

Susceptibility of a number of bivalve species to the protozoan parasite *Bonamia ostreae* and their ability to act as vectors for this parasite

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ABSTRACT: The protozoan parasite *Bonamia ostreae* (Pichot et al., 1980) is a serious pathogen of the native European flat oyster *Ostrea edulis*. Field and laboratory based experiments were carried out in Ireland and Spain to investigate the susceptibility to this protozoan of the Pacific oyster *Crassostrea gigas*, the mussels *Mytilus edulis* and *Mytilus galloprovincialis*, the European native clam *Ruditapes decussatus* and the Manila clam *Ruditapes philippinarum*. Their ability to act as vectors or intermediate hosts was also investigated. The study corroborated previous findings that demonstrated that flat oysters can be successfully infected both naturally in the field and experimentally in the laboratory, and the pathogen can be transmitted directly between flat oysters in the laboratory. However, the other bivalves tested could not be infected with the parasite either naturally or experimentally, and these bivalves did not appear to act as vectors or intermediate hosts for the parasite. These results have implications for movements of bivalves between areas within the European Union.

KEY WORDS: *Bonamia ostreae* · Bonamiasis · *Ostrea edulis* · *Crassostrea gigas* · *Mytilus edulis* · *Mytilus galloprovincialis* · *Ruditapes decussatus* · *Ruditapes philippinarum* · Transmission experiments · European Union directives

INTRODUCTION

The flat oyster *Ostrea edulis* L. is a valuable bivalve mollusc species that is harvested in all major oyster growing areas throughout Europe. A drop in production in Europe since the 1960s has been mainly attributed to 2 parasites that have affected this species with devastating results, namely *Marteilia refringens* (Grizel et al. 1974) and *Bonamia ostreae* (Pichot et al., 1980). These protozoans have been responsible for significant mortalities of up to 90 % in some areas, and for a general drop in production, that has caused some oyster farmers to turn to alternative species to offset their losses.

In Europe, *Bonamia ostreae* was first detected in France in the late 1970s (Pichot et al. 1980). The com-

plete life cycle of this parasite remains uncertain. However, it appears that transmission can occur directly from oyster to oyster and that even when an area has been left fallow for a number of years the parasite persists, infecting flat oysters after they are reintroduced to the area (van Banning 1985, 1987). Three species of flat oyster, *Ostrea edulis*, *Tiostrea chilensis* (Hutton), and *Ostrea puelchana*, have been tested and have been found to be susceptible to this disease (Grizel et al. 1983, Bucke et al. 1984, Bougrier et al. 1986).

As direct transmission of this parasite occurs and uncertainties exist related to its life cycle, legislation has been implemented to restrict the movement of oysters within the European Union particularly from areas known to contain the disease (EU Directive 91/67). The susceptibility of other bivalves to this parasite has also been questioned: particularly those bivalves found within the same growing areas where oyster losses due

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to *Bonamia ostreae* have been recorded. Furthermore, the possibility that bivalves other than oysters could act as vectors or intermediate hosts of *B. ostreae* needs to be investigated, to ensure that this parasite is not transferred from one country to another within the European Union.

This study had 2 main objectives. Firstly, to determine whether a number of bivalve species such as the Pacific oyster *Crassostrea gigas* (Thunberg), the European native clam *Ruditapes decussatus* (L.), the Manila clam *R. philippinarum* (Adams and Reeve), and the mussels *Mytilus edulis* (L.) and *M. galloprovincialis* (Lmk.) are susceptible to *Bonamia ostreae* following either natural or experimental exposure; and secondly to determine whether these bivalve species, after exposure to *B. ostreae*, could act as vectors or intermediate hosts for *B. ostreae* and thus infect co-habiting uninfected flat oysters.

MATERIALS AND METHODS

Sites. Bivalve molluscs for the trials were obtained from a number of sites in Ireland including Cork Harbour (*Bonamia ostreae* present for over 10 yr), Tralee, Bantry Bay, Fountainstown and Sligo Bay (*B. ostreae* never detected in these areas), as well as Norfolk, England (*B. ostreae* never detected) and Villagarcia, Galicia, Spain (*B. ostreae* present for over 14 yr) (Fig. 1). Experiments were carried out in Cork (Ireland) and Vigo (Spain).

Diagnosis of *Bonamia ostreae*. Diagnosis of *B. ostreae* was carried out using heart, gill or hemolymph smears or examination of histological sections (Bachère et al. 1982, Bucke & Feist 1985). Ventricular heart tissues or pieces of gill tissue were excised, placed onto tissue paper and then dabbed onto a clean dry slide. Slides were allowed to air dry and were then fixed in methanol and stained using Hemacolor 2 and 3 (Merck). For histological examination, tissues were fixed in Davidson's solution, processed using standard techniques and 5 µm sections were stained using haematoxylin and eosin. For examination of hemolymph smears, a drop of hemolymph was removed from the adductor muscle sinus, placed on a slide and the blood cells allowed to settle. Smears were fixed in methanol and stained with Hemacolor 2 and 3.

A sub-sample of 30 oysters was taken from each population before the trials began to determine initial prevalence of infection.

Holding conditions. Field: In Cork Harbour bivalves were placed in oyster bags (1 × 0.5 m) on trestles in an area that is exposed at low spring tide only. The trestles held the bags approximately 40 cm off the bed. Larger mesh size bags were used as the shellfish grew.

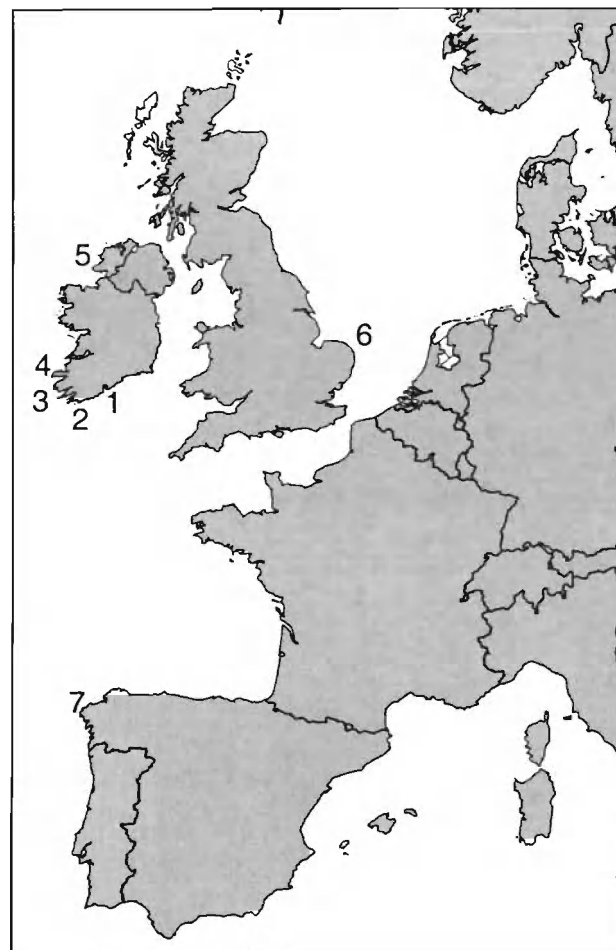


Fig. 1. Areas where trials took place and oysters were obtained. 1: Cork Harbour; 2: Fountainstown; 3: Bantry Bay; 4: Tralee; 5: Sligo Bay; 6: Norfolk; 7: Vigo

Laboratory: In Cork, bivalves were held in the laboratory in 500 l static tanks that were continuously aerated. Seawater was changed completely 3 times per week. The water was at ambient temperature and varied over the trials from 8 to 15°C. Bivalves were fed on *Tetraselmis* daily. They were monitored twice daily and any moribund individuals removed. In Vigo oysters were held in the laboratory in 75 l tanks and fed daily with *Tetraselmis*. Seawater was filtered and passed through a UV system before going into the tanks. Tanks were monitored twice a day and moribund individuals removed.

Infection of bivalves. Laboratory based experimental infections: To obtain high numbers of parasites for experimental infections oysters were obtained from areas where the pathogen had been present for a number of years. Prevalence and intensity of infection in the oysters was diagnosed using heart smears. Heavily infected oysters were retained and tissues homo-

Table 1. Expts 1, 2.1–2.2, 3.1–3.2 and 4 summarising trials involving natural and experimental exposure of bivalve molluscs to the parasite *Bonamia ostreae* and their objectives

Expt	Location	Laboratory/ field based	Objective
1	Cork	Field	Determine if a number of bivalve mollusc species could be infected with <i>B. ostreae</i> following transfer to an oyster growing area where the pathogen has been present for a number of years
2.1–2.2	Cork/Vigo	Laboratory	Determine if a number of bivalve molluscs could be infected with <i>B. ostreae</i> by experimental injection of a known number of viable parasites in the laboratory
3.1–3.2	Cork/Vigo	Laboratory	Determine if bivalves naturally exposed to <i>B. ostreae</i> in the field could act as vectors or intermediate hosts for this pathogen when held in the laboratory with <i>Ostrea edulis</i> from an oyster growing area where <i>B. ostreae</i> has never been detected
4	Cork	Laboratory	Determine if bivalves which have been injected with <i>B. ostreae</i> can act as vectors or intermediate hosts for the pathogen when held in the laboratory with <i>O. edulis</i> from an oyster growing area where the pathogen has never been detected

genised and parasites isolated according to the method of Mialhe et al. (1988). Parasite cell viability was assessed using Trypan blue exclusion. Cells that excluded the dye were regarded as viable and were counted using a Neubauer counting chamber. Using this method for determining viability, cell concentration of viable parasites was adjusted using filtered seawater (0.45 µm).

In Cork, the number of viable parasites injected into each oyster was adjusted to 5×10^6 *Bonamia ostreae* in 500 µl filtered seawater (FSW). In Vigo the number of injected parasites was 4.15×10^6 *B. ostreae* cells in 500 µl FSW.

For injection of the parasites the bivalves were first anaesthetised using 3.5% $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$. The time required for anaesthesia varied from 1 to 3 h depending on the bivalve species. When the bivalve was anaesthetised and the shell was gaping the inoculum was injected into the mantle cavity. Animals were kept out of the water for approximately 1 h following injection and were then returned to aerated seawater where they were allowed to recover. The control animals were anaesthetised in a similar manner and injected with filtered seawater only.

Field based natural challenge experiments: Bivalves to be infected naturally were placed in Cork Harbour where the pathogen had been present for a number of years. They were held for up to 10 mo. Sub-samples of 30 oysters were removed after 0, 1, 2, 5 and 10 mo to screen for prevalence of infection.

Experiments. The following series of experiments was carried out:

Table 1 lists the experiments carried out during the study and their objectives. Table 2 lists the transmis-

sion experiments carried out by natural challenge in the field and experimental infection in the laboratory. Table 3 lists the different co-habitation trials carried out between the different bivalve molluscs that had been exposed to *Bonamia ostreae* either naturally in the field or experimentally in the laboratory and *Ostrea edulis* from areas where bonamiasis has never been diagnosed.

RESULTS

Isolation of parasites, assessment of viability

Approximately 8×10^7 parasites were isolated from each heavily infected oyster in approximately 1 ml of FSW. Assessment of viability indicated that 95% of the cells were viable following the isolation process in all cases.

Experiment 1

Natural transmission: to determine if a number of bivalve mollusc species could be infected with Bonamia ostreae following transfer to an area where B. ostreae has been present for a number of years.

Five bivalve species, *Ostrea edulis*, *Crassostrea gigas*, *Ruditapes decussatus*, *R. philippinarum*, and *Mytilus edulis*, were monitored over a 10 mo period after being placed in a *B. ostreae* infected area (Table 4). No infection with *B. ostreae* was found in any of the sub-samples of each population taken

Table 2. Expts 1, 2.1 and 2.2 outlining transmission experiments carried out by natural infection in the field and experimental injection in the laboratory

Expt	Location	Mode of transmission	Bivalves exposed (number)	Source of bivalves	Method of holding	When sampled	Method of diagnosis
1.1	Cork Harbour – Field	Natural	<i>O. edulis</i> (327) <i>M. edulis</i> (900) <i>R. decussatus</i> (375) <i>R. philippinarum</i> (336) <i>C. gigas</i> (300)	Tralee, Ireland Bantry, Ireland Bantry, Ireland Sligo, Ireland Cork, Ireland	Bags on trestles: 327/bag 300/bag 125/bag 112/bag 75/bag	After 0, 1, 2, 5 and 10 mo (30 bivalves from each group on each occasion)	Heart smears
2.1	Cork – Laboratory	Experimental injection: 5×10^6 <i>B. ostreae</i> in 500 µl FSW/oyster Controls: 500 µl FSW/oyster	<i>O. edulis</i> (180) <i>R. decussatus</i> (180) <i>R. philippinarum</i> (180) <i>C. gigas</i> (180)	Tralee, Ireland Bantry, Ireland Sligo, Ireland Fountainstown, Ireland	500 l aerated tanks; fed on <i>Tetraselmis</i>	30 animals removed 2 wk post-inoculation all remaining animals removed after 6 mo	Heart smears
2.2	Vigo – Laboratory	Experimental injection: 4.15×10^4 <i>B. ostreae</i> in 500 µl FSW/oyster Controls: 500 µl FSW/oyster	<i>O. edulis</i> (19) <i>M. galloprovincialis</i> (20) <i>O. edulis</i> (10)	Norfolk, UK Norfolk, UK Norfolk, UK	75 l aerated tanks (water filtered and passed through UV system); fed on <i>Tetraselmis</i>	Hemolymph tested after 7 wk, all animals removed by 6 mo	Hemolymph smears and histology

Table 3. Expts 3.1, 3.2 and 4 outlining co-habitation trials carried out between *Ostrea edulis*, *Mytilus edulis*, *M. galloprovincialis*, *Ruditapes decussatus*, *R. philippinarum* and *Crassostrea gigas* that had been exposed to *Bonamia ostreae* either naturally in the field or experimentally in the laboratory and *O. edulis* from areas where bonamiasis has never been diagnosed

Expt	Location	Method of transmission	Bivalves exposed	Method of exposure	Held with	Source	Holding facilities	Sampling	Method of diagnosis
3.1	Cork – Laboratory	Co-habitation	<i>O. edulis</i> (150) <i>M. edulis</i> (150) <i>R. decussatus</i> (150) <i>R. philippinarum</i> (150) <i>C. gigas</i> (150)	Natural – Field	<i>O. edulis</i> (87) <i>O. edulis</i> (87) <i>O. edulis</i> (87) <i>O. edulis</i> (87) <i>O. edulis</i> (87)	L. Foyle, Ireland	500 l aerated tanks, at ambient temperature and fed on <i>Tetraselmis</i>	Moribund animals removed throughout trial, all remaining animals removed after 6 mo	Heart smears
3.2	Vigo – Laboratory	Co-habitation	<i>M. galloprovincialis</i> (21)	Natural – Field	<i>O. edulis</i> (25)	Norfolk, UK	75 l tanks	"	Hemolymph smears and histology
4	Cork – Laboratory	Co-habitation	<i>O. edulis</i> (150) <i>R. decussatus</i> (150) <i>R. philippinarum</i> (150) <i>C. gigas</i> (150)	Experimental injection 5×10^6 <i>B. ostreae</i> / 500 µl FSW/oyster	<i>O. edulis</i> (100) <i>O. edulis</i> (100) <i>O. edulis</i> (100) <i>O. edulis</i> (100)	Tralee, Ireland	500 l aerated tanks, at ambient temperature and fed on <i>Tetraselmis</i>	"	Heart smears

Table 4. Monitoring (% prevalence of infection and % cumulative mortality) of *Ostrea edulis*, *Mytilus edulis*, *Ruditapes decussatus*, *R. philippinarum*, and *Crassostrea gigas* at 0, 1, 2, 5, and 10 mo after transfer to an oyster growing area (Cork Harbour, Ireland) where *Bonamia ostreae* has been present for over 10 yr

Species	N	0 mo		1 mo		2 mo		5 mo		10 mo	
		Prev. of infection (%)		Prev. of infection (%)	Cum. mortality (%)	Prev. of infection (%)	Cum. mortality (%)	Prev. of infection (%)	Cum. mortality (%)	Prev. of infection (%)	Cum. mortality (%)
Expt 1											
<i>O. edulis</i>	327	0		0	9.1	0	14.3	0	ND	25.0	24.7
<i>M. edulis</i>	900	0		0	1.6	0	2.0	0	ND	0	7.2
<i>R. decussatus</i>	375	0		0	1.6	0	2.7	0	ND	0	19.3
<i>R. philippinarum</i>	336	0		0	92.3 ^a	0	2.9	0	ND	0	10.0
<i>C. gigas</i>	300	0		0	0	0	0.3	0	0.3	0	0.3

^aFollowing heavy mortalities this group was replaced with a new sample after several weeks in the field

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before relaying took place. Over the 10 mo period 5 sub-samples of the bivalves were taken to monitor prevalence of infection. After 1 mo the Manila clams suffered very heavy mortalities of 92.3% and had to be replaced with a new consignment of clams. The cause of these mortalities was not determined but similar losses occurred in the hatchery of origin at the time. Up to and including 5 mo after transfer none of the bivalves showed any infection with *B. ostreae* following monitoring with heart smears. However, when screened after 10 mo the flat oysters that had survived showed a 25% prevalence of infection. All other species remained uninfected. The flat oysters also showed the highest cumulative mortality at 24.7% over the 10 mo trial period. Cumulative mortalities in the other bivalves varied from 0.3 to 19.3%.

Experiments 2.1–2.2

Experimental transmission: to determine if a number of bivalves could be infected with Bonamia ostreae following injection with the parasite.

In parallel experiments in Cork and in Vigo, *Ostrea edulis*, *Mytilus galloprovincialis*, *Ruditapes decussatus*, *R. philippinarum* and *Crassostrea gigas*, obtained from areas where the disease has never been diagnosed, were held in the laboratory and individuals injected with a known dose of viable parasites in filtered seawater (Table 5). No infection with *Bonamia ostreae* was found in any of the sub-samples taken at the beginning of the trial. Moribund animals were sacrificed during the course of the experiments and examined for *B. ostreae*. The moribund flat oysters showed

Table 5. Monitoring over a 6 mo period (% prevalence of infection and % cumulative mortality) of *Ostrea edulis*, *Mytilus galloprovincialis*, *Ruditapes decussatus*, *R. philippinarum*, and *Crassostrea gigas*, following injection of each animal with a known number of viable *Bonamia ostreae* cells

Species	N	Moribund animals (during trial)		Survivors	
		Cumulative mortality (%)	Prevalence of infection (%)	Survivors (%)	Prevalence of infection (%)
<i>O. edulis</i>					
Expt 2.1 (Cork)	150	53.0	31.2	46.7	80.4
Expt 2.2 (Vigo)	19	5.5	100	94.5	29.4
<i>M. galloprovincialis</i>					
Expt 2.2 (Vigo)	20	40.0	0	60.0	0
<i>R. decussatus</i>					
Expt 2.1 (Cork)	150	25.0	0	75.0	0
<i>R. philippinarum</i>					
Expt 2.1 (Cork)	150	85.8	0	13.3	0
<i>C. gigas</i>					
Expt 2.1 (Cork)	150	4.2	0	95.8	0

31.2 and 100.0% prevalence of infection respectively in the Cork and Vigo laboratories. At the end of the 6 mo trial the remaining live oysters had 80.4 and 29.4% prevalence of infection respectively. No *B. ostreae* were found in the mussels, clams and Pacific oysters either during or at the end of the trials. High cumulative mortalities occurred in Cork in the Manila clams at 85.8% and were lowest in one group of flat oysters at 5.5% and Pacific oysters at 4.2%. The high mortalities in the Manila clams appeared to be a result of the stress induced by the holding conditions and frequent handling.

Experiments 3.1–3.2

Cohabitation experiments: to determine if naturally exposed bivalves can act as vectors for Bonamia ostreae.

In Expt 3.1, flat oysters, mussels, clams and Pacific oysters that were held in an area in Cork Harbour where *Bonamia ostreae* is present were returned to the laboratory in Cork after 10 mo (Table 6). The flat oysters had a 25% prevalence of infection on being returned to the laboratory but *B. ostreae* was not diagnosed in the other species (Table 4). These bivalves were held with groups of flat oysters from an area not previously diagnosed with the disease. After 6 mo co-habitation the flat oysters that had been naturally infected in the wild showed a 66.7% prevalence of infection. The group from an area free of *B. ostreae* held with them showed a 46.6% prevalence of infection. *B. ostreae* was not detected in the other bivalves that had been held in the infected area nor in the flat oysters held with them.

Expt 3.2 took place in the laboratory in Vigo. Naturally exposed *Mytilus galloprovincialis* were held with

Ostrea edulis from an area where the disease has never been diagnosed. No infection was found in either group during or at the end of the 6 mo experimental period.

Experiment 4

Cohabitation experiment: to determine if experimentally injected bivalves can act as vectors or intermediate hosts for Bonamia ostreae.

In Expt 4, flat oysters, clams and Pacific oysters were injected with a known number of parasites and held with groups of flat oysters that came from an area where *Bonamia ostreae* has never been detected (Table 7). All species had been screened at the beginning of the trial and no infection was detected. After 6 mo of co-habitation the experimentally infected oysters had an 80.4% prevalence of infection and the flat oysters held with them had a 22.1% prevalence. *B. ostreae* was not detected in any of the other species injected with the parasite nor in flat oysters held with them.

DISCUSSION

It has been known for a number of years that the flat oyster *Ostrea edulis* can be successfully infected with *Bonamia ostreae* following either experimental injection in the laboratory or natural transmission in the field, and that transmission occurs directly when infected and uninfected oysters are held together (Bachère et al. 1982, Poder et al. 1982, Tigé et al. 1982, Tigé & Grizel 1984, Elston et al. 1986, Montes 1991). These results were corroborated by the present study.

Table 6. Results of laboratory-based monitoring of co-habitation trials involving *Ostrea edulis*, *Mytilus edulis*, *Ruditapes decussatus*, *R. philippinarum*, *Crassostrea gigas*, and *Mytilus galloprovincialis*, all exposed to the pathogen *Bonamia ostreae* in the field and held with groups of *O. edulis* from an area where bonamiasis has never been detected. ND: no data

Species	N	Cumulative mortality (%)		Prevalence of infection during the trial (%)		Prevalence of infection at the end of 6 mo (%)	
		Uninfected <i>O. edulis</i>	Infected bivalve	Uninfected <i>O. edulis</i>	Infected bivalve	Uninfected <i>O. edulis</i>	Infected bivalve
Expt 3.1 (Cork)							
<i>O. edulis</i>	120	19.5	91.6	ND	56.5	46.6	66.7
<i>M. edulis</i>	120	17.0	25.8	0	0	0	0
<i>R. decussatus</i>	120	9.2	8.1	0	0	0	0
<i>R. philippinarum</i>	120	2.3	95.0	0	0	0	0
<i>C. gigas</i>	120	2.5	0	0	0	0	0
Expt 3.2 (Vigo)							
<i>M. galloprovincialis</i>	21	ND	0	0	0	0	0

Table 7. Results of laboratory-based monitoring (% prevalence of infection and % cumulative mortality) of co-habitation trials involving *Ostrea edulis*, *Ruditapes decussatus*, *R. philippinarum*, and *Crassostrea gigas* all experimentally injected with the pathogen *Bonamia ostreae* and held with groups of *O. edulis* from an area where bonamiasis has never been detected

Injected species	N	Cumulative mortality (%)		Prevalence of infection during the trial (%)		Prevalence of infection at the end of the trial (%)	
		Injected bivalve	Uninfected <i>O. edulis</i>	Injected bivalve	Uninfected <i>O. edulis</i>	Injected bivalve	Uninfected <i>O. edulis</i>
Expt 4 (Cork)							
<i>O. edulis</i>	150	53.3	32.0	31.2	12.5	80.4	22.1
<i>R. decussatus</i>	150	25.0	31.0	0	0	0	0
<i>R. philippinarum</i>	150	85.8	10.0	0	0	0	0
<i>C. gigas</i>	150	4.2	5.0	0	0	0	0

The susceptibility of a number of bivalve species including *Crassostrea gigas*, *Mytilus edulis*, *M. galloprovincialis*, *Ruditapes decussatus* and *R. philippinarum* to *Bonamia ostreae* was investigated. Following natural exposure or experimental injection of parasites the disease did not develop in any of these species. These results would indicate that culturing any of these species in close proximity to *B. ostreae* infected flat oysters is possible and these species will not develop the disease. The basis of this 'resistance' or lack of susceptibility needs to be investigated further. Several authors have previously indicated that *B. ostreae* has not been detected in a number of bivalve species such as mussels *M. edulis*, the cockle *Cerastoderma edule* (L.), the native clam, *R. decussatus* and the Manila clam *R. philippinarum* (Balouet & Poder 1983, van Banning 1985, Tigé et al. 1987, Grizel et al. 1988, van Banning 1990). Figueras & Robledo (1994) found that *B. ostreae* in flat oysters in Galicia does not infect neighbouring *M. galloprovincialis*. Renault et al. (1995) were unable to experimentally induce an infection by inoculation of purified parasites into Pacific oysters.

Furthermore, it appears that these bivalve species cannot act as either vectors or intermediate hosts for this parasite, as no infection occurred in any flat oysters that were held with these species for up to 6 mo following either natural or experimental exposure to *Bonamia ostreae*. This would indicate that these species would not be responsible for the spread of the disease.

These results are relevant to the movement of a number of bivalve species within the European Union, particularly those originating from areas where *Bonamia ostreae* is present. In this study under a variety of conditions, in both the laboratory and the field, a number of bivalve species did not act as vectors for the protozoan parasite *B. ostreae* nor did they appear to be susceptible to this parasite. The fact that questions still remain in relation to certain aspects of the life cycle of *B. ostreae* and presently available diagnostic techniques may have their limitations should be taken into

consideration. However, when shellfish are cultured and moved under conditions comparable to those used in these experiments it is unlikely that the spread of *B. ostreae* will occur.

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