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Differences in gametogenic cycle among strains of the European flat oyster *Ostrea edulis* and relationship between gametogenesis and bonamiosis

Patricia Mirella da Silva, José Fuentes, Antonio Villalba *

Centro de Investigacións Mariñas, Consellería de Pesca e Asuntos Marítimos, Xunta de Galicia, Aptdo. 13, E-36620 Vilanova de Arousa, Spain

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ABSTRACT

An experiment was carried out to compare the performance of various cohorts of oysters *Ostrea edulis* from each of four different geographic origins that were born and cultured in the same environment. This paper reports the variation of gonad condition of the oysters through on growing and analyses of the relationship between gametogenesis and infection by *Bonamia ostreae*. Nineteen cohorts of spat were produced in a hatchery from four oyster populations: Irish, Greek, and two Galician: Ortigueira and Coroso; the spat were transferred to a raft in the Ría de Arousa, an area affected by bonamiosis, for grow-out for two years. Significant differences were observed in the temporal pattern of gonad condition between oysters from different geographic origins. Irish oysters showed a short period of gonad activity and a long gonad resting phase. By contrast, Greek oysters had the longest period of gonad activity and the shortest gonad resting phase, as well as the highest percentage of male oysters compared to oysters from other origins. Galician oysters, from both origins, showed a pattern of gonad development intermediate between Irish and Greek, with a higher percentage of females during the second reproductive period. Significant differences were also observed between cohorts within Greek, Coroso and Irish origin, but not from Ortigueira, whose cohorts showed a similar temporal pattern of gonad condition. The differences between oysters from different origins would indicate adaptation to different environments; nevertheless the significant differences that were found between cohorts within origins would indicate that there is variability that eventually could allow adaptation to new environments. Considering sex categories, heavy infections by *B. ostreae* were significantly more frequent in oysters with a predominant female component; considering gonad stage, heavy infections were significantly more frequent in ripe and partially spawned oysters. The results suggest a hypothetical enhancement of progression of infection by *B. ostreae* associated with female maturation.

1. Introduction

Bonamia ostreae infection — bonamiosis — is an important constraint upon flat oyster *Ostrea edulis* production in Europe since the 1980s (Carnegie and Cochennec-Laureau, 2004). A number of studies in different places have shown that high mortalities due to this infection occur when the oysters are close to reaching (or have reached) market size; at that size both the prevalence and the infection intensity are higher than at previous stages (Tigé et al., 1982; Montes et al., 1989, 1991; Figueras, 1991; Robert et al., 1991; Culloty and Mulcahy, 1996). Lynch et al. (2005) have demonstrated that oysters less than one year old can be infected by the parasite. What factors control this oyster age/size related pattern of disease? Indeed the older and the larger the oyster, the higher probability of having captured infective particles, but some physiological change depending on oyster age could favour the progression of the infection. van Banning (1990) suggested that the oyster gonad cycle influences the infection and that *B. ostreae* has an incubation period in the oyster ovary before the next stage develops in the haemocytic phase; therefore, the infection would not progress before the development of the female gametogenesis. Later investigations, however, did not find differential susceptibility to infection depending on oyster sex (Cáceres-Martínez et al., 1995; Culloty and Mulcahy, 1996). Some authors have suggested that after spawning, oysters become weaker, which may favour progression of the disease caused by *Bonamia* spp. (Hine, 1991; Culloty and Mulcahy, 1996; Jeffs and Hickman, 2000). Despite numerous studies on bonamiosis in flat oysters (see review of Carnegie and Cochennec-Laureau, 2004), there are still doubts on the effects of reproduction on bonamiosis susceptibility.

O. edulis has an unusual reproductive biology; it is a larviparous and protandric species, maturing first as a male and then undergoing a regular rhythm of alternating female and male sexual phases (Sparck, 1925; Orton, 1927, 1933; Cole, 1942). Gametogenesis of both sexes in a single follicle is a common phenomenon in flat oysters, resulting from the changing of sex (Galtsoff, 1964; Pascual et al., 1989). Thus, it is not straightforward to ascertain the reproductive sexual phase of oysters,

* Corresponding author. Tel.: +34 986500155; fax: +34 986506788.
E-mail address: villalba@cimacoron.org (A. Villalba).

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considering that hermaphrodites represent transitional sexual phases (Loosanoff, 1962). Therefore, up to six categories can be considered when assessing the sex condition of oysters at a particular moment: undifferentiated, male, female, predominantly male, predominantly female, and oysters with both sexes represented equally (Mann, 1979; Siddiqui and Ahmed, 2002). When maturity is reached in flat oysters, they can spawn as both male and female during the same reproductive period (Orton, 1927, 1933). Mature spermatozoa are released through the exhalant current; however, mature ovocytes are transported from the exhalant to the inhalant chamber where they are kept, eventually become fertilised and the resulting embryos and larvae are brooded for 7–10 days before they are finally released. Román (1984) studied gametogenesis in *O. edulis* cultured on rafts in Ría de Arousa in Galicia, but he used gonad imprints to determine the oyster gonad condition, which is considered less accurate than using histological sections. Reproduction of bivalve molluscs has been shown to be seriously distressed by some protozoan parasites, which mainly inhibit gametogenesis, such as *Marteilia refringens* in flat oysters *O. edulis* (Robert et al., 1991) and in the mussel *Mytilus galloprovincialis* (Villalba et al., 1993), *Haplosporidium nelsoni* (Ford and Figueras, 1988) and *Perkinsus marinus* (Choi et al., 1994; Kennedy et al., 1995; Paynter, 1996; Dittman et al., 2001) in the American oyster *Crassostrea virginica*, and *Perkinsus olseni* (=*atlanticus*) in *Ruditapes decussatus* (Casas, 2001; Villalba et al., 2004).

In Galicia (NW Spain), bonamiosis is considered a serious obstacle to the development of the oyster industry. Accordingly, the Centro de Investigacións Mariñas (CIMA) decided to develop a selective breeding programme to produce oysters *O. edulis* with increased tolerance to bonamiosis. As a previous stage to this programme, evaluation of the variability of productive traits, disease susceptibility, physiological features and immune capability through oyster populations was performed, because particular populations could be favourable for the programme. Four geographic origins were chosen for variability assessment, including extreme and intermediate locations in the European flat oyster geographic range: Greece (Eastern Mediterranean), Ireland (Northern Atlantic), and two areas in Galicia, Spain. Oysters from those locations were used as broodstocks and 19 cohorts were produced (4 to 5 cohorts from each origin). The evaluation of productive traits and disease susceptibility showed significant differences in growth, mortality and susceptibility to bonamiosis and other diseases, both between origins and between cohorts within origins; these results, with emphasis on bonamiosis, have been published elsewhere (da Silva et al., 2005). Evaluation of the reproductive physiology was also considered in the experimental design because it could contribute to explain the differences in performance that could be found through on growing. This article analyses the differences in gonad cycle between flat oysters with different geographic origins and between cohorts within the same origin, with emphasis in the relationship between gonad condition and bonamiosis.

2. Materials and methods

2.1. Production of oyster cohorts

Four oyster populations were selected as broodstock for the experiments: one in the North of Ireland (IR), where bonamiosis had never been detected (Culloty and Mulcahy, 2001), one in Greece (GR) that has neither been affected (http://www.ices.dk/marineworld/fish-diseases/map8_5.htm), one in Ría de Ortigueira (OR) (Galicia, NW Spain), where the disease occurs since 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 (RF Conchas, pers. comm.). Oysters from each origin were transported to the hatchery facilities of Centro de Investigacións Mariñas (CIMA) in December of 2000 and distributed into 5 trays per origin, with 15–20 individuals per tray (20 oyster trays, total). Oysters were conditioned for 2–3 months in running seawater as described by da Silva et al. (2005), and larvae and spat were reared according to the procedure described by Román (1992). Nineteen oyster cohorts were produced, 5 from each origin, except for IR, from which only 4 cohorts were obtained. Every cohort was kept identified through the experiment and all of them shared a common environment. In September 2001, spat >1 cm in shell height were transferred to a raft located near Cambados (Ría de Arousa, Galicia, NW Spain), an area heavily affected by bonamiosis since the 1980's (Montes et al., 1989), for grow-out. Each month, 10 oysters per cohort were sampled haphazardly until June 2002, and after that date, 6 oysters were sampled monthly (da Silva et al., 2005). The experiment ended in September 2003, after 2 years of raft-culture sampling. Three Irish cohorts (IR1, IR2 and IR4) reached 100% cumulative mortality before the end of the experiment, thus samples from those families were not available in the last months, when bonamiosis was most prevalent.

Values of seawater temperature recorded weekly at the area of raft culture were provided by the "Instituto Tecnolóxico para o Control do Medio Mariño en Galicia" (INTECMAR).

2.2. Light microscopy

Oysters were sampled by first withdrawing haemolymph from the adductor muscle sinus using a 21-gauge needle attached to a 2 ml syringe. Soft tissues were then excised to produce a histological section as follows: each oyster was shucked, and a sagittal, approximately 5-mm thick section containing gill, gonad, digestive gland, and mantle

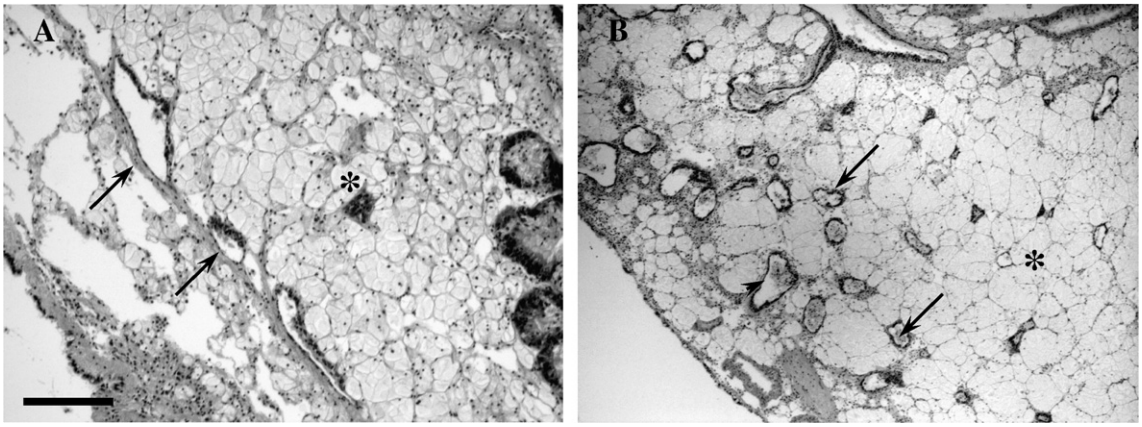

Fig. 1. Micrographs of histological sections of *Ostrea edulis* showing the gonad area in indeterminate sexual phase, gonad inactive or in resting phase. A. Predominance of connective tissue (*); some gonad ducts (arrows) appeared in the periphery of the body. Bar = 100 µm. B. Few small gonad follicles (arrows) surrounded by predominant connective tissue (*). Some phagocytes (arrowheads) appear inside follicles. Bar = 250 µm.

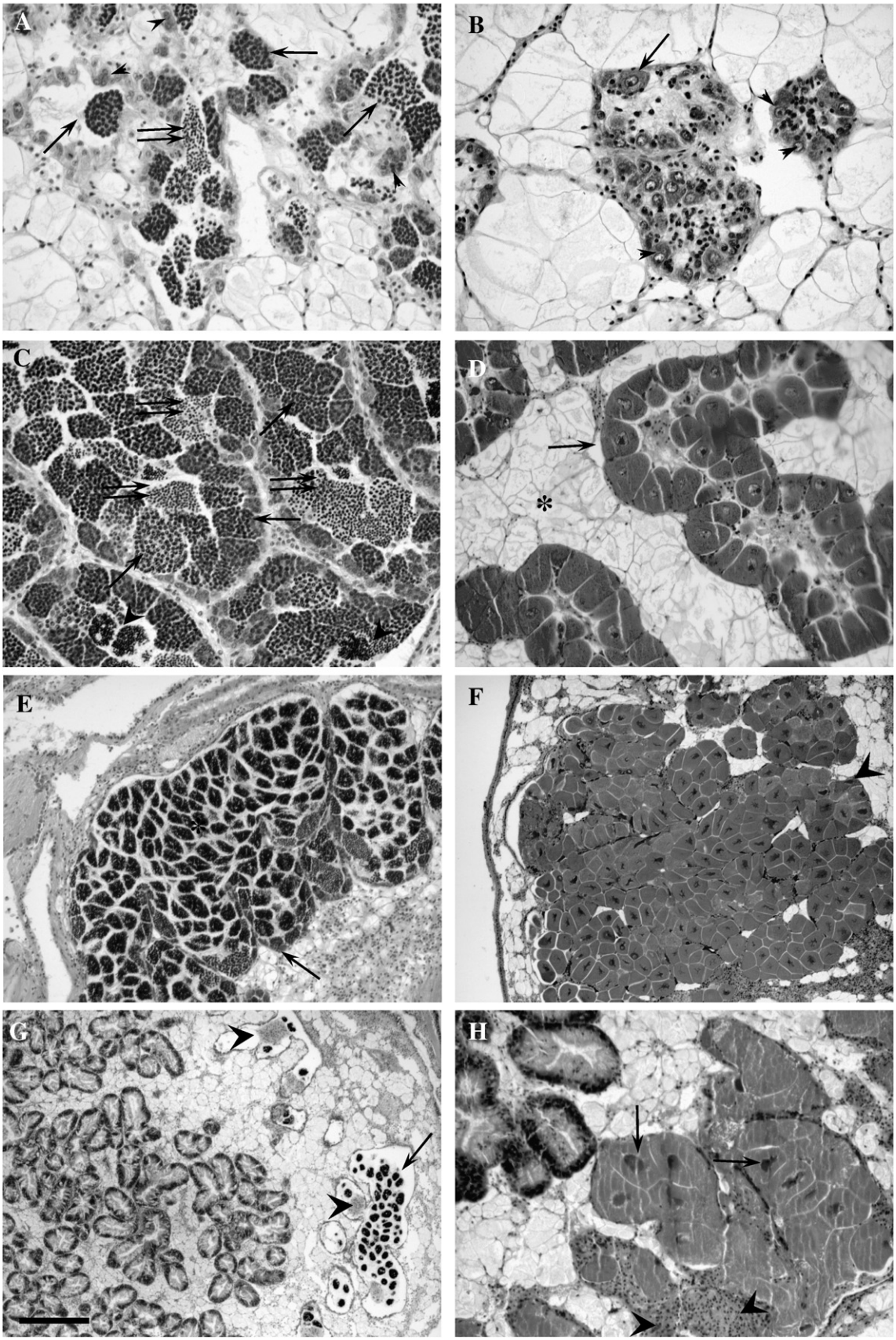

Fig. 2. Micrographs of histological sections of *Ostrea edulis*, showing the gonad area of different sex categories in different gonad stages. A. Male, early gametogenesis. Spermatogonia (arrowhead), spermatocyte balls (arrow), spermatid balls (double arrows). Bar = 50 μm. B. Female, early gametogenesis. Ovogonia (arrowheads), Ovocytes (arrow). Bar = 50 μm. C. Male, advanced gametogenesis. Spermatocyte balls (arrows), spermatid balls (double arrows), spermatozoa balls (arrowhead). Bar = 50 μm. D. Female, advanced gametogenesis. Ovocytes (arrow). Bar = 100 μm. E. Male, ripe gonad. Spermatozoa balls (*), spermatogonia (arrow). Bar = 100 μm. F. Female, ripe gonad. Bar = 100 μm. G. Male, partially spawned. Spermatozoa balls (arrow), phagocytes (arrowhead). Bar = 250 μm. H. Female, partially spawned. Residual ovocytes (arrows), phagocytes (arrowhead). Bar = 100 μm.

lobes was fixed in Davidson's solution and embedded in paraffin; finally, 5-μm thick sections were stained with Harris' haematoxylin and eosin (Howard and Smith, 1983). Haemolymph cell monolayers were prepared by cytocentrifugation (92 ×*g*, 5 min, 4 °C); haemocyte slides were fixed and stained with the Hemacolor kit (Merck) (da Silva and Villalba, 2004). Gonad analysis was done on the histological sections; each oyster was allocated to a sex category and to a gonad development stage. The following sex category scale was used: *indeterminate* (I), when just collapsed or empty follicles occurred (Fig. 1); *male solely* (M), when follicles contained only male gonad material (Figs. 2A, C, E, G and 3A); *female solely* (F), when follicles contained only female gonad material (Figs. 2B, D, F, H and 3B); *hermaphrodite with both sexes equally represented* (HBS), when follicles contained approx. half male and half female gonad material (Fig. 3C); *hermaphrodite predominantly male* (HPM), when follicles contained predominantly male but also some female gonad material (Fig. 3D); and *hermaphrodite predominantly female* (HPF), when follicles contained predominantly female but also some male gonad material (Fig. 3E).

Five stages of gonad development were considered as follows:

Inactive or resting gonad (0) (Fig. 1): no evidence of developing or ripe gametes, which included juvenile, sexually-immature animals and animals that had already undergone a previous reproductive period. In the juvenile oysters the gonads were limited to gonad ducts close to the mantle; in section, the epithelium of the ducts had ciliated cells in the mantle side and germinal cells in the opposite side. In resting animals after spawning, the gonad consisted of dilated and empty

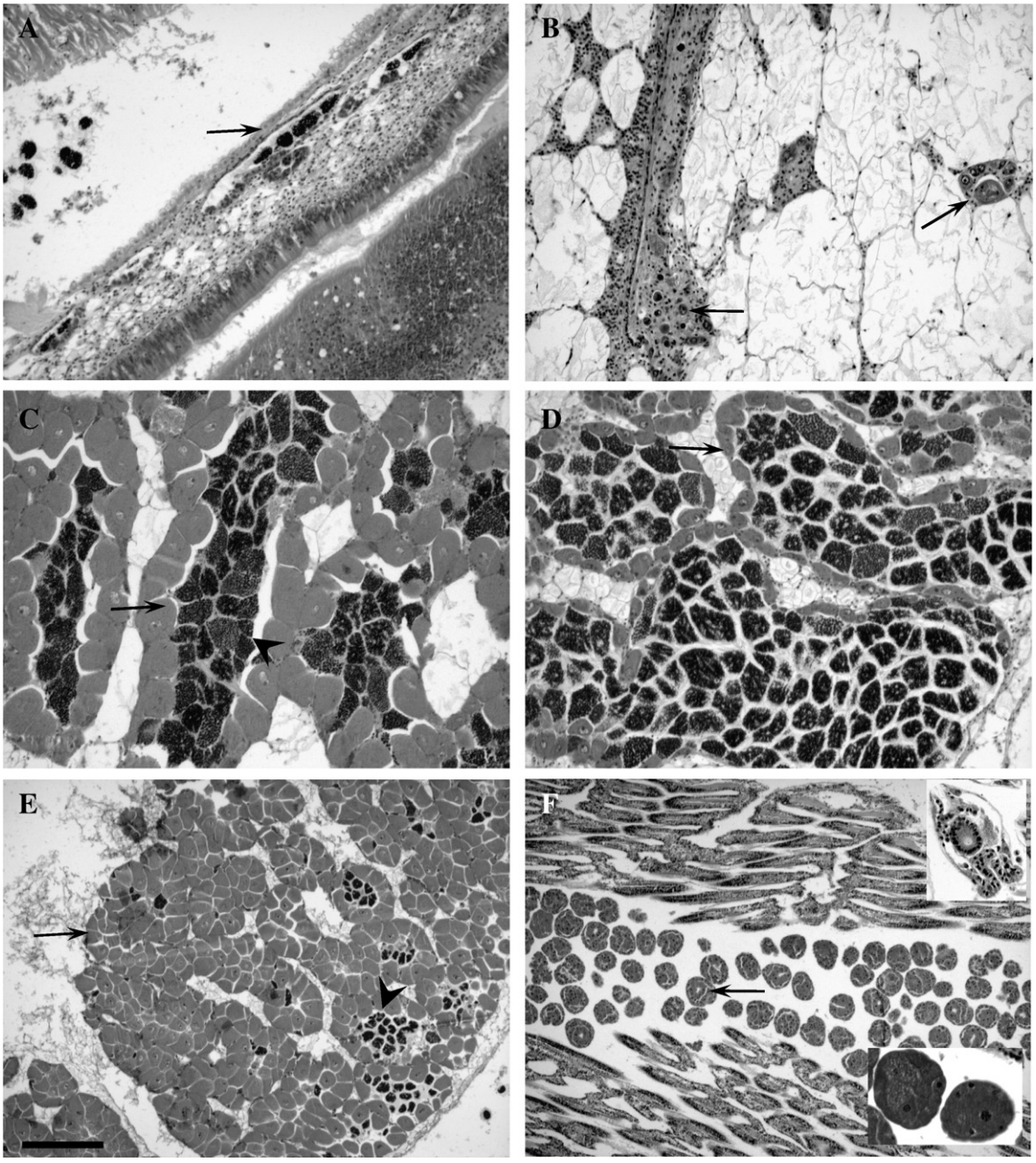

Fig. 3. Micrographs of histological sections of *Ostrea edulis*, showing the gonad area of different sex categories in different gonad stages. A. Male, reabsorbing gonad. Scarce residual spermatozoa balls (arrow). Bar = 100 μm. B. Female, reabsorbing gonad. Residual ovocytes (arrow). Bar = 100 μm. C. Hermaphrodite with both sexes equally represented, advanced gametogenesis. Ovocytes (arrow), spermatocytes (arrowhead). Bar = 100 μm. D. Hermaphrodite predominantly male, ripe gonad. Ovocytes (arrow). Bar = 100 μm. E. Hermaphrodite predominantly female, Ripe gonad. Ripe ovocytes (arrow), spermatozoa balls (arrowhead). Bar = 250 μm. F. Presence of larvae (arrow) in pallial cavity close to branchial lamellae. Bar = 250 μm. Upper inset (bar = 50 μm) and lower inset (bar = 100 μm) represent magnifications of a larvae and embryos of flat oysters, respectively.

Table 1
Distribution (%) of flat oysters *Ostrea edulis* from each origin examined through the study into sex categories

Sex category	Origin				
	IR	GR	CO	OR	Total
Indeterminate	48.3	29.2	43.5	44.3	40.9
Male	31.1	46.1	27.8	26.2	32.6
Hermaphrodite predominantly male	8.2	9.6	7.0	9.9	8.7
Female solely	4.4	4.8	12.2	10.1	8.4
Hermaphrodite predominantly female	6.2	8.3	7.6	7.6	7.5
Hermaphrodite with both sexes equally represented	1.8	2.0	1.9	1.8	1.9

CO: Coroso; GR: Greece; IR: Ireland; OR: Ortigueira.

follicles located between the mantle and the digestive gland, surrounded by abundant connective tissue. Presence of remaining haemocytes inside the follicles could be observed.

Early gametogenesis (1) (Fig. 2A and B): gonad follicles more spread into the connective tissue, and even closer to the digestive gland than in the previous phase. Ovogonia and spermatogonia were mostly attached to the follicle wall. In the male developing line spermatocytes and few spermatid balls were present; in the female developing line there was a predominance of attached, developing ovocytes (15–30 µm in diameter).

Advanced gametogenesis (2) (Fig. 2C and D): gonad follicles were larger than in the previous phase, filling a large area between mantle and digestive gland; connective tissue was already present. Every cell type of the germ line was present. In the male developing line few spermatogonia occurred, spermatocytes and spermatid balls were dominant, and few spermatozoa balls appeared in the follicle lumen; in the female developing line, attached medium-sized ovocytes (30–80 µm in diameter) in vitellogenesis were dominant, and ovocytes in post-vitellogenesis were less abundant and located free in the follicle lumen.

Ripe gonad (3) (Fig. 2E and F): Juxtaposed large follicles occupied the entire area between the mantle and the digestive gland, and only a vestige of connective tissue was observed. In both male and female developing lines, follicles contained mature gametes and sometimes a thin layer of primary germ cells. Abundant spermatozoa balls and mature ovocytes (90–110 µm in diameter) filled the follicle lumen, in male line and female line, respectively.

Partially spawned gonad (4) (Fig. 2G and H): Gonad follicles were smaller than in the previous phase and separated by some connective tissue. Gametes had been released but a large amount of residual mature gametes remains in the follicle lumen. In some cases phagocytes were observed within follicles to re-absorb residual gametes.

Reabsorbing gonad (5) (Fig. 3A and B): Few gonad follicles appeared which were mostly located in the proximity of the mantle and connective tissue was abundant. Residual mature ovocytes and sperm balls could be observed in the follicle lumen; sometimes a thin layer of primary germ cells appear in the follicle wall. Phagocytes were often observed in the follicle lumen.

HBS, HPM and HPF oysters (Fig. 3C–E) could not be precisely allocated into the stages 1, 4, and 5 of gonad development, because of the lack of synchrony between female and male developing lines. Therefore, those oysters were considered as *not classified hermaphrodites* (HNC).

Brooded embryos or larvae were observed in some sections, in the pallial cavity, indicating a shortly previous release of ripe ovocytes (Fig. 3F).

2.3. Diagnosis of B. ostreae

B. ostreae was detected by examining histological sections and haemolymph cell monolayers; the prevalence of the parasite was calculated as the percentage of infected oysters in each monthly sample. Estimation of *B. ostreae* infection intensity involved ranking each oyster after examination of histological sections using the scale proposed by da Silva and Villalba (2004): null (N), light (L), moderate (M), and heavy infection (H). The oysters in which infection was detected in the haemolymph cell monolayer but was not in histological section were categorized in the *light* infection class. Results corresponding to histopathological aspects and temporal variation of bonamiosis in each cohort were reported and discussed elsewhere (da Silva et al., 2005).

2.4. Statistical analysis

Comparisons of oyster distribution in gonad condition classes (sex and gonad development) between origins and cohorts were analysed using a contingency table and a χ^2 test. The same procedure was applied to test the association of gonad condition classes (sex and gonad development) and intensity of infection by *B. ostreae*, in which the sex or gonad development categories were organized into columns, and the *B. ostreae* intensity classes into rows. Oysters with both sexes represented equally were excluded as they were not represented in all infection-intensity categories, rendering the analysis impossible. MINITAB 14 software was used for all statistical analysis. Differences were considered statistically significant when P values were lower than 0.05.

3. Results

A total of 3096 oysters were analysed during the two years of grow-out. The percentage of oysters in each sex category is shown in Table 1.

3.1. Temporal variation of oyster gonad condition

Oyster broodstock in the hatchery spawned between March and May 2001 and the resulting seed were transferred to the raft for on growing in September 2001. Two months later, a few oysters had already started gametogenesis. Considering all oysters together, the monthly distribution of oysters in sex categories and gonad development classes is shown in Figs. 4 and 5, respectively. The percentage of immature individuals decreased progressively from November 2001 to July 2002, when the reproductive activity of the oysters was maximal (95%) and males predominated (Figs. 4 and 5). From April

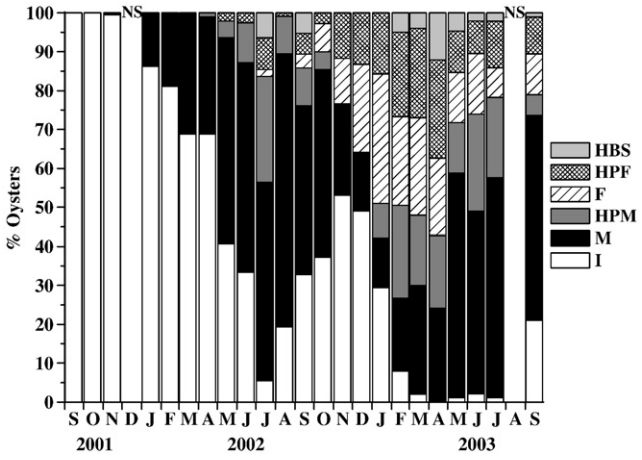

Fig. 4. Distribution of the oysters *Ostrea edulis* of each monthly sample in sex categories. NS: No sample collected in December 2001 and August 2003. I: indeterminate; M: male solely; HPM: hermaphrodite predominantly male; F: female solely; HPF: hermaphrodite predominantly female; HBS: hermaphrodite with both sexes equally represented.

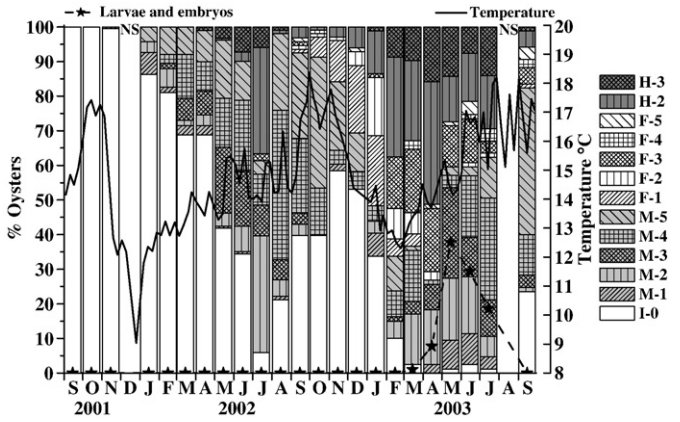

Fig. 5. Distribution of the oysters *Ostrea edulis* of each monthly sample according to gonad stages. NS: no sample collected in December 2001 and August 2003. 0: resting gonad; 1: early gametogenesis; 2: advanced gametogenesis; 3: ripe gonad; 4: partially spawned gonad; 5: Reabsorbing gonad. I: indeterminate; M: male solely; F: female solely; H: hermaphrodite. Curves depict weekly variation of seawater temperature and the percentage of oysters brooding embryos or larvae in each monthly sample.

2002, the percentage of hermaphrodites predominantly male increased, and in the following months hermaphrodites predominantly female and hermaphrodites with both sexes equally represented also appeared (Fig. 4). The first oysters functioning as female solely were detected in July 2002, at a very low percentage, increasing from September 2002 through the following spring when both females solely and hermaphrodites predominantly female percentages increased over indeterminate oysters (Fig. 4). In turn, male gametogenesis also occurred during the same period, but in lower proportion than female gametogenesis, reaching the maximum in May 2003. Finally, in September 2003, indeterminate oysters started to appear, indicating the beginning of resting phase (Figs. 4 and 5).

Gonads were predominantly inactive from September 2001 to April 2002 (Fig. 5). Nevertheless, early gametogenesis was detected as soon as December 2001. Male gametogenesis progressed and a peak of ripe oysters was observed in May and June 2002. Postspawning stages increased from May 2002 up to a maximum in August 2002; since then the percentage of resting gonad increased and in November 2002 more than half of the oysters were in resting phase (Fig. 5). This autumn resting phase separated two reproductive periods, the first mainly involving male gametogenesis and the next one with predominance of female gametogenesis, at least in the beginning. Maximum percentage of ripe female oysters occurred in March and April 2003 and males in May of 2003; ripe hermaphrodites were detected from February to July 2003 (Fig. 5). The main spawning periods covered from April to August 2002 and from April to September 2003; oysters brooding embryos and larvae were detected from March to July 2003, with a peak in May (Fig. 5).

Water temperature in spring 2002 varied from 13.5 to 15.5 °C and from 13.5 to 17.5 °C in spring 2003; the highest values (18 to 18.5 °C) were recorded in September–October 2002 and July–September 2003; the lowest value (9 °C) was recorded in December 2001 (Fig. 5).

3.2. Comparison of gonad condition between oysters from different geographic origins

Oysters from Ireland, Greece, and Galicia showed significantly different distributions in sexual-phase categories (χ^2=227.44; df=15;

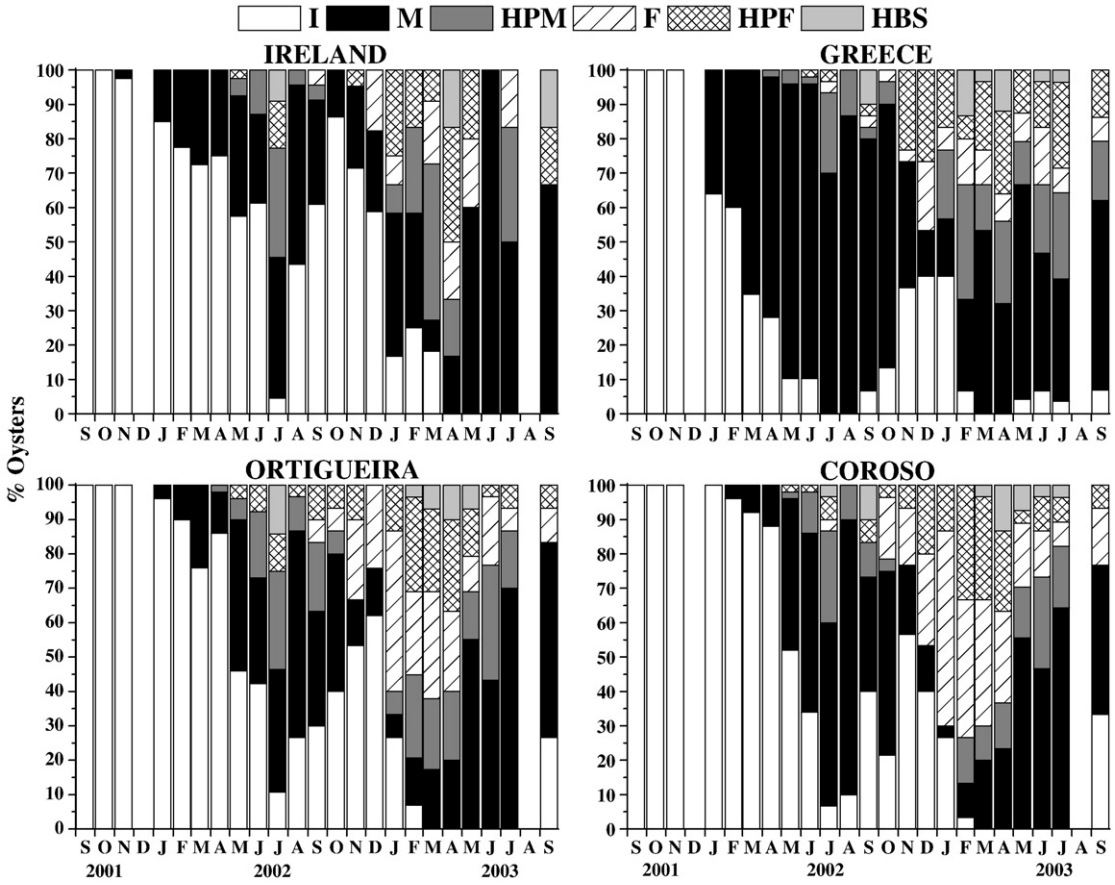

Fig. 6. Distribution of the oysters *Ostrea edulis* from every geographic origin in each monthly sample according to sex categories. No sample was collected in December 2001 and August 2003. I: indeterminate; M: male solely; HPM: hermaphrodite predominantly male; F: female solely; HPF: hermaphrodite predominantly female; HBS: hermaphrodite with both sexes equally represented.

$P<0.001$), but no significant differences were observed between oysters from Galician origins ($\chi^2=4.45$; df=5; $P=0.487$) (Table 1, Fig. 6). Irish oysters had the highest mean percentage of indeterminate individuals, whereas Greek oysters had the lowest percentage in this category. Conversely, oysters from both Galician origins showed a similar pattern (Table 1, Fig. 6).

The oysters of Irish origin had a high percentage of indeterminate gonads, both in the first reproductive period (from September 2001–July 2002) and in the transitional period (autumn resting phase), which was more extended than in oysters from other origins. The first time that most of the IR oysters of a monthly sample showed active gonads occurred in July 2002 (Fig. 6).

Greek oysters, during the first reproductive period, got ripeness earlier than oysters from other origins, mainly as males solely. The autumn resting phase in oysters from Greece was shorter than in oysters from other origins and was seen in a low percentage of the population. The percentage of females solely and hermaphrodites predominantly females increased during the second reproductive period, but were never more abundant than males solely and hermaphrodites predominantly male. Indeed, the percentage of ripe males and females remained balanced from March to September 2003 (Fig. 5).

Galician oysters, as IR oysters, had a high percentage of indeterminate individuals during the first reproductive period. However, during the next autumn resting phase, the percentage of indeterminate oysters was intermediate between those from IR and GR origins. In the beginning of the second reproductive period, gametogenesis had evolved into major F and HPF components, resulting in two clear phases: one (November 2002 to April 2003) with predominance of female gametogenesis (F and HPF), and the other (May 2003 to September 2003) with predominance of male gametogenesis (M) (Fig. 6). This pattern of the second reproductive period was not observed in IR and GR oysters.

3.3. Comparison of gonad condition between oysters from different cohorts within origins

The most marked differences between cohorts within origins in distribution of oysters in sexual-phase categories were observed between Irish cohorts ($\chi^2=175.66$; df=6; $P=0.000$) (Fig. 7). Three cohorts (IR1, IR2, IR4) had percentages higher than 80% of indeterminate oysters, whereas the IR3 cohort showed a much lower percentage (Fig. 7); in fact IR3 had the lowest percentage of indeterminate oysters of all cohorts from any origin. However, very few oysters from IR1, IR2 and IR4 were present during the second reproductive period because of high mortalities, thus the expected decrease of indeterminate oysters during that second reproductive cycle could not be seen. Even during the first or in the beginning of the second reproductive period,

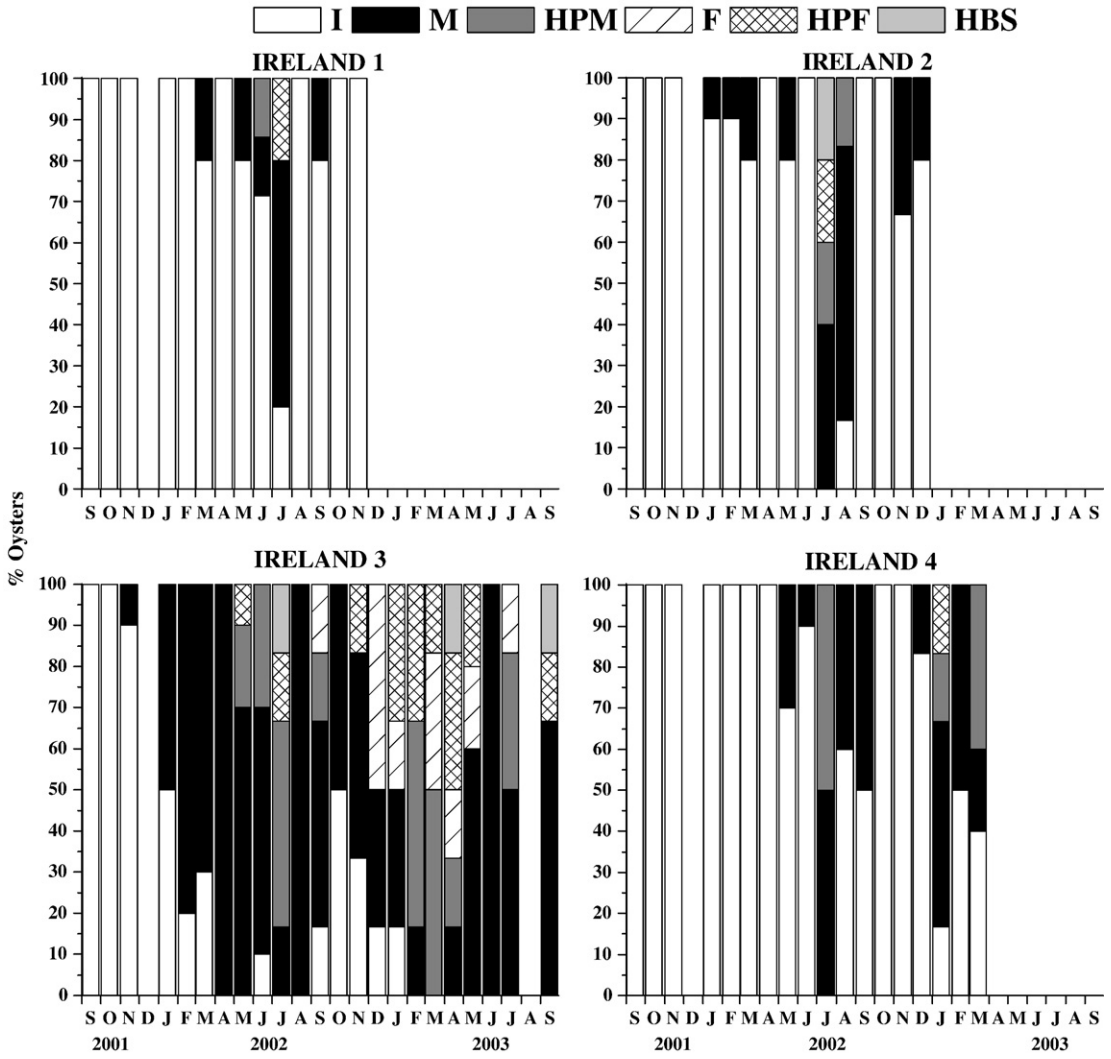

Fig. 7. Distribution of the oysters *Ostrea edulis* from every Irish cohort in each monthly sample according to sex categories. No sample was collected in December 2001 and August 2003; no oyster of the cohorts IR1, IR2 and IR4 was available from December 2002, January 2003 and April 2003 onwards, respectively. I: indeterminate; M: male solely; HPM: hermaphrodite predominantly male; F: female solely; HPF: hermaphrodite predominantly female; HBS: hermaphrodite with both sexes equally represented.

those cohorts had retarded the beginning of gonad activity compared to IR3 (Fig. 7).

The differences between cohorts were less evident in oysters with Greek origin (χ^2=27.18; df=16; P=0.040) (Fig. 8). The GR2 cohort had the highest percentage of indeterminate oysters. In GR4 and GR1, there was a predominance of males solely and hermaphrodites predominantly male, even during the second reproductive period (Fig. 8).

Differences between cohorts were significant in CO oysters (χ^2=47.14; df=16; P<0.001) (Fig. 9). CO4 and CO5 had a higher percentage of males than the other three cohorts. The CO3 cohort had a strong predominance of female component (both female solely and hermaphrodite predominantly female) that was the highest of all cohorts studied. In the second reproductive period, the percentages of CO3 oysters in the categories female solely and hermaphrodite predominantly female were very high (especially ripe oysters, data not shown), and few male and hermaphrodite predominantly male oysters were recorded (Fig. 9).

Oyster cohorts deriving from Ortigueira were quite homogeneous in their pattern of sex category distribution (χ^2=22.98; df=16; P=0.114) (Fig. 10).

3.4. Brooding of embryos and larvae

Considering all the oysters examined in the study, the presence of embryos and larvae was detected in the pallial cavity of 84 oysters

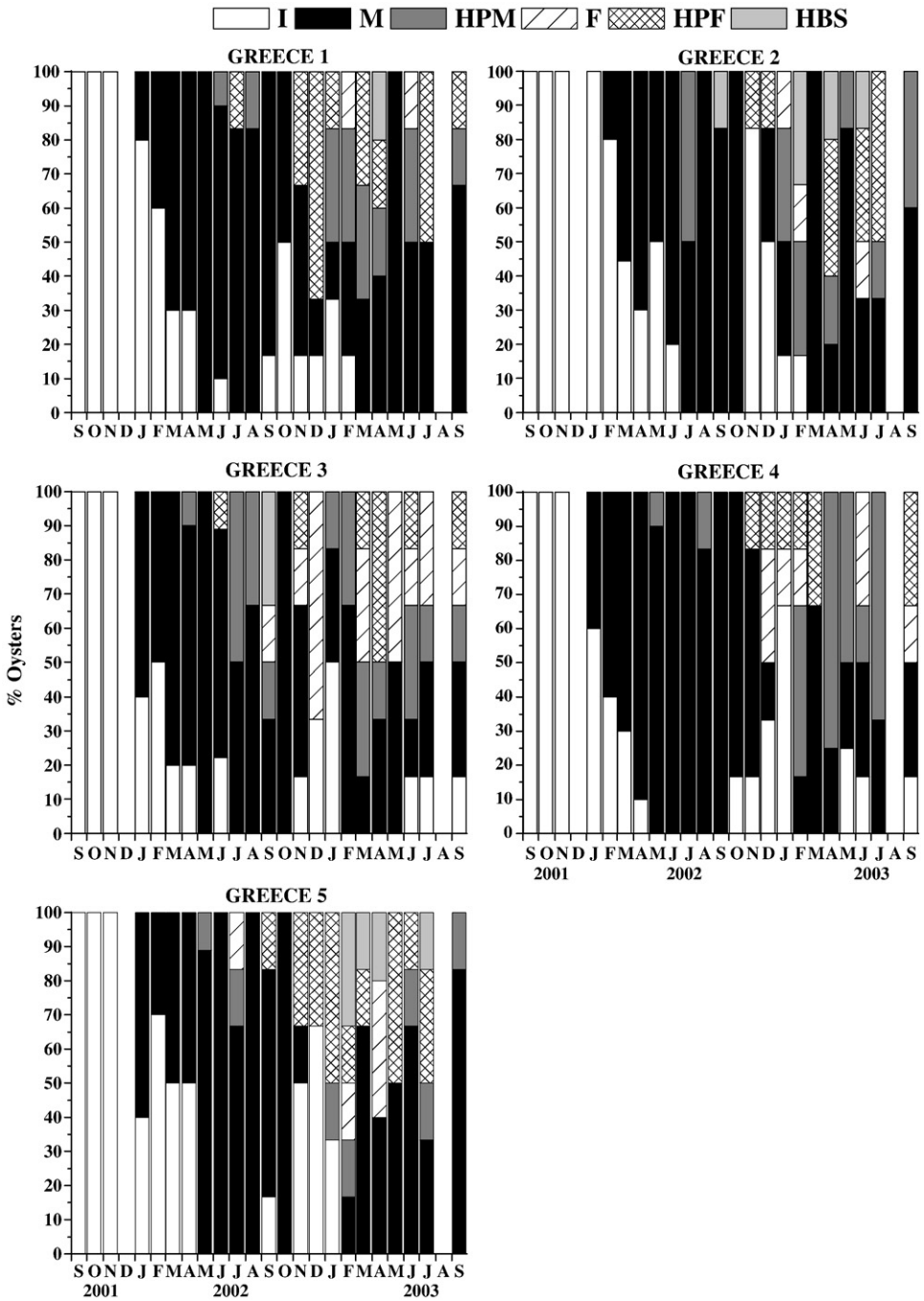

Fig. 8. Distribution of the oysters *Ostrea edulis* from every Greek cohort in each monthly sample according to sex categories. No sample was collected in December 2001 and August 2003. I: indeterminate; M: male solely; HPM: hermaphrodite predominantly male; F: female solely; HPF: hermaphrodite predominantly female; HBS: hermaphrodite with both sexes equally represented.

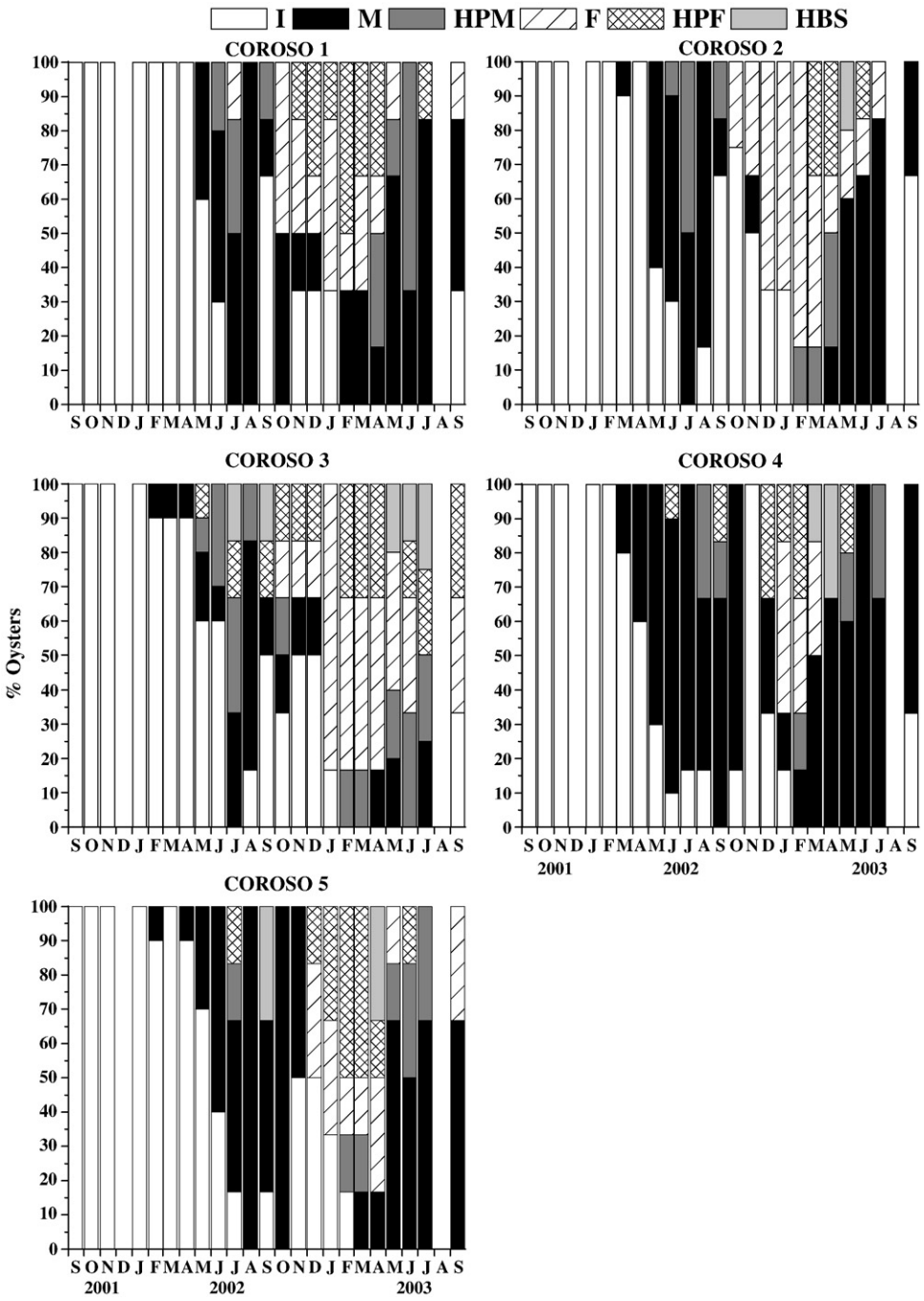

Fig. 9. Distribution of the oysters *Ostrea edulis* from every Coroso cohort in each monthly sample according to sex categories. No sample was collected in December 2001 and August 2003. I: indeterminate; M: male solely; HPM: hermaphrodite predominantly male; F: female solely; HPF: hermaphrodite predominantly female; HBS: hermaphrodite with both sexes equally represented.

during the second reproductive period, from March 2003 (one case) to July 2003, May 2003 being the month with the highest percentage (37.6%) (Fig. 5). Larvae and embryos were observed in oysters 46–85 mm in shell height, more frequently in males (63%) than in hermaphrodites predominantly male (18%), females (8%), hermaphrodites predominantly female (11%), or in hermaphrodites with both sexes equally represented (only one case).

Oysters brooding embryos or larvae were observed in all cohorts, except the GR2, IR1, IR2 and IR4, which suffered high mortalities during the corresponding period; consequently, IR3 was the only IR cohort with brooding oysters (4.5%) during this period. GR5 had the highest percentage (7.5%) of brooding oysters of all cohorts, but GR1 had only 3.0%, GR3 0.6% and GR4 2.4%. Also CO1 had a higher percentage (6.3%), compared to the other cohorts within the CO origin, i.e., CO2 (1.2%), CO3 (2.3%), CO4 (4.6%) and CO5 (0.6%). OR cohorts had closer percentages of brooding oysters; OR1 (3.4%), OR2 (3.4%), OR3 (2.4%), OR4 (4.8%) and OR5 (1.8%).

3.5. Infection of oysters by *B. ostreae*

B. ostreae was detected in 144 oysters through the study after examining histological sections and haemolymph monolayers. Spherical 2 to 4 μm *B. ostreae* cells with an eccentric nucleus were found inside haemocytes and free in the connective tissue of the infected oysters. Different degrees of haemocytic infiltration were observed according to disease intensity. Early infections were mostly located

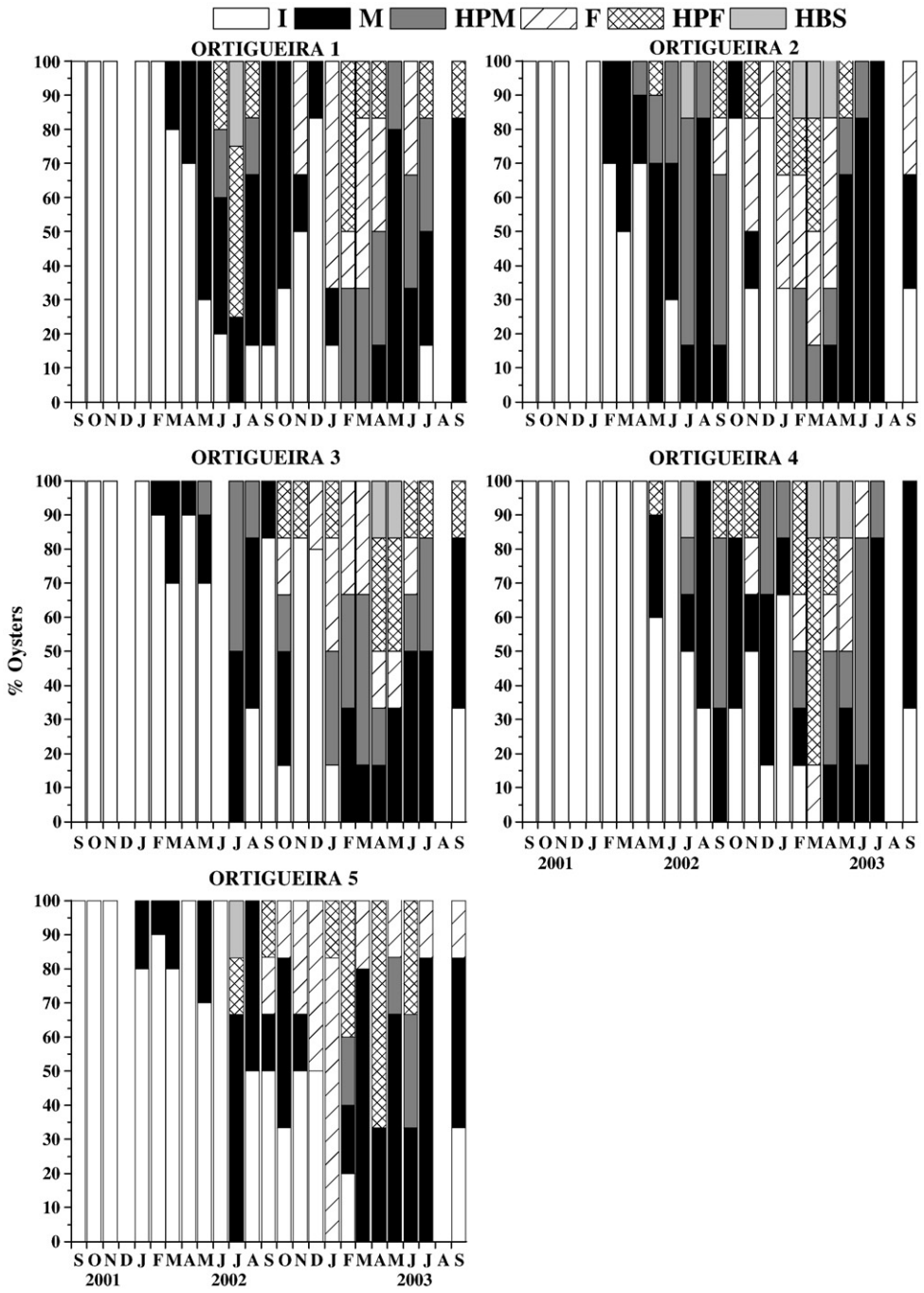

Fig. 10. Distribution of the oysters *Ostrea edulis* from every Ortigueira cohort in each monthly sample according to sex categories. No sample was collected in December 2001 and August 2003. I: indeterminate; M: male solely; HPM: hermaphrodite predominantly male; F: female solely; HPF: hermaphrodite predominantly female; HBS: hermaphrodite with both sexes equally represented.

in the gills. In advanced stages, the parasite was found within haemocytes throughout the connective tissue of every organ and invading gonad follicles (both in female or male gametogenic stages) and digestive epithelia. The monthly variation of the mean prevalence of *B. ostreae* in every origin through on growing is shown in Fig. 11. The 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 ones. 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 (Table 2). Remarkable differences between cohorts within GR and CO origins were detected (Table 2). The cohort 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). The cohorts OR1 and CO5 showed the lowest value of average prevalence (Table 2). The lack of samples of the cohorts IR1, IR2 and IR4 from the months with higher prevalence of *B. ostreae* prevents from including those cohorts in any comparison on susceptibility to bonamiosis.

3.6. Gonad condition of oysters vs infection by B. ostreae

B. ostreae was observed in oysters at every sex category. Namely, 36.8% of all cases occurred in males solely, 20.7% in females solely, 15.5% in hermaphrodite predominantly male, 13.5% in hermaphrodite

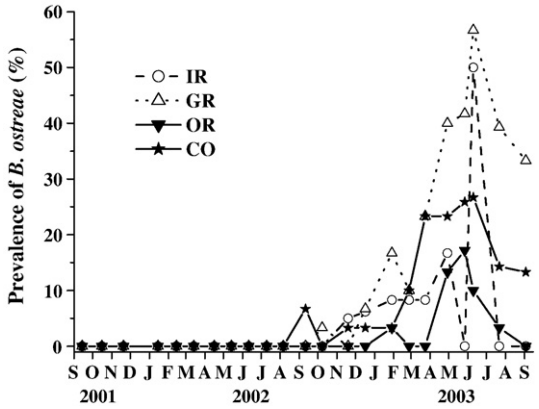

Fig. 11. Monthly variation of the mean prevalence of *Bonamia ostreae* in the oysters from each geographic origin through on growing. CO: Coroso; OR: Ortigueira; GR: Greece; IR: Ireland.

predominantly female, 10.3% in indeterminate oysters, and 3.2% in hermaphrodite with both sexes equally represented.

Significant differences (χ^2=29.26; df=12; P=0.004) in the distribution of oysters according to sex category between classes of *B. ostreae* infection intensity were found (Fig. 12). Both uninfected and heavily infected oysters had higher percentages of indeterminate gonads than lightly or moderately infected oysters (Figs. 12 and 13). In the light and moderate infection classes, male components predominated in follicles (as M and HPM). The heavy infection class had the highest percentage of females solely, this sex category being dominant in oysters heavily infected by *B. ostreae* (Fig. 12). The female component (including both F and HPF categories) increased with parasite burden (Fig. 12). In the month with the highest prevalence of infection (June 2003, Fig. 11), the percentage of males solely (47%) was higher than that of females solely (15%), but heavy infections were more frequent in females (50%) than in males (33%). The cohort CO3 was the one with the highest percentages of females solely and hermaphrodites predominantly female from all the cohorts involved in the study

Table 2
Occurrence of *Bonamia ostreae* in the oysters *Ostrea edulis* of different origins and cohorts through the study

	IR1	IR2	IR3	IR4		Mean IR
N	110	116	179	143		548
A	0	2.8	4.4c	1.0		2.0
B	0	25	50	16.7		
C	0	2	4	1		7
	GR1	GR2	GR3	GR4	GR5	Mean GR
N	169	173	171	170	173	856
A	21.4	8.3	12.7	13.8	3.8	12.0
B	83.3	66.7	75	83.3	50	
C	10	6	6	8	3	10
	CO1	CO2	CO3	CO4	CO5	Mean CO
N	174	170	171	173	174	862
A	3.6	2.9	22.9	2.9	1.4	6.8
B	50	33.3	100	33.3	16.7	
C	3	3	11	2	2	11
	OR1	OR2	OR3	OR4	OR5	Mean OR
N	172	174	164	166	168	844
A	1.4	2.2	1.5	2.2	2.9	2.0
B	16.7	16.7	16.7	33.3	33.3	
C	2	3	2	2	3	5

Twenty three monthly samples were analysed per cohort, except for cohorts IR1, IR2 and IR4, for which 14, 15 and 18 samples were analysed, respectively. A: mean prevalence (%) through the study period; B: highest monthly record of prevalence (%); C: number of monthly samples in which the parasite was detected; N: number of oysters analysed. CO: Coroso; GR: Greece; IR: Ireland; OR: Ortigueira.

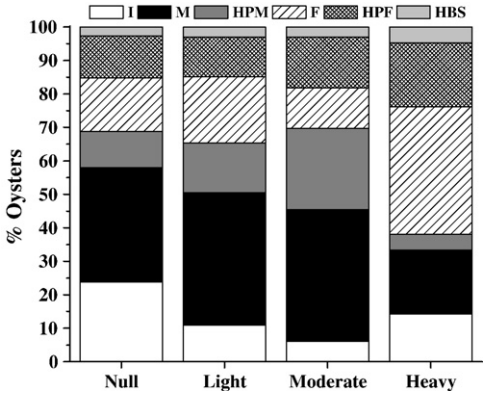

Fig. 12. Distribution of oysters *Ostrea edulis* from each class of infection intensity by *Bonamia ostreae* (abscissas) into sex categories (patterns). This graph includes the oyster samples taken in the period of parasite detection (September 2002 to September 2003). The number of oysters included in each class of infection intensity was: null=1113; light=101; moderate=33; heavy:=21. I: indeterminate; M: male solely; HPM: hermaphrodite predominantly male; F: female solely; HPF: hermaphrodite predominantly female; HBS: hermaphrodite with both sexes equally represented.

(Figs. 7–10); this cohort was also the one that showed the highest prevalence of bonamiosis (Table 2).

The distribution of oysters according to gametogenesis stages was significantly-different (χ^2=49.05; df=15; P<0.001) between classes of *B. ostreae* infection intensity (Fig. 13). Moderately and heavily infected oysters had the highest percentages of ripe and partially spawning gonads.

4. Discussion

The study of the temporal variation of gonad condition of various flat oyster cohorts from different geographic origins, through on growing in the Ría de Arousa, showed a general gametogenic pattern in agreement with those described for *O. edulis* (Cole, 1942; Loosanoff, 1962; Leonard, 1969; Mann, 1979) and other larviparous oyster species, e.g. *Ostrea nomades* (Siddiqui and Ahmed, 2002). However, a significant inter and intra population variability in the gonad condition was found. Greek oysters showed active gonads earlier than the others and exhibited a male predominance; very likely, a percentage of Greek oysters, after first maturing as males in spring, developed again as males without passing through a female phase. On the other hand, three Irish cohorts (IR1, IR2 and IR4) did not show gonad activity until summer, although IR3 was the most precocious of all studied cohorts and showed more similarities with cohorts from GR, CO and

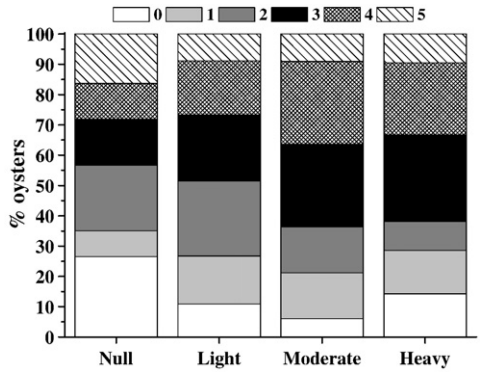

Fig. 13. Oysters *Ostrea edulis* from each intensity class of infection by *Bonamia ostreae* (bars) distributed into gonadal development categories (patterns). Oysters sampled during parasite infection period (September 2002 to September 2003). The number of oysters included in each class of infection intensity was: null=1113; light=101; moderate=33; heavy:=21. 0: resting gonad; 1: early gametogenesis; 2: advanced gametogenesis; 3: ripe gonad; 4: partially spawned gonad; 5: reabsorbing gonad.

OR than with the other IR cohorts. Marked differences were found between cohorts within geographic origins, except in OR cohorts, which had marked similarity in the temporal pattern of gonad condition. Similarity between OR cohorts was also observed in growth, survival, susceptibility to diseases (da Silva et al., 2005, 2008a) and immune related parameters (da Silva et al., 2008b). This low variability in oysters from OR origin may be attributable to the longer (over 20 years) selective bonamiosis pressure that drastically decreased the number of individuals in the population. Moreover, flat oyster culture activities based upon importation of foreign seed to the Ría de Ortigueira ceased in the early 1980s (Polanco et al., 1984), thus limiting the gene flow.

The differences in temporal pattern of gonad condition between oysters from different geographic origins that were born and cultured in the same environment could be explained by the existence of *physiological races* adapted to different environmental conditions. It is well known that gametogenesis and spawning in bivalves are influenced by temperature (Giese, 1959; Sastry, 1979). This variable seems to have a great effect on development of the female germinal cell line (Sparck, 1925; Loosanoff, 1962; Leonard, 1969). Loosanoff (1962) hypothesised the existence of races in *O. edulis* with different temperature requirements for gametogenesis. Ruiz et al. (1992) presented a table showing the temperatures that triggered gametogenesis in *O. edulis* originating from different regions. In general, animals located in regions closer to the equator have earlier and longer lasting gametogenesis than animals located in higher latitudes (Thompson et al., 1996).

Our results seem to agree with the physiological races hypothesis, as seawater temperatures are lower on the coast of Ireland than in Galician coastal waters or in the Mediterranean Sea, at least for a period of the year. According to Wilson and Simons (1985), gametogenesis in Irish oysters starts in spring, spawning occurs in summer only, and a resting phase predominates during the rest of the year, as observed in the present study for three Irish cohorts. Moreover, in the Mediterranean Sea, where temperature is higher than in Galicia, earlier gonad activation, longer period of gonad activity, and shorter resting phase would be expected (Leonard, 1969), as it was observed in Greek cohorts. The lower percentage of female gametogenesis observed in GR, compared to CO and OR oysters, could be due to temperature values below an hypothetical threshold in the Eastern area of the Mediterranean Sea, to which Greek oysters would be adapted. Nevertheless, the trend that would correspond to each physiological race would allow a significant plasticity (variability), because significant differences were observed between cohorts within origins; this variability involved that the best adapted cohort (IR3) of the IR origin had a much higher survival rate and higher success in production and release of gametes of both sexes, thus enhancing reproduction success.

Thompson et al. (1996) stated that food availability affects gametogenesis, inducing development and favouring one sex over the other. Production of spermatozoa is energetically less costly than production of ovocytes (Thompson et al., 1996); consistently, more severe impairment of female than male gametogenesis due to nutritional stress would be expected. Food availability in the Ría de Arousa was unlikely a serious limiting factor during the study period and the growth of Galician oysters (especially OR) was much quicker than that of oysters from foreign origins (da Silva et al., 2005). Likely, foreign oysters had less energy available for growth and even gametogenesis, which could be a consequence of an incomplete adaptation to the Galician environment. This could explain that gametogenesis was limited in three Irish cohorts and female gametogenesis was reduced in Greek oysters compared to the better adapted Galician oysters.

Results on susceptibility to bonamiosis of origins and cohorts within origins were discussed elsewhere (da Silva et al., 2005). *B. ostreae* was detected in oysters from all sex categories, more often in males solely because this was the most frequent sexual phase in the period in which bonamiosis was detected. Two important associations were observed between intensity of infection and oyster gonad condition. First, heavy infections were significantly more frequent in oysters with a predominant female component (females solely and hermaphrodites predominantly female). Second, heavy infections were significantly more frequent in ripe and partially spawned oysters. These results suggest that the probability of the infection to reach an advanced stage is significantly higher in females solely or hermaphrodites predominantly female than in other sex categories, especially when the oysters have got gonad ripeness (either they are ripe or have spawned recently). The association does not unequivocally involve a cause–effect relationship between gonad condition and infection progression and indirect association cannot be discarded. Other pathological conditions were also detected in oysters from all stocks (da Silva et al., 2005), but no apparent association between them and gonad condition was detected. A hypothesis to explain a linkage between the progression of female gametogenesis and the advance of *B. ostreae* infection could be that ovocyte production consumes considerable energy and thus oysters would have less energy available to defend against the parasite. Progression of female gametogenesis is associated with changes in hormone levels, which could influence the immune response; the immune system of bivalve molluscs is modulated by the endocrine system (Ottaviani et al., 1999; Lacoste et al., 2001a,b), including sexual hormones (Canesi et al., 2004).

Lynch et al. (2005) demonstrated that oysters less than one year old can be infected by *B. ostreae*, but in most cases the infections remain subclinical at that age (only detected by PCR). When the oysters are close to reach (or have reached) market size, both the prevalence and the infection intensity are higher than at previous stages (Tigé et al., 1982; Montes et al., 1989, 1991; Figueras, 1991; Robert et al., 1991; Culloty and Mulcahy, 1996). Thus, female maturation might play a role contributing to the progression of bonamiosis to clinical level. Previous studies did not detect any significant association between sex of *O. edulis* and infection by *B. ostreae*, in Ría de Vigo (Cáceres-Martínez et al., 1995) and in Ireland (Culloty and Mulcahy, 1996). Hawkins et al. (1993) studied responses to stress in *O. edulis* from different origins (Scotland, Wales and Ireland) that were maintained in a common environment affected by bonamiosis. The authors detected significant differences in prevalence of *B. ostreae* and oyster survival between oysters from different origins, suggesting differences in immunological capacity or reproductive effort, as Scottish oysters, with higher survival and lower prevalence of *B. ostreae*, showed less gonad activity. Hine (1991) reported that infection by *Bonamia exitiosa* in *Ostrea* (=*Tiostrea*) *chilensis* was more frequent in females than in males and that the increase in intensity of infection was associated with the infection of the gonad; the author reported that the proliferation of *B. exitiosa* within the host occurs when oysters develop female gametogenesis and suggested that the parasite utilizes the lipid reserves of the host ovocytes, as haemocytes phagocytose the residual ovocytes. If the hypothesis of that female gametogenesis favours progression of *B. ostreae* infection were true, any method to elude the female phase in the oysters could reduce the effects of bonamiosis in the oysters, which would open new perspectives for breeding programmes or changes of culture methods. Tun et al. (2006) demonstrated that in the case of the infection by the protozoan parasite *Marteilioides chungmuensis*, which is clinical only when the oyster *Crassostrea gigas* is in the female phase, the prevalence of the disease can be reduced by controlling the sex ratio through changing the culture method: intertidal oysters showed significantly lower female ratio than subtidal oysters.

In conclusion, significant differences in the temporal pattern of gonad condition were found between oysters from different origins that would indicate adaptation to different environments; nevertheless the significant differences that were found between cohorts

within origins would indicate that there is variability that eventually could allow adaptation to new environments. The results suggest a hypothetical enhancement of progression of infection by *B. ostreae* associated with female gametogenesis.

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References

Cáceres-Martínez, J., Robledo, J.A.F., Figueras, A., 1995. Presence of *Bonamia* and its relation to age, growth rates and gonadal development of the oyster, *Ostrea edulis*, in the Ría de Vigo, Galicia (NW Spain). Aquaculture 130, 15–23.

Canesi, L., Ciacci, C., Betti, M., Lorusso, L.C., Marchi, B., Burattini, S., Falcieri, E., Gallo, G., 2004. Rapid effect of 17β-estradiol on cell signalling and function of *Mytilus* hemocytes. Gen. Comp. Endocrinol. 136, 58–71.

Carnegie, R.B., Cochennec-Laureau, N., 2004. Microcell parasites of oysters: recent insights and future trends. Aquat. Living Resour. 17, 519–528.

Casas, S.M., 2001. Estudio de la perkinsosis en la almeja fina, *Tapes decussatus* (Linnaeus, 1758), de Galicia. Ph.D. Thesis. Universidad de Santiago de Compostela, Spain.

Choi, K.S., Powell, E.N., Lewis, D.H., Ray, S.M., 1994. Instantaneous reproductive effort in female American oysters, *Crassostrea virginica*, measured by a new immunoprecipitation assay. Biol. Bull. 186, 41–61.

Cole, H.A., 1942. Primary sex-phases in *Ostrea edulis*. Q. J. Microsc. Sci. 83, 317–356.

Culloty, S.C., Mulcahy, M.F., 1996. Season-, age-, and sex-related variation in the prevalence of bonamiasis in flat oysters (*Ostrea edulis* L.) on the south coast of Ireland. Aquaculture 144, 53–63.

Culloty, S.C., Mulcahy, M.F., 2001. Living with bonamiasis: Irish research since 1987. Hydrobiologia 465, 181–186.

da Silva, P.M., Villalba, A., 2004. Comparison of light miscroscopic techniques for the diagnosis of the infection of the European flat oyster *Ostrea edulis* by protozoan *Bonamia ostreae*. J. Invertebr. Pathol. 85, 97–104.

da Silva, P.M., Fuentes, J., Villalba, A., 2005. 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). Mar. Biol. 147, 965–977.

da Silva, P.M., Renault, T., Fuentes, J., Villalba, A., 2008a. Herpesvirus infection in European flat oysters *Ostrea edulis* obtained from brood stocks of various geographic origins and grown in Galicia (NW Spain). Dis. Aquat. Org. 78, 181–188.

da Silva, P.M., Comesaña, P., Fuentes, J., Villalba, A., 2008b. Variability of haemocyte and haemolymph parameters in European flat oyster *Ostrea edulis* families obtained from brood stocks of different geographical origins and relation with infection by the protozoan *Bonamia ostreae*. Fish Shellfish Immunol. 24, 551–563.

Dittman, D.E., Ford, S.E., Padilla, D.K., 2001. Effects of *Perkinsus marinus* on reproduction and condition of the eastern oyster, *Crassostrea virginica*, depend on timing. J. Shellfish Res. 20, 1025–1034.

Figueras, A., 1991. *Bonamia* status and its effects in cultured flat oysters in the Ria de Vigo, Galicia (N.W. Spain). Aquaculture 93, 225–233.

Ford, S.E., Figueras, A., 1988. Effects of sublethal infection by the parasite *Haplosporidium nelsoni* (MSX) on gametogenesis, spawning, and sex ratios of oysters in Delaware Bay, USA. Dis. Aquat. Org. 4, 121–133.

Galtsoff, P.S., 1964. The American oyster *Crassostrea virginica* Gmelin. U.S. Fish Wildl. Serv., Fish. Bull. 64, 480.

Giese, A.C., 1959. Comparative physiology: annual reproductive cycles of marine invertebrates. Annu. Rev. Physiol. 21, 547–576.

Hawkins, L.E., Hutchinson, S., Brooks, J.D., Bucke, D., 1993. Differential responses of three populations of *Ostrea edulis* to environmental stress. In: Aldrich, J.C. (Ed.), Quantified Phenotypic Responses in Morphology and Physiology. JAPAGA, Ashford, UK, pp. 207–214.

Hine, P.M., 1991. The annual pattern of infection by *Bonamia* sp. in New Zealand flat oysters, *Tiostrea chilensis*. Aquaculture 93, 241–251.

Howard, D.W., Smith, C.S., 1983. Histological techniques for bivalve mollusks. NOAA technical memorandum NMFS-F/NEC-25. U.S. Dept. Commerce, Woods Hole, MA, USA.

Jeffs, A.G., Hickman, R.W., 2000. Reproductive activity in a pre-epizootic wild population of the Chilean oyster, *Ostrea chilensis*, from southerm New Zealand. Aquaculture 183, 241–253.

Kennedy, V.S., Newell, R.I.E., Krantz, G.E., Otto, S., 1995. Reproductive capacity of the eastern oyster *Crassostrea virginica* infected with the *Perkinsus marinus*. Dis. Aquat. Org. 23, 135–144.

Lacoste, A., Malham, S.K., Cueff, A., Poulet, S.A., 2001a. Noradrenaline modulates oyster hemocyte phagocytosis via a β-adrenergic receptor-cAMP signalling pathway. Gen. Comp. Endocrinol. 122, 252–259.

Lacoste, A., Malham, S.K., Cueff, A., Poulet, S.A., 2001b. Noradrenaline modulates hemocyte reactive oxygen species production via β-adrenergic receptors in the oyster *Crassostrea gigas*. Dev. Comp. Immunol. 25, 285–289.

Leonard Jr, V.K., 1969. Seasonal gonadal changes in two bivalve mollusks in Tomales Bay, California. Veliger 11, 382–390.

Loosanoff, V.L., 1962. Gametogenesis and spawning of the European oyster, *O. edulis*, in waters of Maine. Biol. Bull. 122, 86–94.

Lynch, S.A., Armitage, D.V., Wylde, S., Mulcahy, M.F., Culloty, S.C., 2005. The susceptibility of young prespawning oysters, *Ostrea edulis*, to *Bonamia ostreae*. J. Shellfish Res. 24, 1019–1026.

Mann, R., 1979. Some biochemical and physiological aspects of growth and gametogenesis in *Crassostrea gigas* and *Ostrea edulis* grown at sustained elevated temperatures. J. Mar. Biol. Assoc. U.K. 59, 95–110.

Montes, J., Acosta, C.P., Guerra, A., 1989. Oyster mortality in Galicia (NW Spain). In: de Paw, N., Jaspers, E., Ackeford, H., Wilkins, N. (Eds.), Aquaculture a Biotechnology in Progress. European Aquaculture Society, Bredene, UK, pp. 941–948.

Montes, J., Villalba, A., López, M.C., Carballal, M.J., Mourelle, S.G., 1991. Bonamiasis in native flat oysters (*Ostrea edulis* L.) from two intertidal beds of the Ortigueira Estuary (Galicia, N.W. Spain) with different histories of oyster culture. Aquaculture 93, 213–224.

Orton, J.H., 1927. Observations and experiments on sex-change in the European oyster (*O. edulis*). Part I. The change from female to male. J. Mar. Biol. Assoc. 14, 967–1045.

Orton, J.H., 1933. Observations and experiments on sex-change in the European oyster (*O. edulis*). Part III. On the fate of unspawned ova. Part IV. On the change from male to female. J. Mar. Biol. Assoc. 19, 1–53.

Ottaviani, E., Franchini, A., Genedani, S., 1999. ACTH and its role in immune-neuroendocrine functions. A comparative study. Curr. Pharm. Des. 5, 673–681.

Pascual, M.S., Iribarne, O.O., Zampatti, E.A., Bocca, A.H., 1989. Female–male interaction in the breeding system of the puelche oyster *Ostrea puelchana* d'Orbigny. J. Exp. Mar. Biol. Ecol. 132, 209–219.

Paynter, K.T., 1996. The effects of *Perkinsus marinus* infection on physiological processes in the eastern oyster, *Crassostrea virginica*. J. Shellfish Res. 15, 119–125.

Polanco, E., Montes, J., Outon, M.J., Melendez, M.I., 1984. Situation pathologique du stock d'huîtres plates en Galice (Espagne) en relation avec *Bonamia ostreae*. Haliotis 14, 91–95.

Robert, R., Borel, M., Pichot, Y., Trut, G., 1991. Growth and mortality of the European oyster *Ostrea edulis* in the bay of Arcachon (France). Aquat. Living Resour. 4, 265–274.

Román, G., 1984. Estudio sobre la reproducción de la ostra (*Ostrea edulis* L.) cultivada en bateas en la Ría de Arousa. Cuadernos da Área de Ciencias Mariñas, Seminario de Estudos Galegos 1, 405–425.

Román, G., 1992. Efecto del ciclo reproductivo y el acondicionamiento en el desarrollo larvario, la fijación y el posterior crecimiento de la semilla de *Ostrea edulis* Linné 1758. Publ. Espec. - Inst. Esp. Ocean. 1–172.

Ruiz, C., Martínez, D., Mosquera, G., Abad, M., Sánchez, J.L., 1992. Seasonal variations in condition, reproductive activity and biochemical composition of the flat oyster, *Ostrea edulis*, from San Cibran (Galicia, Spain). Mar. Biol. 112, 67–74.

Sastry, A.N., 1979. Pelecypoda (excluding Ostreidae). In: Giese, A.C., Pearse (Eds.), Reproduction in Marine Invertebrates. Molluscs: Pelecypods and Lesser Classes. Academic Press, New York, NY, pp. 113–292.

Siddiqui, G., Ahmed, M., 2002. Gametogenic patterns of the larviparous oyster *Ostrea nomades* from Karachi, Pakistan (northern Arabian Sea). Aquac. Res. 33, 1049–1058.

Sparck, R., 1925. Biology of the oyster (*Ostrea edulis*) in Limfjord, with special reference to the influence of temperature and the sex-change. Rep. Dan. Biol. Sta. 30, 1–84.

Thompson, R.J., Newell, R.I.E., Kennedy, V.S., Mann, R., 1996. Reproductive processes and early development. In: Kennedy, V.S., Newell, R.I.E., Eble, A.F. (Eds.), The Eastern Oyster *Crassostrea virginica*. Maryland Sea Grand, Maryland, USA, pp. 335–370.

Tigé, G., Grizel, H., Rabouin, M., Cochennec, N., Audic, G., Langlade, A., 1982. Maladie hemocitaire de l'huître plate causée par *Bonamia ostreae*: évolution de la situation epizootiologique en Bretagne au cours de l'année 1981. Bull. Inst. Pêches Marit. 328, 3–13.

Tun, K.L., Itoh, N., Komiyama, H., Ueki, N., Yoshinaga, T., Ogawa, K., 2006. Comparison of *Marteilioides chungmuensis* infection in the Pacific oyster *Crassostrea gigas* cultured in different conditions. Aquaculture 253, 91–97.

van Banning, P., 1990. The life cycle of the oyster pathogen *Bonamia ostreae* with a presumptive phase in the ovarian tissue of the European flat oyster, *Ostrea edulis*. Aquaculture 84, 189–192.

Villalba, A., Mourelle, S.G., Carballal, M.J., López, C., 1993. Effects of infection by the protistan parasite *Marteilia refringens* on the reproduction of cultured mussels *Mytilus galloprovincialis* in Galicia (NW Spain). Dis. Aquat. Org. 17, 205–213.

Villalba, A., Reece, K.S., Ordás, M.C., Casas, S.M., Figueras, A., 2004. Perkinsosis in molluscs: a review. Aquat. Living Resour. 17, 411–432.

Wilson, J.H., Simons, J., 1985. Gametogenesis and breeding of *Ostrea edulis* on the west coast of Ireland. Aquaculture 16, 307–321.