respectively) and families ($F = 3.38$, $P < 0.001$ and $F = 1.78$, $P = 0.035$, respectively). Growth in weight was faster in summer and autumn 2002. It slowed down or ceased in winter and even weight decrease was observed in some families. Growth was reassumed in spring and continued through summer 2003 in OR and some CO families, whereas it was slower in GR families. IR oysters had the slowest growth when they were represented, except family IR3, which showed a growth pattern similar to the GR families. The final mean size of all OR families was similar and corresponded to 16 oysters per kg in market terms (Table 1). Three CO families attained a mean size corresponding to 20 oysters per kg, but that of the other 2 CO families was below the minimum market size (MMS, 60 mm). The final mean size of GR families was either slightly above or below the MMS. Family IR3 hardly exceeded the MMS, whereas the growth of the other IR families was almost negligible. Differences in height and weight were significant between origins ($F = 6.39$, $P = 0.008$ and $F = 12.84$, $P < 0.001$, respectively) and families ($F = 8.34$, $P < 0.001$ and $F = 4.86$, $P < 0.001$, respectively) at the end of the study.

Marked differences in temporal mortality patterns between origins and between families under origins were recorded through the study (Fig. 2). High mortality affected early IR families, more heavily in the period April–August 2002 and again in April 2003, and thus three of them reached 100% cumulative mortality before the end of the study. Mortality of GR oysters was high in the period winter-spring 2002 and throughout 2003. Two OR families (OR1 and OR2) suffered much higher mortalities in the first on-growing year than the other three OR families, whereas the differences were not so marked in the 2nd year. Mortality was higher in the periods mid-spring/summer in the CO oysters, being higher in 2002 for families CO1, CO2 and CO4 and in 2003 for CO3. Families IR3, CO2, CO3 and GR5 showed marked deviations from the other families of their respective origins (Fig. 2). At the end of the study, the mean cumulative mortality corresponding to each origin was 93.4% (IR), 72.2% (GR), 59.4% (CO) and 51.2% (OR). The lowest cumulative mortality after 2 years of on-growing corresponded to family CO5 (25%). Three families of each of the Galician origins showed cumulative mortalities below 50%, whereas only one GR family and no IR did. Differences between origins and families were significant ($F = 3.71$, $P = 0.035$ and $F = 26.77$, $P < 0.001$, respectively).

Pathological conditions

B. ostreae was detected in 144 oysters through the study, from which 28 (19.4%) were found infected by HCM only. Spherical 2- to 4-μm *B. ostreae* cells with an eccentric nucleus were found inside haemocytes and free in the connective tissue (HS) and haemolymph (HCM) of the infected oysters. Different degrees of haemocytic infiltration were observed according to disease intensity. The monthly variation of the prevalence of *B. ostreae* in the families and that of the mean prevalence of every origin through on-growing are shown in Figs. 3 and 4, respectively. This parasite was first detected in CO oysters in September 2002. The earliest detection in GR oysters occurred in October 2002, November 2002 in IR oysters, and January 2003 in OR oysters. The prevalence increased through winter in GR oysters, but stayed low in oysters from the other origins, and reached high levels in spring and summer 2003. On average, prevalence was higher in GR oysters, followed by CO, IR and OR oysters. Differences were significant both between origins ($P < 0.001$) and families ($P < 0.001$), including remarkable differences between families under GR and CO origins. The paired comparisons allowed us to allocate the families into three groups (Table 2), CO3 being the family with the highest mean prevalence of *B. ostreae*, followed by GR families, except GR5, and then the remaining families (Table 2). Family CO3 showed the earliest case of infection and the highest values of average prevalence, monthly record of prevalence and number of monthly samples in which the parasite was detected (Table 2). Conversely, families OR1 and CO5 showed the lowest value of average prevalence (Table 2). Families IR1, IR2 and IR4 should not be considered in this context because of the lack of samples from the months with higher prevalence of *B. ostreae*. Paired comparisons between origins showed that differences were significant in each case, except between IR and OR. Considering the infected oysters only, over the study

Table 1 Mean oyster size (height and whole weight, ±SE) corresponding to each origin (*CO* Coroso; *GR* Greece; *IR* Ireland; *OR* Ortigueira) at four sampling times: the beginning of on-growing, immediately before and after removing "tails" and the end of the study

Sampling time	Origin			
	IR	GR	CO	OR
September 2001	17.6 ± 0.82 mm	18.1 ± 0.95 mm	13.4 ± 0.76 mm	13.9 ± 0.61 mm
(transference of seed to raft)	0.57 ± 0.061 g	0.50 ± 0.047 g	0.33 ± 0.046 g	0.30 ± 0.030 g
June 2002	23.3 ± 1.05 mm	28.4 ± 1.02 mm	32.5 ± 0.89 mm	32.6 ± 1.03 mm
(before removing "tails")	1.80 ± 0.261 g	2.71 ± 0.255 g	3.76 ± 0.259 g	3.42 ± 0.299 g
June 2002	36.0 ± 1.58 mm	41.0 ± 1.01 mm	40.3 ± 1.07 mm	42.8 ± 1.22 mm
(after removing "tails")	5.54 ± 0.656 g	7.09 ± 0.503 g	6.44 ± 0.485 g	7.83 ± 0.650 g
September 2003	60.5 ± 1.96 mm	58.8 ± 0.71 mm	63.5 ± 1.29 mm	73.0 ± 0.89 mm
	34.77 ± 3.65 g	32.61 ± 1.05 g	44.81 ± 1.92 g	61.4 ± 1.96 g

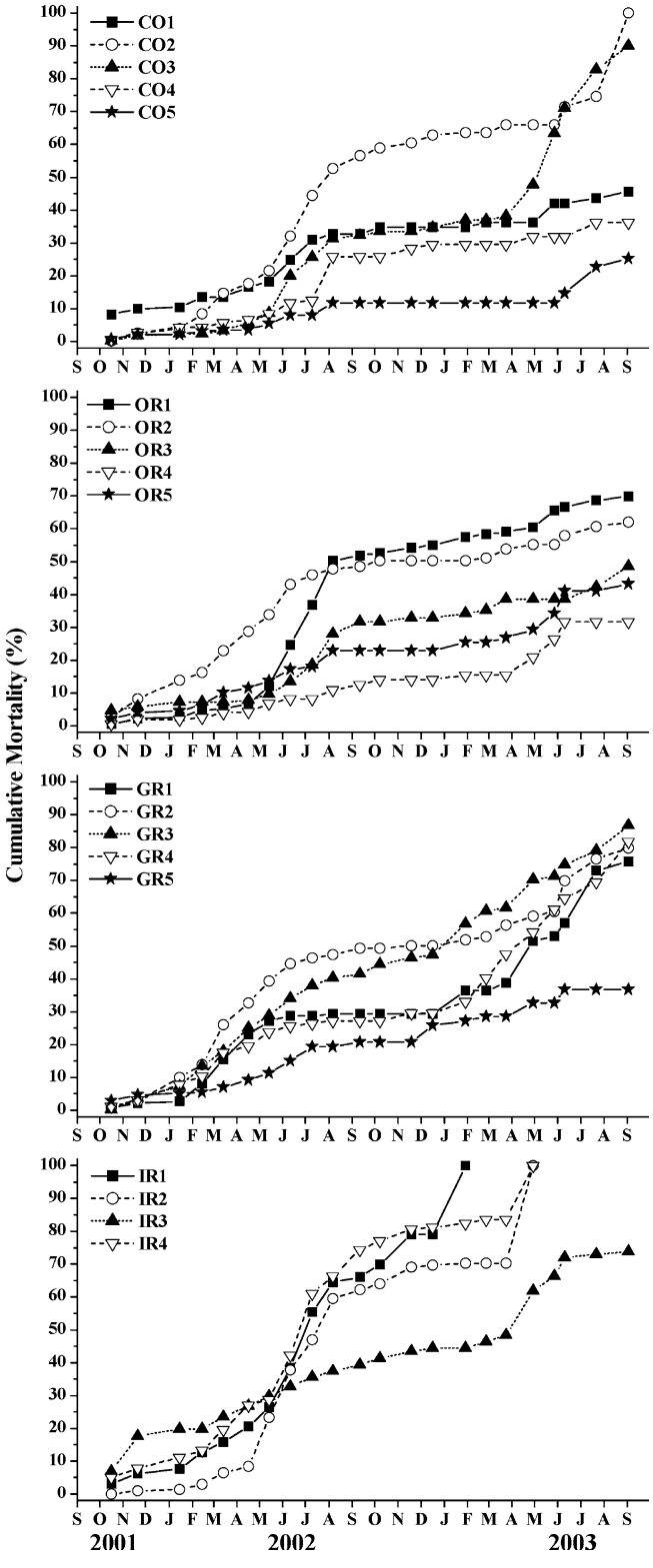

Fig. 2 Progression of cumulative mortality corresponding to each family from every origin through on-growing. Families of each origin are identified by numbers (CO Coroso; OR Ortigueira; GR Greece; IR Ireland)

period, the lowest percentage of light infections and the highest of heavy infections corresponded to CO oysters whereas the highest percentage of light infections corresponded to OR oysters (Fig. 5). However, differences in infection intensity between origins were not statistically significant, likely because of the low number of individuals of some origins in some infection-intensity classes.

Various symbionts and pathological conditions different from bonamiosis were also found through the study (Table 2). One of the pathological conditions involved the occurrence of abnormal cells in the connective tissue of different organs, with an intranuclear eosinophilic large inclusion and marginated chromatin in the nucleus. The intranuclear inclusions were Feulgen-positive. Those abnormal cells were very abundant, which led to the total disappearance of vesicular cells, but without triggering a heavy haemocytic infiltration. This condition was only detected in the first 11 months of on-growing, except for one case in March 2003. It was found in oysters from every origin but not from every family, IR1 and CO2 being the most affected families (Table 2).

Colonies of Rickettsia-like organisms (RLO) were found in cells of the digestive diverticula. They appeared in sections as oval or kidney-shaped intracytoplasmic inclusions, 10–50 μm long, with different degrees of basophilia and granulated texture. Few RLO inclusions were observed in the sections of affected oysters and no haemocytic reaction was detected. RLOs were more frequent through the 2nd year of on-growing. They were observed in oysters from every origin but not from every family (Table 2). Remarkably, RLOs were detected in one oyster from OR in the first sampling and never again in oysters from that origin.

Plasmodia of *Haplosporidium armoricanum* were found scattered in the connective tissue of the visceral mass and gills. They appeared as spherical to elongated, eosinophilic cells, 3–30 μm long, and enclosing 2–20 nuclei. Infection intensity was low in most cases. In one oyster of the family GR4, typical ovoid haplosporidian spores, with an operculum, filled the connective tissue of all organs, thus replacing the normal tissue components. Abundant spores (3×5 μm), each with two filaments in opposition at each end, were observed in the haemolymph of that oyster. The prevalence was low and most cases were found in spring 2002 and 2003. Infection by *H. armoricanum* was more frequent in IR oysters and no case was found in CO families (Table 2).

Two types of ciliates were found. Ellipsoidal 20- to 40-μm-long ciliates were observed in the lumen of the digestive tubules. Only a few of them per oyster section were observed, sometimes in the same tubule, and they did not evoke haemocytic reaction. These ciliates were found every month through the study and the highest prevalence corresponded to September 2001 samples, just before deployment at the on-growing site of the oyster spat from the nursery. These ciliates were detected in every family and, on average, they were more prevalent in GR oysters (Table 2). In addition, pear-shaped to oval, ca. 20-μm-long ciliates were found wandering on gill filaments, gill water tubes and labial palps. They

were detected in all but the three first samplings and affected all the families, except GR5. On average, their prevalence was lower than that of ciliates occurring in digestive tubules (Table 2).

Copepods were observed in the intestine lumen of some oysters. More than one copepod were seldom seen in the same oyster section. The prevalence was low through the study, and even lower in the periods May-September 2002 and 2003. No copepod was found in the families OR3 and CO5 (Table 2).

Numerous cases of disseminated neoplasia were found in every family. This pathological condition was characterised by the proliferation of abnormal enlarged cells with a high nucleus/cytoplasm ratio infiltrating the connective tissue of different organs. The neoplastic cells were round with a round or pleomorphic, 4.5- to 7-μm-long nucleus enclosing a patent nucleolus. Different stages of disease progression were detected, from a few abnormal cells occurring in the connective tissue below the stomach or intestine epithelium to heavy infiltration of the connective tissue of every organ by concurrent neoplastic cells with abundant mitotic figures. Disseminated neoplasia was detected in all but the three first samplings. The prevalence was higher (up to 100% in CO3) in the periods April to July of 2002 and 2003, especially in the latter. All the families were affected (Table 2).

Another pathological condition involved extensive gill lesions, characterised by the presence of unusually abundant pycnotic nuclei (showing chromatin condensation or karyorrhexis) in the branchial connective and epithelial tissues, associated with haemocytic infiltration of the affected area. This condition was also observed in the mantle in a few cases. No etiological agent was identified. This condition was detected in some families of every origin, but in few (up to three) monthly samples of each family (Table 2) that were distributed from May to August 2002 and April 2003.

Unspecific histopathological symptoms of stress were also observed in every family (Table 2). One of them involved haemocytic infiltration of tissues, mainly the connective tissue, of different organs. Another symptom involved concentration of infiltrating haemocytes in foci with destruction of normal tissue and organ structure, forming the so-called granulocytomas.

Trophozoites of the flagellate protistan *Hexamita* sp. were detected in the haemolymph of eight oysters, of which seven were Greek *B. ostreae*-infected oysters and 1 was an Irish *B. ostreae*-free oyster. However, *Hexamita* sp. has not been included in comparisons because haemolymph examination was only performed from September 2002.

The OIPCs corresponding to each origin and family are shown in Table 2. Differences in OIPC between origins and between families were significant ($P = 0.050$ and $P = 0.023$, respectively). OIPCs of the oysters corresponding to foreign origins were higher than those of the Galician oysters. Paired comparisons grouped GR families (except GR5) and CO3 as families with higher OIPC. The correlation between the cumulative mortality of each family at the end of the study and the OIPC was statistically significant ($r_s = 0.81$, $P < 0.001$).

Discussion and conclusions

The comparison of the growth and mortality of oysters of 19 families derived from 4 different origins through on-growing in a *B. ostreae*-affected area in the Ría de Arousa, revealed significant differences between origins and between families under origins. Oysters of Galician origin grew faster, specially those of OR families. The poor growth of all families before size sorting was probably due to a too-high stocking density and poor water renewal because of the small mesh size of the spat containers. It could have been improved by decreasing stocking density. A broad variability in the final cumulative mortality and in the temporal pattern of mortality progression was observed between origins and families. The high mortality and poor growth of oysters derived from foreign origins reveals a lack of adaptation to the Galician coast environment, with the exception of family GR5, which showed much lower mortality but slow growth. The lower average mortality and faster growth of oysters from Galician origins would indicate a better adaptation. Nevertheless, two families of each Galician origin showed high cumulative mortality, especially CO2 and CO3. Conversely, the final cumulative mortality after 2 years of on-growing was below 50% in three families of each Galician origin, which could mean a profitable survival for the Galician oyster industry.

Best performance, that is, higher growth and lower mortality, corresponded to three families of Galician origin (OR3, OR4 and OR5). Their performance was similar to those reported from hatchery-produced oyster spat that were on-grown in the Ría de Ares-Betanzos (Galicia, Spain) in 1979–1980 (Otero 1984) and in the Ría de Arousa in 1980–1981 (González and González 1985), when bonamiosis had not yet been established in those areas. Montes et al. (2003) evaluated the performance of various batches of hatchery-produced oyster spat (using Coroso oysters as broodstock) that were on-grown in 1999–2000 near Cambados (the same area as in our study, i.e. a *B. ostreae*-affected area) and in an area of the Ría de Pontevedra (southwest of Galicia, Spain) free of *B. ostreae* at that time. These authors reported slower growth and much higher mortality in the affected area (96–100% vs 20–36% cumulative mortality, in the last 18 months of on-growing). The performance of the three best OR families recorded in our study was similar to that reported by Montes et al. (2003) from the *B. ostreae*-free area.

The results of this study support the association of bonamiosis with oyster mortality stated in previous studies (Tigé et al. 1982; Montes et al. 1989; Hudson and Hill 1991; van Banning 1991; Culloty and Mulcahy 2001). Mortality increased when the prevalence of

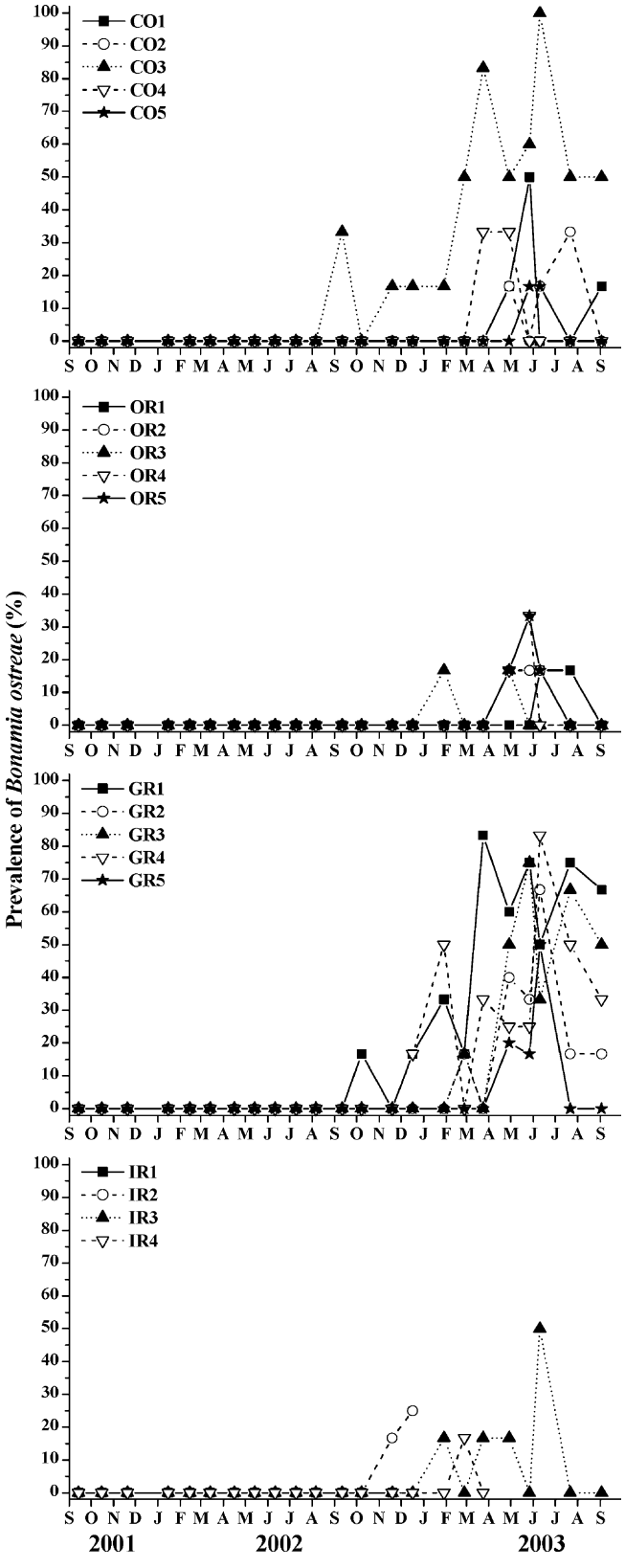

Fig. 3 Monthly variation of the prevalence of *Bonamia ostreae* in each family from every origin through on-growing. Families of each origin are identified by numbers (CO Coroso; OR Ortigueira; GR Greece; IR Ireland)

B. ostreae rose in the families with higher prevalence from each origin (IR3, GR1–GR4, OR4, OR5, CO2, CO3). Furthermore, in the families suffering longer periods of high prevalence (GR1–GR4 and CO3), the cumulative mortality corresponding to those periods was very high. The infection dynamics involved initial detection after 12–16 months of on-growing (18–24 months old), depending on origins; the highest values of mean prevalence in each origin were recorded around the end of the following spring. The decrease of prevalence in summer was probably due to the death of heavily infected oysters. This temporal dynamics is similar to that reported previously from Galicia (Montes et al. 1989, 2003). Tigé et al. (1982) detected the infection with histological techniques in less than 18-months-old oysters (that they called "naissan"). No obvious seasonality of the infection dynamics has been found in Irish oyster beds; instead, continual cycles of infection and mortality occurred throughout the year (Culloty and Mulcahy 2001), which is in agreement with the report of occurrence of infestation all year round in Brittany (France) (Tigé and Grizel 1984). Once again significant differences were found between origins and between families under origins, families CO3 and GR5 being markedly different from the other families of their respective origins. The GR families, except GR5, exhibited a high susceptibility to bonamiosis. In contrast, all the OR families evidenced low susceptibility since they showed low prevalence but also low intensity in the infected oysters. The families with the highest (CO3) and the lowest (CO5) mean prevalence in the study had the same origin, which confirms that there can be a broad range of variability in susceptibility to bonamiosis even within an oyster population. Excluding CO3, prevalence of CO families was not far from that of OR families. No definitive conclusion on the susceptibility of Irish oysters could be obtained because of the lack of family replicates through most of the period of occurrence of bonamiosis. Family IR3, which was the best-performing Irish family, showed a mean prevalence higher but not significantly different from those of Galician families (excluding CO3). The lower susceptibility of OR families could be due to the longer exposure of the original population to bonamiosis, which could have allowed natural selection to operate, favouring less susceptible oysters. Bonamiosis has been detected in that area since 1982 (Polanco et al. 1984). Elston et al. (1987) suggested naturally acquired resistance to bonamiosis in a population of *Ostrea edulis* as a consequence of long exposure (at least 20 years) to the disease. Conversely, bonamiosis has not been reported from Greece thus far (Anonymous 2002), in spite of oyster pathological surveys that have been performed looking for causes of oyster mortality (Virvilis et al. 2003). Therefore, there is no evidence that the population from which the GR families derived has been exposed to bonamiosis.

This study revealed the occurrence of other symbionts and pathological conditions affecting oysters through on-growing in the Ría de Arousa. They are

categorised according to their pathogenicity. The first group consisted of symbionts and an abnormal condition with an unnoticeable (or very mild at most) pathogenic effect: colonies of RLO in the digestive gland, ciliates, copepods and gill lesions. The RLO detected in the digestive gland strongly resembled those reported from flat oysters by Comps et al. (1979) and from other bivalve species (Harshbarger et al. 1977; Bower et al. 1994). Nevertheless, the lack of an ultrastructural study prevents a precise taxonomic allocation. The effects of RLO located in the digestive gland of molluscs have been found to be very mildly detrimental or undetectable, and were restricted to degeneration of the infected cell (Comps et al. 1979), with the exception of *Candidatus Xenohaliotis californiensis*, a rickettsia blamed for the withering syndrome in abalones (Moore et al. 2001). However, rickettsia-like infections have been associated with mortalities in bivalve populations (Gulka and Chang 1984; Elston 1986; Le Gall et al. 1988; Norton et al. 1993; Villalba et al. 1999).

The ciliates observed in the filter-feeding and digestive organs could be considered as commensals rather than parasites, although they could have an effect on oysters under adverse growing conditions (Bower et al. 1994). Those occurring in digestive tubules resembled *Ancistrocoma*-like ciliates described in various oyster species (van Banning 1979a; Bower et al. 1994). Their high prevalence recorded in the spat before being deployed in the Ría de Arousa could be a signal of high stress conditions in the nursery facilities. The ciliates occurring on gills and labial palps resembled *Sphenophrya*-like ciliates found in various oyster species (Bower et al. 1994).

Copepods were frequently observed in the digestive system of *O. edulis* cultured in France (His 1979). However, the occurrence of copepods was not mentioned by Figueras (1991) in his report of a pathological survey of oysters cultured in the Ría de Vigo (Galicia, Spain). If copepods had occurred in his samples, they could not have gone unnoticed. That absence in Figueras's report and the low copepod prevalence recorded in this study contrast with the higher prevalence of the copepod *Mytilicola intestinalis* in cultured mussels *Mytilus galloprovincialis* in both Rías de Vigo and Arousa (Villalba et al. 1997). The susceptibility of *O. edulis* to *Mytilicola intestinalis* was experimentally demonstrated by Dare (1982).

The occurrence of unusually abundant picnotic nuclei in the gill lesions detected in the study could be interpreted as a manifestation of an abnormally high number of apoptotic cells. Apoptosis was further confirmed through transmission electron microscopy and in-situ TUNEL technique, which is dealt with elsewhere (da Silva et al., unpublished work). The cause of the increase of apoptotic cells remains unknown.

The other group of pathological conditions consisted of a viral infection, the parasitisation by *Haplosporidium armoricanum* and a neoplastic condition. The extent of histological lesions in the heavy-intensity cases suggests that these three conditions could be fatal. The morphological features of the abnormal cells with intranuclear eosinophilic Feulgen-positive inclusions closely resembled those of the herpes-like virus-infected cells that were reported from the oyster species *O. edulis* (Comps and Cochennec 1993) and *O. angasi* (Hine and Thorne 1997). Transmission electron microscopy has recently confirmed that the abnormal cells contained herpes virus-like particles (da Silva et al., unpublished work). This pathological condition was mostly detected in young oysters, with higher prevalence in the IR origin. Looking at each origin, the families with higher prevalence of this condition showed higher cumulative mortality at the end of the 1st year of on-growing. It was specially noticeable in the case of family CO2, which showed markedly higher prevalence and cumulative mortality at the end of the 1st year than the other families of that origin. Conversely, the families in which the condition was not detected showed lower cumulative mortality at the end of the 1st year than the other families of the same origin. Possibly, this pathological condition was responsible for oyster mortality to some extent throughout the 1st year of on-growing, while bonamiosis was not detected. Herpes-like viruses have been blamed for mortality in larvae and spat of *O. edulis* (Comps and Cochennec 1993; Renault et al. 2000) and other bivalve mollusc species (Renault et al. 1994; Hine et al. 1998; Arzul et al. 2001a, 2001b). This pathological condition had not been reported previously in oysters *O. edulis* from the Spanish coast, and thus it can be considered as a new threat for the oyster industry of the country.

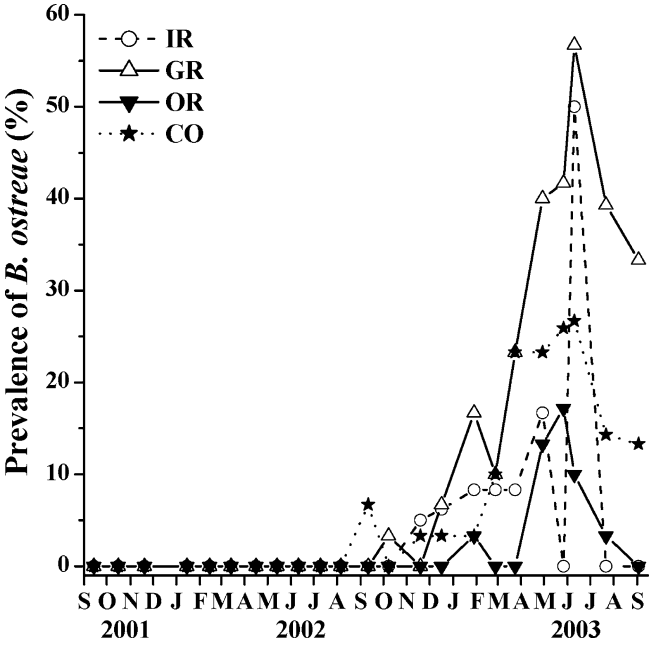

Fig. 4 Monthly variation of the mean prevalence of *Bonamia ostreae* in each origin through on-growing (CO Coroso; OR Ortigueira; GR Greece; IR Ireland)

Table 2 Symbionts and pathological conditions found in the different origins and families of *Ostrea edulis* through the study (*A* mean prevalence (%) through the study period; *B* highest monthly record of prevalence (%);*C* number of monthly samples in which each symbiont and pathological condition was detected). The overall incidence of pathological conditions (*OIPC*) is shown at the bottom (*ms* number of monthly samples examined from each origin (CO Coroso; GR Greece; IR Ireland; OR Ortigueira) and family (*1–5*); number of oysters examined from each origin and family). Paired comparisons for prevalence of *Bonamia ostreae* and OIPC: different *lower-case letters* indicate significant ($P < 0.05$) differences between families and different *upper-case letters* indicate significant ($P < 0.05$) differences between origins. The families IR1, IR2, and IR4 were not included in the comparison of the prevalence of *B. ostreae* and OIPC because they reached 100% cumulative mortality before the end of the study

		Families and origins																					
		GR1 ms = 23 n = 169	GR2 ms = 23 n = 173	GR3 ms = 23 n = 171	GR4 ms = 23 n = 170	GR5 ms = 23 n = 173	GR n = 856	IR1 ms = 14 n = 110	IR2 ms = 15 n = 116	IR3 ms = 23 n = 179	IR4 ms = 18 n = 143	IR n = 548	OR1 ms = 23 n = 172	OR2 ms = 23 n = 174	OR3 ms = 22 n = 164	OR4 ms = 23 n = 166	OR5 ms = 23 n = 168	OR n = 844	CO1 ms = 23 n = 174	CO2 ms = 23 n = 170	CO3 ms = 23 n = 171	CO4 ms = 23 n = 173	CO5 ms = 23 n = 174	CO n = 862
Bonamia ostreae	A	21.4 ab	8.3b	12.7b	13.8b	3.8c	12.0 A	0	2.8	4.4c	1.0	2.0 C	1.4c	2.2c	1.5c	2.2c	2.9c	2.0 C	3.6c	2.9c	22.9a	2.9c	1.4c	6.8 B
	B	83.3	66.7	75	83.3	50		0	25	50	16.7		16.7	16.7	16.7	33.3	33.3		50	33.3	100	33.3	16.7	
	C	10	6	6	8	3	10	0	2	4	1	7	2	3	2	2	3	5	3	3	11	2	2	11
Intranuclear inclusions	A	0.4	0	1.3	0.4	0	0.4	6.2	1.7	0	1.7	2.4	1.3	1.3	1.2	0	0	0.8	0.9	5.4	0.9	0	0	1.3
	B	10	0	20	10	0		30	25	0	20		10	20	16.7	0	0		10	30	10	0	0	
	C	1	0	2	1	0	3	6	1	0	2	7	3	2	2	0	0	5	2	6	2	0	0	7
Ricketti sia-like organisms	A	0	3.5	1.9	2.2	0	1.5	1.4	0	1.9	0	0.8	0	0	0	0.4	0	0.1	1.4	4.3	0	4.3	1.2	2.2
	B	0	20	16.7	25.0	0		10	0	16.7	0		0	0	0	10	0		16.7	33.3	0	33.3	16.7	
	C	0	5	3	3	0	7	2	0	3	0	5	0	0	0	1	0	1	2	5	0	4	2	8
Haplos poridium armori canum	A	0	1.4	0	1.8	0	0.6	2.1	1.3	0	4.1	1.9	0.7	2.2	0	0	0	0.6	0	0	0	0	0	0
	B	0	16.7	0	25	0		30	20	0	20		16.7	16.7	0	0	0		0	0	0	0	0	
	C	0	2	0	2	0	4	1	1	0	5	5	1	3	0	0	0	3	0	0	0	0	0	0
Ciliates in digestive gland lumen	A	19.0	7.0	21.2	24.0	15.2	17.3	15.3	16.4	4.1	22.2	14.5	13.0	9.1	15.3	15.8	16.2	13.9	11.7	2.6	3.8	13.6	15.1	9.4
	B	75	20	100	66.7	50		50	66.7	40	60		50	50	90	100	80		66.7	22.2	40	66.7	50	
	C	15	10	17	18	15	22	8	8	4	14	17	13	8	12	11	11	21	10	3	4	11	14	19
Ciliates in gills	A	8.0	6.2	6.0	1.8	0	4.4	16.1	11.3	6.2	4.6	9.6	6.0	9.7	6.7	7.1	4.1	6.7	5.1	2.6	2.9	14.3	5.5	6.1
	B	50	33.3	33.3	25	0		75	50	33.3	30		33.3	33.3	33.3	33.3	16.7		33.3	16.7	16.7	50	33.3	
	C	7	7	7	2	0	12	7	7	8	4	15	8	13	8	7	6	17	8	4	4	11	7	16
Copepods	A	1.2	1.2	2.3	2.2	5.8	2.5	2.9	3.8	1.2	2.0	2.5	2.0	2.3	0	0.7	1.2	1.2	2.2	0.9	2.0	0.4	0	1.1
	B	16.7	16.7	16.7	25	33.3		20	16.7	16.7	16.7		20	16.7	0	16.7	16.7		16.7	10	16.7	10	0	
	C	2	2	4	3	7	12	3	5	2	3	6	3	4	0	1	2	8	3	2	4	1	0	8
Disseminated neoplasia	A	12.8	16.6	8.6	10.4	6.4	11.0	3.9	4.3	4.9	12.8	6.5	10.4	4.8	7.0	7.4	8.7	7.7	7.4	13.9	17.2	6.7	7.2	10.5
	B	60	66.7	33.3	66.7	33.3		20	30	33.3	70		50	33.3	66.7	66.7	50		50	66.7	100	40	50	
	C	8	11	10	8	6	16	3	3	7	8	12	8	6	6	6	7	14	7	10	11	7	7	16
Gill lesions	A	0.7	0.7	0.7	2.2	1.4	1.2	2.4	1.1	2.9	0	1.6	0.7	0.9	2.7	1.4	0.7	1.3	0	0	1.4	0	1.4	0.6
	B	16.7	16.7	16.7	50	33.3		33.3	16.7	33.3	0		16.7	10	33.3	33.3	16.7		0	0	33.3	0	16.7	
	C	1	1	1	1	1	2	1	1	2	0	3	1	2	3	1	1	5	0	0	1	0	2	2
Haemocytic infiltration	A	32.2	25.3	31.0	23.7	16.8	25.8	21.6	23.0	15.5	36.1	24.1	18.4	21.2	28.2	13.5	18.1	19.9	17.7	24.4	34.1	20.6	11.9	21.7
	B	100	70	100	83.3	83.3		100	50	50	83.3		66.7	83.3	66.7	100	100		66.7	66.7	100	80	66.7	
	C	16	16	19	15	15	21	8	11	12	16	21	13	14	16	11	9	21	12	17	17	16	10	22
Granulo cytomas	A	14.2	6.1	6.5	11.0	2.0	8.0	0.7	3.8	3.3	11.1	4.7	4.6	4.5	2.3	1.4	3.6	3.3	2.9	2.6	17.5	0	2.9	5.2
	B	75	33.3	50	66.7	16.7		10	20	16.7	50		33.3	50	33.3	16.7	33.3		33.3	16.7	83.3	0	16.7	
	C	8	7	6	9	4	13	1	4	5	7	13	5	3	2	2	3	7	3	4	9	0	4	9
OIPC		10.77 abc	10.09 abcde	11.36 ab	11.86 a	6.64 ef	3.00 A	-	-	7.54 cdef	-	2.91 A	8.54 abcdef	9.27 abcdef	7.91 bcdef	6.82 def	7.09 ef	1.91 B	7.41 cdef	7.82 bcdef	10.27 abcd	6.82 def	5.79 f	2.18 AB

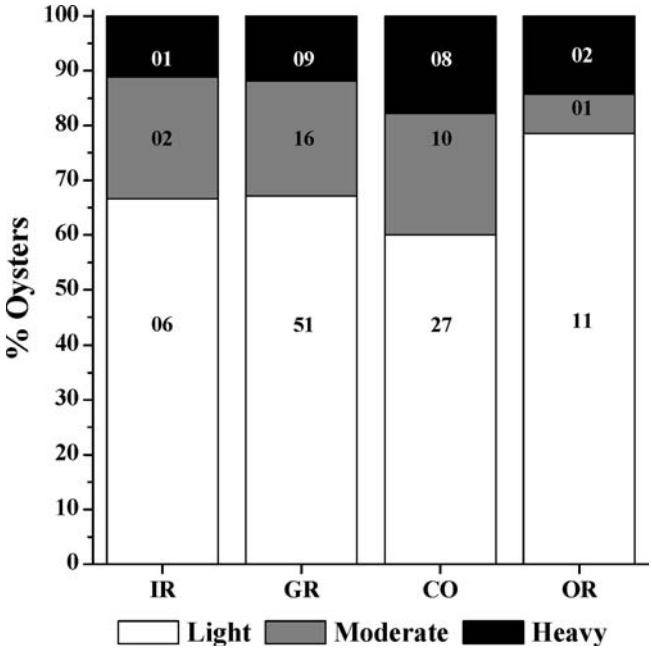

Fig. 5 Distribution of the oysters infected with *Bonamia ostreae* of each origin in classes of infection intensity. The number of oysters in each class of infection intensity is shown in the *bars*. Infection-intensity classes are distinguished with different patterns (CO Coroso; OR Ortigueira; GR Greece; IR Ireland)

The low intensity of the infections by plasmodia of *H. armoricanum* detected through the study would suggest a mild effect, if any, of this parasite on the oyster. However, the only detected case of sporulation involved extreme tissue disruption indicating morbidity. The co-generic species *Haplosporidium nelsoni* and *H. costale* have been blamed for mass mortalities of oysters *Crassostrea virginica* in the Atlantic coast of the United States (Ford and Haskin 1982; Andrews 1984). The first haplosporidian parasite found in *O. edulis* was described as *Minchinia armoricana* (van Banning 1977), and later redescribed as *H. armoricanum* (Azevedo et al. 1999). Since then, other haplosporidian parasites found in *O. edulis* have been reported as *Haplosporidium* sp. (Pichot et al. 1979; Vivarès et al. 1982; Bachère and Grizel 1983), arguing morphological differences with the other species of the genus. The morphological features observed with light microscopy and the histological location of the plasmodia and the spores found in this study match the description of *H. armoricanum* (van Banning 1977). Transmission electron microscopy has recently confirmed that the spores found in this study match the detailed ultrastructural description of *H. armoricanum* reported by Azevedo et al. (1999). The prevalence recorded in the study was low (higher in the IR origin), but seasonality could be detected. If prevalence continues at the same level, this parasite will not threaten oyster farming in Galicia. Very low prevalence was also reported by van Banning (1979b) and Bachère and Grizel (1983) for *O. edulis* infection by *H. armoricanum* and *Haplosporidium* sp., respectively.

The morphological characteristics of the disseminated neoplasia detected in the study were similar to those described in cases of this disease referred to by different names (haemocytic sarcoma, diffuse sarcoma, leukaemia) from various bivalve mollusc species (reviewed by Peters 1988; Elston et al. 1992), including *O. edulis* (Alderman et al. 1977; Balouet et al. 1986). This pathological condition reached epizootic levels and was associated with high mortality in populations of various mollusc species (Peters 1988; Elston et al. 1992). This condition was detected in every family, with high prevalence in some of them, through the study. Disseminated neoplasia probably contributed to oyster mortality to some extent. High prevalence of this neoplastic condition had been detected already in oysters (*O. edulis*) on-grown in Galicia (Alderman et al. 1977; Figueras 1991), whereas its prevalence was much lower in Brittany (France) (Balouet et al. 1986).

Histological symptoms of stress were more frequent in oysters with a foreign origin, which would support the idea that oysters from those origins suffered higher stress through on-growing in Galicia. The flagellate *Hexamita* sp. was consistently only detected in the haemolymph of GR and IR oysters. Flagellates of several *Hexamita* species are free-living saprobic organisms, affecting oysters as facultative parasites only when the latter are weak or stressed (van Banning 1979a; Lauckner 1983). The OIPC of GR and IR oysters were also significantly higher than those of CO and OR oysters. Again, significant differences were also found between families with the same origin. A significant correlation between the OIPC and the cumulative mortality of the families was noted.

No case of infestation by trematode larvae was detected through the on-growing period. Nor was parasitisation by *Marteilia refringens* found, in spite of the high prevalence (80%) of this protozoan parasite in a sample ($n = 20$) taken from the batch of Greek oysters used as brood stock. Therefore, vertical transmission did not occur. Virvilis et al. (2003) had reported 60% prevalence of *Marteilia refringens* in Greek populations of *O. edulis*. Occurrence of this parasite in oysters in Galicia is rare (Figueras 1991), except when the oysters come already parasitised from affected areas.

In conclusion, the occurrence of variability in productive characters and disease susceptibility of both inter- and intra-oyster populations was proved. This, together with the strong indications of occurrence of natural selection of bonamiosis less-susceptible oysters, should encourage the development of selective breeding programmes to produce resistant strains. On average, oysters from autochthonous origins showed a significantly better performance through on-growing. Nevertheless, the significant variability under origins resulted in some CO families with high mortality and high disease susceptibility and some GR families with low mortality and low disease susceptibility. The results obtained with the best-performing families suggest that the profitability of oyster farming in Galicia would

improve, even under bonamiosis pressure, by using appropriate oyster spat. Bonamiosis caused high mortality in the late stage of oyster on-growing but herpes-like viral infections and disseminated neoplasia could also cause mortality and should be considered in selection programmes.

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