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Epidemiology of *Bonamia ostreae* infecting European flat oysters *Ostrea edulis* from Lake Grevelingen, The Netherlands

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ABSTRACT: Production of European flat oysters *Ostrea edulis* in the Netherlands has been hampered by the presence of the haplosporidian parasite *Bonamia ostreae*, which is now an enzootic species following its establishment after 1980. We analyzed histopathological data from annual shellfish disease monitoring from 1988 to 2006 to quantify prevalence of *B. ostreae* in flat oyster stocks of the marine Lake Grevelingen. In addition, we estimated prevalence of *B. ostreae* on a monthly basis with field surveys in 2003. The parasite was detected with PCR, using specific primers for *B. ostreae*. Prevalence of *B. ostreae* was analyzed relative to *O. edulis* density, biomass and a range of environmental parameters. *B. ostreae* was detected in flat oysters throughout the year with a higher prevalence in spring than in autumn, possibly due to termination of spawning and the onset of oyster growth in autumn. Although *B. ostreae* was detected in all oyster weight classes, prevalence was highest in the largest oysters in spring and declined disproportionately in autumn, possibly due to high mortality of large oysters before autumn, suggesting that prevalence depends on oyster age. Parasite prevalence was independent of oyster density and total biomass, but appeared to be higher after a warm autumn. Abundance of the flat oyster (infected or non-infected with *B. ostreae*) was negatively related to the temperature of the preceding period, suggesting that mortality in flat oysters increased at higher water temperatures. Furthermore, *O. edulis* appeared to be more susceptible to *B. ostreae* after years with lower food availability and lower salinities (<29.5). *B. ostreae* may weaken the competitive ability of *O. edulis* relative to the introduced Pacific oyster *Crassostrea gigas*, particularly in years with high water temperatures.

KEY WORDS: *Bonamia ostreae* · *Ostrea edulis* · Oyster · Disease · Population dynamics · Environmental factors

INTRODUCTION

Production of European flat oysters *Ostrea edulis* in the Netherlands has gradually declined since the mid-twentieth century, partially due to a severe winter (1963) and to food and space competition with the introduced Pacific oyster *Crassostrea gigas*. Moreover, the introduction of the protozoan parasite *Bonamia ostreae* has had a dramatic impact on the *O. edulis* population (Van Banning 1991). *B. ostreae* (Pichot et al. 1980) causes the lethal disease bonamiosis in *O. edulis*, whereas *C. gigas* is not susceptible to this disease (Renault et al. 1995). Similar to the closely related parasite *B. exitiosa* (Hine et al. 2001), it invades the

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haemocytes of its host (Balouet et al. 1983). *B. ostreae* was first reported in 1979 in France (Pichot et al. 1980), possibly after importation of infected *O. edulis* from California (Elston et al. 1986). Subsequently, the parasite was also detected in Ireland, Spain, the UK and the Netherlands (Van Banning 1982, Bucke et al. 1984, Polanco et al. 1984, Rogan et al. 1991).

In the Netherlands, oyster culture is concentrated in the Oosterschelde estuary and in the marine Lake Grevelingen. Lake Grevelingen is an artificially enclosed estuary that is relatively shallow and has limited seawater exchange with the North Sea. After the initial observation of *Bonamia ostreae* in the neighbouring Oosterschelde (Yerseke Bank area) in 1980, attempts to eradicate the pathogen failed (Van Banning 1985, 1987). Strict hygiene measures for shellfish farmers prevented an outbreak of bonamiosis in Lake Grevelingen until 1988, when *B. ostreae* was first observed there (Van Banning 1991). The parasite quickly spread throughout commercial oyster beds and wild oyster stocks. The prevalence of *B. ostreae* in September–October 1989 was up to 46%, while mortality of flat oysters reached 80% in some locations (Van Banning 1991).

Currently, commercial culture of flat oysters in the Oosterschelde is negligible, whereas in Lake Grevelingen it takes place as bottom culture mixed with Pacific oysters at 2 to 8 m water depths. Oysters are produced from natural spat fall using cultch (mussel shell) as settlement substrate. Annual production of flat oysters in Lake Grevelingen ranges from 50 to 100 tonnes (Dutch Fish Product Board unpubl. data).

In shellfish disease monitoring, histopathology is the recommended surveillance method for detection of *Bonamia ostreae* (OIE 2009). With the recent development of molecular techniques, e.g. PCR and *in situ* hybridisation, detection of *B. ostreae* has improved (Carnegie et al. 2000, Cochennec et al. 2000, Corbeil et al. 2006). Consequently, we used species-specific primers for rapid detection of *B. ostreae* by PCR.

Whereas there are many studies on the epizootiology of *Bonamia ostreae* (Tigé et al. 1984, Montes & Meléndez 1987, Montes et al. 1989, Cáceres-Martínez et al. 1995, Conchas et al. 2003), few have investigated the interaction between host, parasite and environment (Culloty & Mulcahy 1996). We investigated the influence of the host population and environmental factors upon prevalence of *B. ostreae* in an area where it has been present for nearly 2 decades. Data from 3 monitoring programmes in Lake Grevelingen were analysed. Survey I was an annual monitoring programme carried out from 1980 to determine the status of bonamiosis and other shellfish diseases in Dutch coastal waters, with an emphasis on prevalence of *B. ostreae*, using histological techniques for detection of the parasite, from the first outbreak in 1988 through 2006. Survey II was a seasonal survey conducted in 2003; prevalence was analysed using specific primers and PCR. In Survey III, data from both monitoring programmes were compared with data on density and biomass of *Ostrea edulis* from a monitoring programme of the macrobenthic assemblages.

MATERIALS AND METHODS

Sample collection. From 1988 to 2006, Survey I monitored *Bonamia ostreae* prevalence in flat oyster stocks in spring (May–June) and autumn (October–November) at 5 or 6 locations selected from a total of 8 locations in Lake Grevelingen (Fig. 1). The sites were natural beds in close proximity to commercial lots. Samples were collected by trawling a 100 m transect with a 1 m wide oyster dredge. At each location, 25 *Ostrea edulis* were randomly sampled for histological examination. In the laboratory, whole wet weight (including shell) and length (maximum shell length) of each oyster were recorded. A transverse section of the tissue, including digestive diverticulum, mantle and gills, was fixed in Davidson solution (Shaw & Battle 1957).

In Survey II, a sample of 100 *Ostrea edulis* was taken from a commercial lot in Lake Grevelingen in each month of 2003. Density of the oyster stock was estimated on each sampling occasion. Whole wet weight and length of each oyster were measured. From each oyster a transverse section of the tissue was fixed in Davidson solution and a second transverse section fixed in 95% ethanol for histology and DNA extraction, respectively.

Within the frame of the Monitoring Programme of the National Water Systems (MWTL), the Centre for Estuarine and Marine Ecology (NIOO-CEME) has monitored the macrobenthic faunal assemblages of the Dutch delta (BIOMON monitoring) biannually since 1990 (spring: April–May and autumn: September–October; Survey III). In 1990–1994, samples were taken randomly within 3 strata, 0–2, 2–6 and >6 m depth, both in the western and eastern regions of Lake Grevelingen. In 1990 and 1991, more than 60 samples were taken per survey, including some from the central region. From autumn 1994, 30 fixed locations were sampled, 15 each in the eastern and western regions. The samples within each region were equally distributed over the 3 strata. As the total area of each of the strata in Lake Grevelingen is equal (16.0 ± 1.6 km^2), average densities and biomass per species for each of the strata were combined to calculate total densities and biomass for the soft sediment substrates for the entire lake. The samples from the 0–2 m stratum were

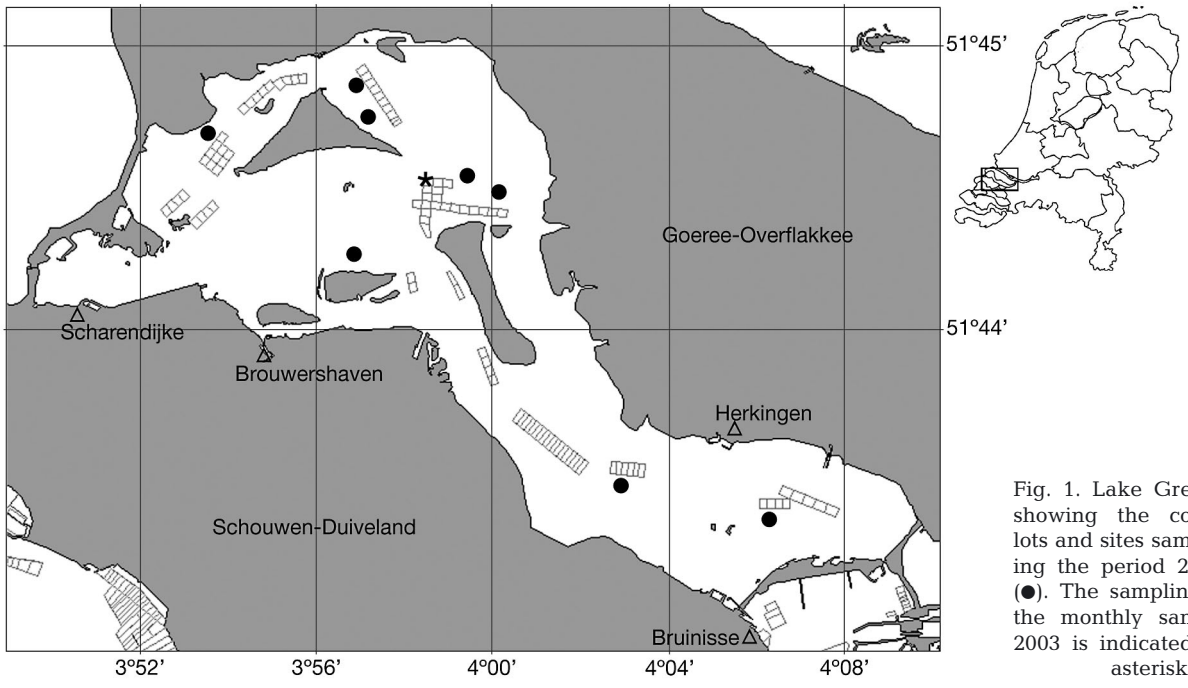

Fig. 1. Lake Grevelingen, showing the commercial lots and sites sampled during the period 2000–2006 (●). The sampling site for the monthly sampling in 2003 is indicated with an asterisk

taken using a 0.020 m^2 flushing sampler, sucking sediment with macrofauna and overlaying water, and sieved over a 1 mm mesh. Deeper samples were taken with a Reineck box corer from which 3 cores of 8 cm diameter (combined surface 0.0150 m^2) were taken to an approximate depth of 25 cm. These samples were also sieved over a 1 mm mesh. All animals, except Oligochaeta, Actinaria and Nemertea, were counted and identified to species in the laboratory. The length (maximum shell length) of all bivalves was recorded, and the ash-free dry weight (AFDW; difference between dry weight after 2 d at 80°C and ash weight after 2 h at 560 to 580°C) was measured, or calculated from length–weight regressions or fresh weight to AFDW conversion factors. From these data, the presence of *Ostrea edulis* (number of samples with *O. edulis* divided by the total number of samples), the average density (no. m^{-2}) and the average biomass (mg AFDW m^{-2}) were calculated. Density and biomass of *Crassostrea gigas* were also determined. All macrofauna data were stored in the local Benthos Information System database (BIS, version 1.20.0).

Several abiotic parameters were extracted from www.waterbase.nl, the online database service from the National Institute for Coastal and Marine Management of the Dutch Ministry of Transport, Public Works and Water Management (RWS-RIKZ) for the surface water near Dreischor (central part of Lake Grevelingen) over 1990 to 2006 on a monthly basis: surface water turbidity (Secchi disc visibility, dm), pH, oxygen content (%), dissolved organic carbon (DOC, mg l^{-1}), nitrate (NO_3^-, mg l^{-1}) and chlorophyll *a* (chl *a*, µg l^{-1}). Salinity and temperature (°C) data from the same period, measured at the same location 1 m below the surface of the water, were kindly provided by RWS-RIKZ.

Histological processing and examination. After at least 24 h fixation in Davidson solution, the oyster tissue was embedded in paraffin blocks. Sections of tissue 4 to 5 µm thick were stained with haematoxylin and eosin (H&E), and the slides examined for the presence of *Bonamia ostreae* infections using light microscopy. The intensity of *B. ostreae* infection was classified semi-quantitatively as adapted from the Bachère et al. (1982) scale: Class 0: no *B. ostreae* observed; Class I: light infection, few haemocytes observed with 1 to 2 parasites in the cytoplasm; Class II: moderate infection, infected haemocytes present in large numbers with multiple parasites in the cytoplasm; and Class III: heavy infection, *B. ostreae* widespread throughout the host and numerous parasites in the cytoplasm of each haemocyte.

DNA extraction and PCR. Material fixed in 95% ethanol was used for PCR detection of *Bonamia ostreae*. Total DNA was extracted from approximately 20 mg of oyster tissue from the digestive diverticula or gills. A commercial kit, QIAamp DNA Mini kit (Qiagen), was used for DNA extraction according to the manufacturer's protocol. The extractions were stored at –20°C.

For detection of *Bonamia ostreae*, specific primers for 18S ribosomal RNA were used (BoosF03 3′-

CAA TGG TGC GTT CAA CGA GT-5′ and BoosR03 3′-GGG TTC GCG GTT GAA TTT TA-5′) with an expected product size of 352 bp. The PCR reaction mixtures consisted of 0.4 µM of each primer, PCR buffer at 1× concentration, 2 mM $MgCl_2$, 0.2 mM of each deoxynucleotide (dNTP mixture, Takara Bio) and 2 units Taq DNA Polymerase (Invitrogen) in distilled water with 0.005% (v/v) Nonidet P-40 (NP-40). Two µl of template DNA was added to the mix to make a final volume of 50 µl. A reaction with 2 µl distilled water added to the mix served as a control for each PCR run. Furthermore, a *Bonamia*-positive field sample confirmed by histology and a known *Bonamia*-negative field sample were used as controls for the reaction. The conditions of the PCR were 94°C for 2 min followed by 40 cycles of 94°C for 30 s, 58°C for 30 s, 72°C for 45 s and finally extension at 72°C for 7 min with a GeneAmp 9700 thermocycler (Applied Biosystems). The products were electrophoresed on a 1.5% agarose gel with ethidium bromide and subsequently visualised using GeneSnape 4.0 (Syngene). Where only a faint band was visible on the agarose gel, the sample was reanalysed; it was processed again from the ethanol-fixed material to exclude a false-positive result due to cross-contamination. To determine the specificity of the PCR, a positive sample for *B. exitiosa* (kindly provided by M. Hine, Fouras, France) and 3 flat oyster samples positive for *Haplosporidium armoricanum* (field samples from Oosterschelde, the Netherlands) were tested with the primer set BoosF03–BoosR03.

As control for the DNA extraction, a PCR with specific primers for *Ostrea edulis* 18S rRNA (OsedF01 3′-GTA AAC CCT TGG TGC TCT TGA CT-5′; OsedR01 3′-CCG AGT CAT TGA AGC AAC TCC-5′) was run for each isolation using the PCR conditions as described above. The expected product size of this primer set is 375 bp.

Statistical analysis. Regression analyses and testing for significant differences in prevalence between size groups and seasons by means of ANOVA were executed in SYSTAT 11 (Systat Software). In order to cope with the bias introduced with multiple testing, a Bonferroni correction on the significance level ($p = 0.05/\alpha$, in which α is the number of tests of the same kind) was additionally applied to identify significant regressions (Sokal & Rohlf 1995). The agreement between the results obtained by histopathology and PCR testing was assessed by kappa statistics (Cohen 1960), calculated using Win Episcope 2.0 (available at www.clive.ed.ac.uk/winepiscope/).

Table 1. Evaluation of histopathology and PCR for detection of *Bonamia ostreae* in a random selected number of samples from the 2003 monitoring

		Histology		Total
		Positive	Negative	
PCR	Positive	14	10	24
	Negative	2	88	90
Total		16	98	114

RESULTS

Evaluation of the PCR assay for the detection of *Bonamia ostreae*

The described PCR was developed to be specific for *Bonamia ostreae*. Testing with the related parasites *B. exitiosa* and *Haplosporidium armoricanum* showed no evidence of cross-reactivity (data not shown). Sequencing of a random number of PCR products confirmed the amplicon to be *B. ostreae* 18S rRNA (n = 7, data not shown). In order to evaluate the PCR test the results were compared with results obtained by histopathology. For this, 10 samples from every month of the monitoring in 2003 were randomly selected and analysed 'blind' by 2 scientists and compared with the PCR results (Table 1). The PCR test showed a higher sensitivity (calculated sensitivity, $[14/16] \times 100 = 88\%$) compared with histopathology (calculated sensitivity, $[14/20] \times 100 = 58\%$). Evaluation of the level of agreement between the histopathology and PCR test resulted in a kappa value of 0.64, indicating a substantial agreement between both tests (Landis & Koch 1977).

Annual monitoring of *Bonamia ostreae* 1988–2006

The number of *Ostrea edulis* infected with *Bonamia ostreae* from the 1988–2006 monitoring in Lake Grevelingen was determined by histopathological screening of H&E stained slides. The average prevalence of oysters infected with *B. ostreae* over the 1989–2006 period in spring (14.8 ± 8.3%) was significantly higher than in autumn (5.6 ± 3.6%; $p = 0.007$; Fig. 2). Two peaks in the prevalence of *B. ostreae* could be distinguished in the spring of 1991 and 1999, respectively, and a lower peak in 2003. In the last 6 yr the percentage of *B. ostreae*-infected flat oysters seemed to have increased, with higher prevalence in autumn in particular, compared to the years before.

Relating *Bonamia ostreae* prevalence to the abundance (presence, density and biomass) of *Ostrea edulis* in Lake Grevelingen as indicated by the BIOMON macrobenthos monitoring revealed no significant patterns (Table 2). Yet *O. edulis* density and biomass

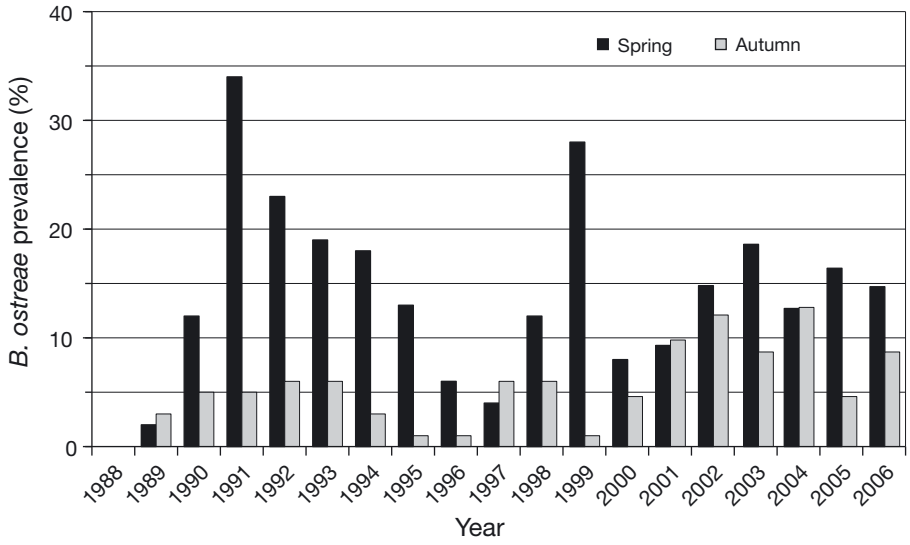

Fig. 2. Prevalence of *Bonamia ostreae* in *Ostrea edulis* from Lake Grevelingen, the Netherlands, in spring and autumn during the period 1988–2006 as detected by histopathology

in autumn were positively significantly related to *B. ostreae* prevalence in spring (the peak) ($p = 0.005$ and $p = 0.001$, respectively) of the year before (Fig. 3, Table 3). A higher prevalence of parasites was thus followed by a year with a higher density and biomass of oysters.

To investigate the relationship between prevalence of *Bonamia ostreae* and oyster size, oysters collected from 2000 to 2006 were divided into length classes of ≤60, 61–75, 76–90 and ≥91 mm and weight classes of ≤30, 31–60, 61–90 and ≥91 g (Fig. 4). In general there was a clear increase in the prevalence of parasites with increasing size of oysters in spring. In autumn, however, this increase in prevalence occurred only in the smaller to intermediate sized oysters, whereas in the largest oysters the prevalence of parasites was significantly reduced (from >20% in spring to <5% in autumn; $p < 0.001$). The distribution of infection intensity varied between years (Fig. 5), but no clear pattern in time (seasonality) could be discerned. The distribution of infection intensity over either the oyster length or weight classes showed that the largest sized oyster classes displayed the lowest percentage of oysters with the highest intensity of infection.

Table 2. Dependence of *Bonamia ostreae* prevalence (%) on *Ostrea edulis* abundance (presence, density and biomass) and environmental conditions. Relations tested for are indicated with an ×; p-values and whether relations are positive or negative are indicated when $p < 0.05$; significant regressions ($p < 0.0083$, after Bonferroni correction) are in **bold**. Year average: average of the year in which the factor is recorded; Spring/Autumn: average of spring/autumn of the year in which the factor is recorded; Year –1: year average of 1 yr before; Spring –1: average of the factor in the spring of 1 yr before; Autumn –1: average of the factor in the autumn of 1 yr before; Autumn –2: average of the factor in the autumn of 2 yr before

	Year average	Year –1	Spring	Spring –1	Autumn –1	Autumn –2
	***Ostrea edulis* presence**					
Year average		×				
Spring			×	×	×	
Autumn			×		×	
	***Ostrea edulis* density**					
Year average		×				
Spring			×	×	×	
Autumn			×		×	
	***Ostrea edulis* biomass**					
Year average		×				
Spring			×	×	×	
Autumn			×		×	
	Secchi (dm)					
Spring	+0.020	×	×	×	**+0.003**	×
	pH					
Spring	×	−0.024	×	×	×	×
	DOC (mg l^{-1})					
Spring	×	×	×	−0.011	×	×
	O_2 (%)					
Spring	+0.027	×	×	×	+0.043	×
	Salinity					
Spring	×	×	×	×	×	**+0.002**
	NO_3^- (mg l^{-1})					
Spring	×	×	×	×	×	×
	Chlorophyll *a* (µg l^{-1})					
Spring	×	−0.022	×	×	×	×

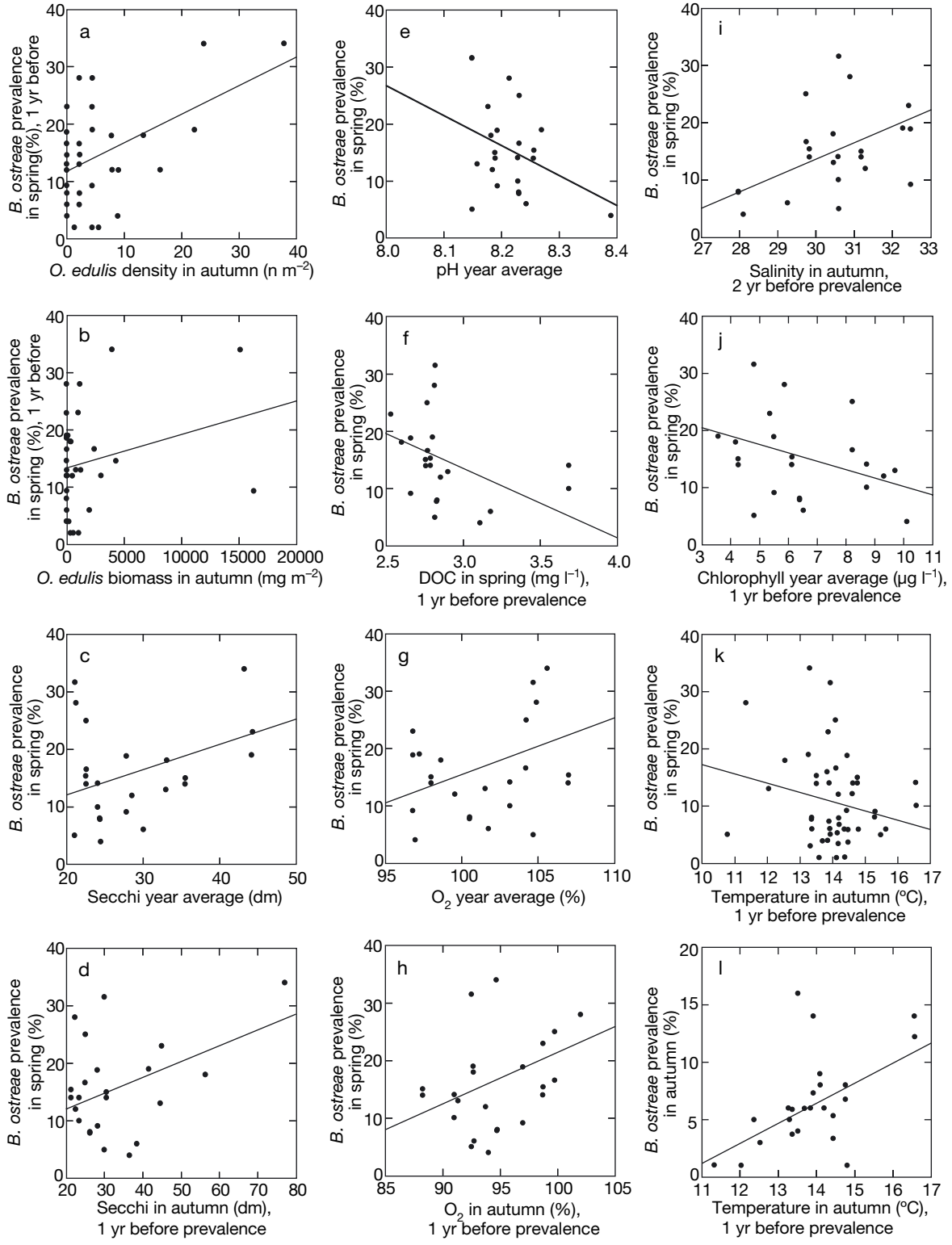

Fig. 3. *Bonamia ostreae* prevalence related to *Ostrea edulis* abundance and environmental factors for Lake Grevelingen during 1989–2006. Regressions with $p < 0.05$ (equations in Table 2 & 3) are shown. *B. ostreae* prevalence (%) in spring related to (a) *O. edulis* density (no. m^{-2}); (b) *O. edulis* biomass (mg m^{-2}); (c) surface water turbidity as Secchi year average (dm) in autumn of the previous year; (d) Secchi in autumn the previous year (dm); (e) pH year average; (f) dissolved organic content of the surface water (mg l^{-1}) the previous year; (g) year average oxygen content (%); (h) oxygen content (%) in autumn the previous year; (i) salinity in autumn 2 yr before; (j) chlorophyll *a* content year average (µg l^{-1}) the previous year; and (k) average autumn water temperature (°C) of the previous year. (l) *B. ostreae* prevalence (%) in autumn related to average autumn water temperature (°C) of the previous year

As *Bonamia ostreae* prevalence or *Ostrea edulis* susceptibility might also be related to environmental conditions, we analysed the data for any possible relationship between prevalence of parasites and the monthly mean levels of various abiotic parameters. A significant positive relationship was found between the prevalence of *B. ostreae* in spring (the peak) and Secchi disc visibility in the autumn of the year before, and salinity in the autumn of 2 yr before (Fig. 3, Table 2). A significant relationship was also observed between prevalence of *B. ostreae* in autumn and temperature in the autumn of the year before (Fig. 3, Table 4). Other environmental factors also tended to show a relationship with parasite prevalence (Table 2), but were not significant.

Throughout the research period 1990–2006, when *Bonamia ostreae* was present in Lake Grevelingen, the percentage of samples in the BIOMON monitoring containing *Ostrea edulis* declined. However, the number of samples containing *Crassostrea gigas* increased, leading to a significant ($p = 0.048$) negative relationship between the presence of the 2 species (Fig. 6). Furthermore, the presence or density of *O. edulis* was negatively related to the water temperature of the preceding seasons, while the presence or density of *C. gigas* was positively related to water temperature (Table 4).

Table 3. Dependence of *Ostrea edulis* abundance (presence, density and biomass) on *Bonamia ostreae* prevalence (%) and *Crassostrea gigas* abundance. Relations tested for are indicated with an ×; p-values and whether relations are positive or negative are indicated when $p < 0.05$; significant regressions ($p < 0.0063$ for effects of *B. ostreae* prevalence or $p < 0.05$ for effects of *C. gigas* abundance after Bonferroni correction) are in **bold**. Year average: average of the year in which the factor is recorded; Spring/Autumn: average of spring/autumn of the year in which the factor is recorded; Year –1: year average of 1 yr before; Spring –1: average of the factor in the spring of 1 yr before; Autumn –1: average of the factor in the autumn of 1 yr before; Autumn –2: average of the factor in the autumn of 2 yr before

	Year average	Year –1	Spring	Spring –1	Autumn –1	Autumn –2
***O. edulis* presence**	***Bonamia ostrea* prevalence (%)**					
Year average	×	×				
Spring				×	×	×
Autumn			×	×	×	
	***Crassostrea gigas* presence, year average**					
Year average	**–0.048**					
***O. edulis* density**	***Bonamia ostrea* prevalence**					
Year average	×	×				
Spring				×	×	×
Autumn			×	**+0.005**	×	
	***Crassostrea gigas* density, year average**					
Year average	×					
***O. edulis* biomass**	***Bonamia ostrea* prevalence**					
Year average	×	×				
Spring				×	×	×
Autumn			×	**+0.001**	+0.047	
	***Crassostrea gigas* biomass, year average**					
Year average	×					

Seasonal prevalence of *Bonamia ostreae* in 2003

Each month from December 2002 to December 2003, a sample of 100 flat oysters was collected from one site in Lake Grevelingen. The mean weight of the collected

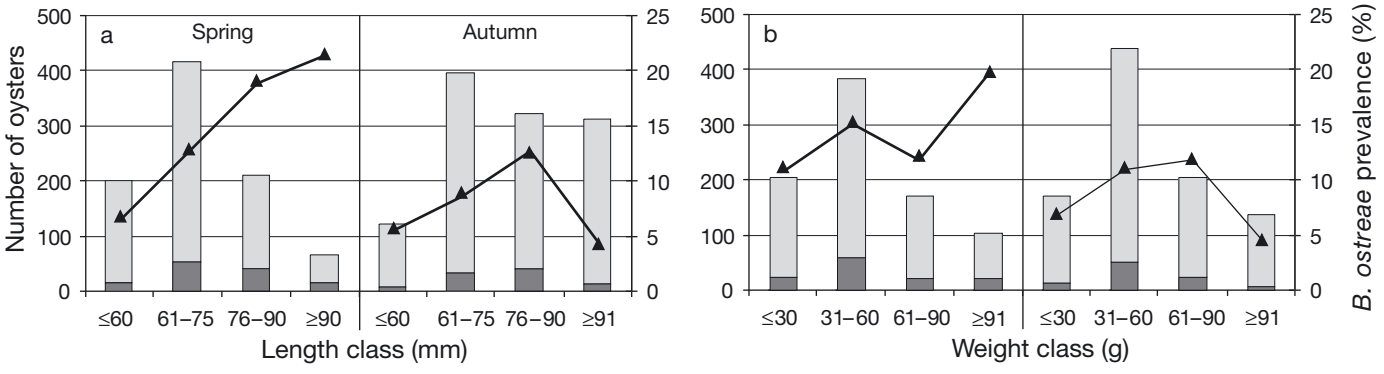

Fig. 4. Distribution of *Bonamia ostreae*-infected specimens of *Ostrea edulis* over size classes in spring and autumn based on data of the 2001–2006 surveys. Number of oysters shown as bars, with infected specimens shown in dark grey. Percentage oysters infected with *B. ostreae* is shown by triangles. *B. ostreae* prevalence related to (a) oyster length (mm) and (b) oyster weight (g)

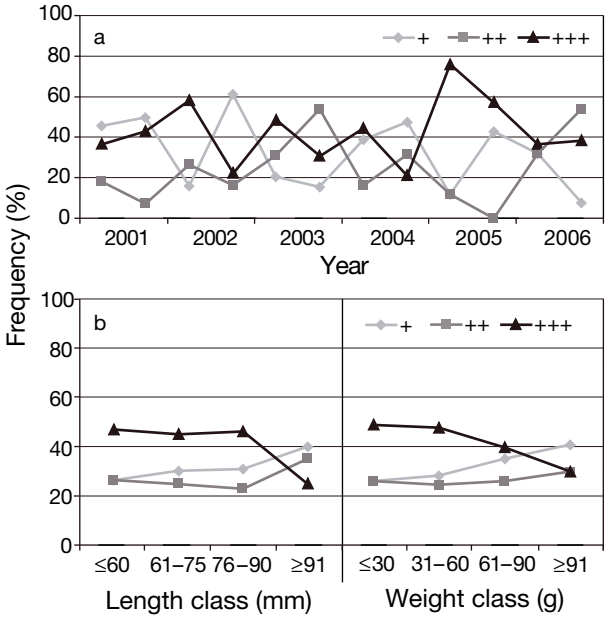

Fig. 5. *Bonamia ostreae* and *Ostrea edulis*. (a) Frequency of *B. ostreae* infection intensity in *O. edulis* during the period 2001–2006 semi-quantitatively assessed by histopathology. (b) Frequency of *B. ostreae* infection intensity related to oyster length (mm) and weight (g)

flat oysters was 66.6 ± 28.5 g. The estimated densities of the flat oysters on the site varied from month to month but, on average were 30 ± 16 flat oysters per 100 m^2.

Each month 60 oysters were analysed for the presence of *Bonamia ostreae* using PCR. The total DNA from each specimen (ethanol fixed tissue) was extracted and *B. ostreae*-infected specimens were identified. Flat oysters infected with *B. ostreae* were found throughout the year. The highest prevalence of *B. ostreae* was in April, with 37.7% of the flat oysters infected, while the lowest prevalence was in June at 13.3% (Fig. 7). In general, *B. ostreae* prevalence seemed to decrease from spring to autumn (from April to September, although not significant). Consequently, irrespective of the weight class, significant differences between the seasons ($p = 0.019$) could be observed in 2003 with a higher prevalence of parasites in spring than in autumn ($p = 0.043$) or summer ($p = 0.031$) (Fig. 8).

DISCUSSION

Two techniques were used for the detection of *Bonamia ostreae* in *Ostrea edulis* tissue: histopatho-

Table 4. Dependence of *Bonamia ostreae* prevalence (%) and *Ostrea edulis* and *Crassostrea gigas* abundance (presence, density and biomass) on temperature. Relations tested for are indicated with an ×; p-values and whether relations are positive or negative are indicated when $p < 0.05$; significant regressions ($p < 0.0042$ for effects of temperature after Bonferroni correction) are in **bold**. Year average: average of the year in which the factor is recorded; Spring/Summer/Autumn: average of spring/summer/autumn of the year in which the factor is recorded; Year –1: year average of 1 yr before; Spring –1: average of the factor in the spring of 1 yr before; Autumn –1: average of the factor in the autumn of 1 yr before

	Year average	Year –1	Spring	Spring –1	Summer	Autumn	Autumn –1
***Bonamia* prevalence**							
Spring	–0.016	×	–0.005	×			–0.045
Autumn	×	×	×		×	×	**+0.000**
***Ostrea edulis* presence**							
Spring	×	×	×	×			×
Autumn	–0.007	–0.009	**–0.001**		×	×	×
***Ostrea edulis* density**							
Spring	–0.012	×	×	×			×
Autumn	–0.027	**–0.001**	–0.018		×	×	×
***Ostrea edulis* biomass**							
Spring	×	×	×	×			×
Autumn	×	×	×		×	×	×
***Crassostrea gigas* presence**							
Spring	+0.046	×	×	×			**+0.000**
Autumn	+0.028	×	×		**+0.004**	**+0.000**	×
***Crassostrea gigas* density**							
Spring	×	×	×	×			**+0.001**
Autumn	×	×	×		**+0.000**		×
***Crassostrea gigas* biomass**							
Spring	×	×	×	×			×
Autumn	×	×	×		×		×

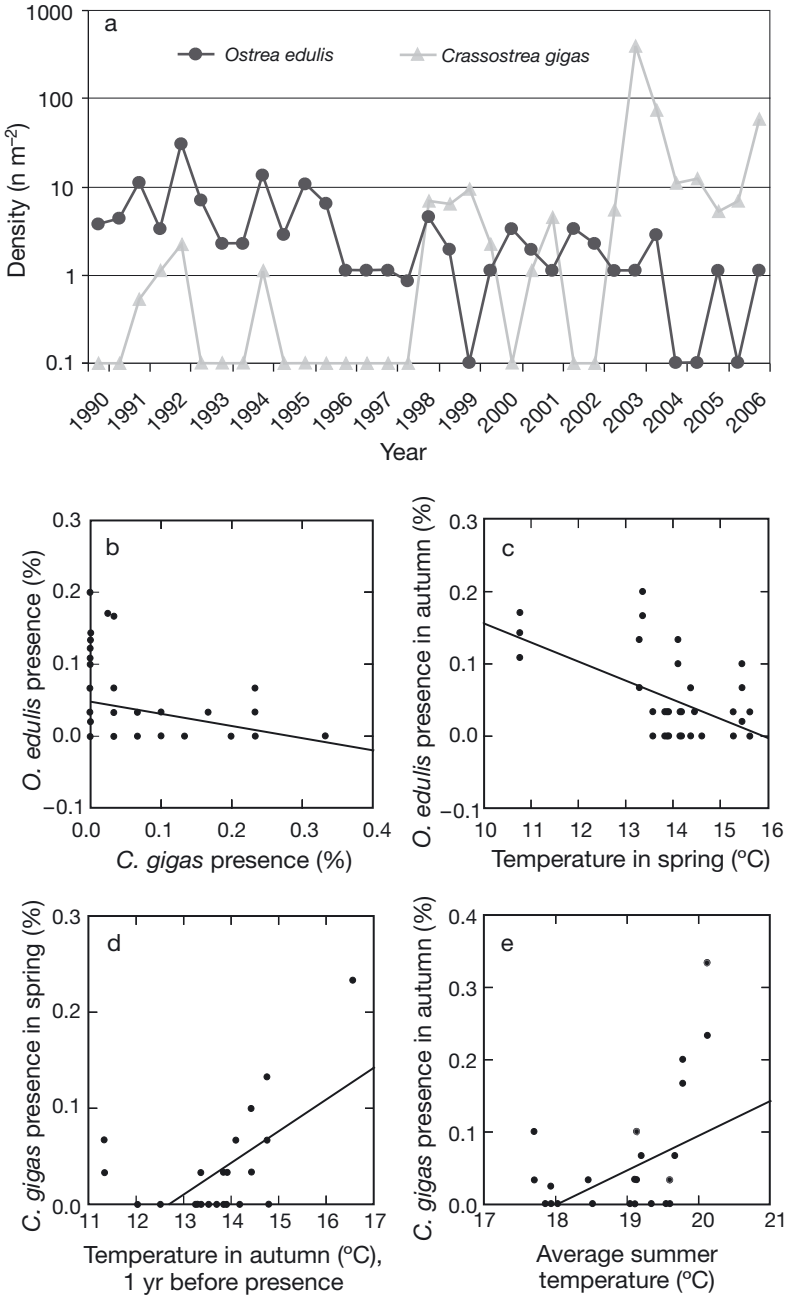

Fig. 6. (a) Densities of *Ostrea edulis* and *Crassostrea gigas* between 1990 and 2006 as calculated on the basis of the BIOMON data for the soft sediment area of Lake Grevelingen. (b) Presence of *O. edulis* related to the presence of *C. gigas*; (c) presence of *O. edulis* related to temperature; and (d,e) presence of *C. gigas* related to temperature

logy based on visible observation of the parasite in the host tissue and a PCR assay based on detection of the genetic material of the parasite. In 2003, comparison of the histopathological data with the May–June and October–November data of the PCR survey showed a higher prevalence of parasites detected by PCR. Discrepancies between the techniques are partly caused by the difference in methodology. This was also shown in the random selection and comparison of 114 samples from the 2003 monitoring, analysed both with histopathology and PCR. In general, PCR is considered more sensitive compared to histology (Balseiro et al. 2006, Marty et al. 2006). A low infection level might be detected by PCR, while no histopathological effects are yet visible. The small number (2) of samples displaying a positive score for *Bonamia* presence from histology observed in combination with a negative score from PCR (Table 1) could be explained by the fact that different pieces of tissue were used for the histopathology and for the DNA extraction. Besides the higher sensitivity, another advantage of PCR is that the technique is less time consuming. The disadvantage of using PCR in routine screening is that the technique selectively detects *B. ostreae*. Using histopathology it is possible to detect other pathogens in the oysters and to gain an impression of the health status of the oyster. Moreover, PCR does not discriminate between a viable or degraded parasite and is not conclusive on whether the actual location of the parasite is within its host.

In the present study, *Bonamia ostreae* was detected in flat oysters throughout the year, which supports findings in previous studies (Tigé et al. 1984, Cáceres-Martínez et al. 1995, Culloty & Mulcahy 1996). Data from the annual monitoring during 1988–2006 showed that in general the prevalence in spring was higher than in autumn. This was confirmed in the additional survey in 2003 with a clear increase during winter and early spring, a peak of *B. ostreae* infection in spring and a decreasing trend in prevalence towards the onset of autumn. Although a number of studies reported the prevalence of *B. ostreae* in flat oyster stocks in Europe (Tigé et al. 1984, Montes & Meléndez 1987, Culloty & Mulcahy 1996, Conchas et al. 2003), only a few focused on the association of the prevalence with season. Culloty & Mulcahy (1996) found fluctuations in prevalence when following 2 age groups for 2 yr. Although no consistent pattern in prevalence was observed, in accordance with the present study, high peaks were observed in winter and spring. From the data presented in Tables 1 & 2 of Tigé et al. (1984), it can be deduced that the peak in *B.*

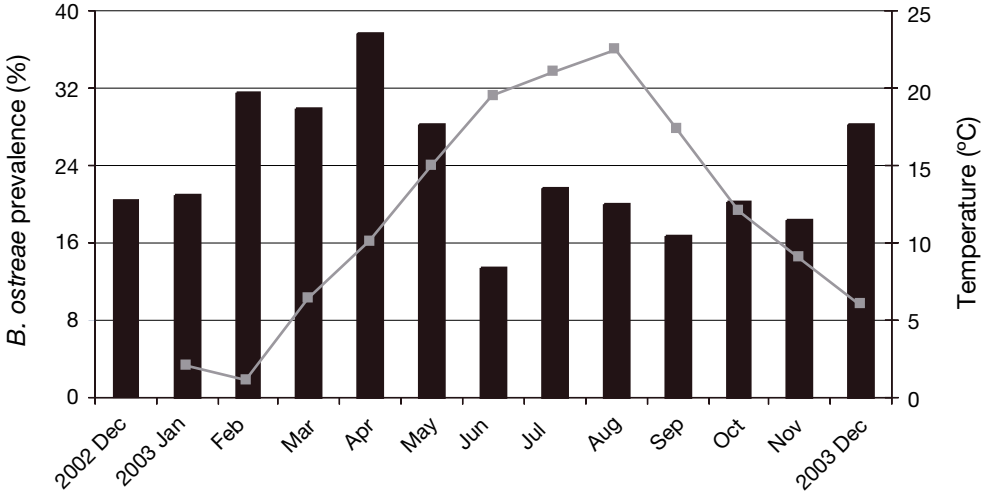

Fig. 7. *Bonamia ostreae*. Prevalence in Lake Grevelingen from December 2002 to December 2003 as detected by PCR (bars). Line graph gives water temperature as measured at 1 m depth at Dreischor at the time of sampling

ostreae prevalence in 1983 in Brittany occurred in April, while the prevalence in autumn was low.

In the present study the infection intensity distribution varied between years. This may indicate that periods of larger mortality are alternated with years of less mortality, and may also indicate that a strong infection is soon followed by mortality of the infected specimens. However, since there was no clear seasonal pattern, no link could be made with, for example, severe winters or hot summers.

With regard to oyster size, *Bonamia ostreae* was detected in oysters weighing as little as 13.8 g (data not shown). However, oysters in the ≥91 g weight class had a significantly higher *B. ostreae* prevalence in spring and significantly lower *B. ostreae* prevalence in autumn in the years 2000–2006. This would suggest that the impact of the disease was more pronounced in oysters with a higher weight. In the 2003 survey, the seasonal fluctuation in prevalence was reflected in all weight classes (Fig. 6). However, the decline in prevalence between spring and summer was most prominent in the 61–90 g weight class. This shows a clear relationship between the oyster size and *B. ostreae* prevalence. A similar observation on the increased prevalence of the parasite in larger individuals was reported by Cáceres-Martínez et al. (1995), though this was also observed in considerably smaller individuals (4 to 6 cm). Also, Culloty & Mulcahy (1996) observed a higher level of infection with increased age. In particular, the larger and hence older oysters appear to be more susceptible to *B. ostreae*. When considering either oyster length or weight classes, the largest sized oyster classes displayed a lower percentage of oysters with heavy intensity of infection. This may be the result of higher mortality rates in the highly infected group of the largest oysters.

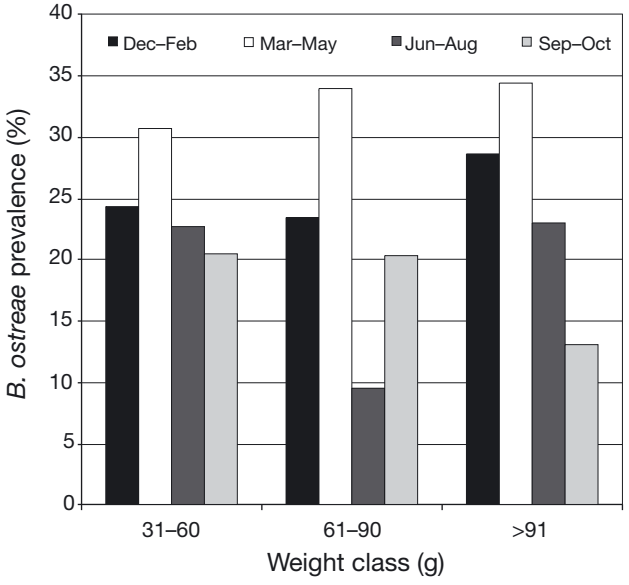

Fig. 8. Relationship between the prevalence of *Bonamia ostreae* with *Ostrea edulis* weight during the sampling from December 2002 to December 2003

Throughout the study, larger oysters appeared to be most affected by the parasite. This suggests that natural resistance to *Bonamia ostreae* is only acquired slowly by the oyster population, as the most susceptible/older oysters have already reproduced before infection develops. Culloty et al. (2004) compared the sensitivity of flat oysters to *B. ostreae* between oysters from different origins. Their study indicated that since 1988 the flat oyster population in Lake Grevelingen acquired limited reduced susceptibility to *B. ostreae*. Interestingly, the Lake Grevelingen population did not do well in terms of prevalence and intensity of infection, but performed well in terms of overall survival.

From the seasonal pattern of infection observed in the present study and the relationship between host size and parasite prevalence the following annual cycle is proposed: during summer and autumn the parasite infection rate remains fairly stable, but in the winter the condition of the oyster decreases and *Bonamia ostreae* spreads through the population, affecting in particular the larger specimens. In Northern Europe, spawning of *Ostrea edulis* starts at water temperatures of 12 to 14°C (Wilson & Simons 1985, Ruiz et al. 1992). On average, these temperatures are reached in Lake Grevelingen in May (1988–2006 data). Hence the spawning of the oysters coincides, especially in the larger, i.e. older, specimens, with the decrease in prevalence. On one hand, this might suggest that the conditional toll of spawning results in a relatively higher mortality in the infected oysters compared to non-infected oysters. On the other hand, the growth of (uninfected) small oysters in spring will lead to a decrease in the number of infected oysters. These phenomena probably co-occur.

A significant increase in oyster biomass, but also in oyster densities, was observed 1 yr after high parasite prevalence. Based on these results, we hypothesised that growth of smaller (uninfected) oysters compensates, or even overcompensates, for the loss of larger (infected) oysters due to mortality. Overcompensation points in the direction of better growing conditions, which might be the case at higher than average water temperatures (Haure et al. 1998, Naciri-Graven et al. 1999). On the other hand, mortality might also be increased at higher peak temperatures. It is also possible that *Bonamia ostreae* profits from other environmental conditions, or that *Ostrea edulis* populations are more susceptible under other environmental conditions. The results shown in Fig. 3 indicate that *B. ostreae* prevalence in spring is higher following an autumn with relatively clear water. These conditions coincide with lower DOC and chl *a* concentrations. These parameters might point to a higher susceptibility of flat oysters to *B. ostreae* infection after lower food availability. Furthermore, a positive relationship was observed between *B. ostreae* prevalence and salinity during the foregoing period. The results for salinity might indicate that a lower salinity is less suitable for *B. ostreae*. This is in agreement with a recent study, which showed that higher salinity supports better parasite survival (Arzul et al. 2009). Furthermore, unpublished observations have shown a correlation between salinity and prevalence of the parasite (I. Arzul pers. comm.). Recent studies on the related parasite *Bonamia* sp. in the Asian oyster *Crassostrea ariakensis* suggest similar results, with a possible affinity of the parasite for a high salinity environment in the field (Bishop et al. 2006), and a decreased prevalence and infection intensity at lower salinities under experimental conditions (Audemard et al. 2008).

As indicated by the significant negative relationship between the presence of *Ostrea edulis* and *Crassostrea gigas* in the monitoring samples (BIOMON results; Fig. 6), the 2 oyster species seem to compete for space and food. It is possible that *Bonamia ostreae* infection plays an important role in the competitive potential of *O. edulis*, and that *C. gigas* benefits from increased mortality of *O. edulis* after high prevalence, while during a period of low prevalence *O. edulis* can compete with the exotic oyster species. However, the percentage of *O. edulis* infected with *B. ostreae* has increased slightly during the last few years, which was temperature related. Yet higher temperatures also lead to higher infection and mortality rates, from which *C. gigas* can profit. At lower temperatures, the competitive power of *O. edulis* at low infection rates may be better, leading to a more suppressed abundance of *C. gigas*. The success of *C. gigas* might thus partly be due to the presence of *B. ostreae* in Lake Grevelingen.

In conclusion, the results of the present study show that nearly 2 decades after its introduction, *Bonamia ostreae* has become enzootic in the flat oyster population of Lake Grevelingen. The parasite infects a substantial number of flat oysters in Lake Grevelingen and shows a seasonality associated with the reproduction of the host. The susceptibility of *Ostrea edulis* to infection with *B. ostreae* seems to be related to the age of the oyster, food availability and water temperature. Furthermore, the prevalence of the parasite *B. ostreae* influences the competitive position of the 2 oyster species, the native *O. edulis* and the introduced *Crassostrea gigas*.

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