



***Bonamia* parasites: a rapidly changing perspective on a genus of important mollusc pathogens**

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ABSTRACT: Organisms of the genus *Bonamia* are intracellular protistan parasites of oysters. To date, 4 species have been described (*B. ostreae*, *B. exitiosa*, *B. perspora* and *B. roughleyi*), although the status of *B. roughleyi* is controversial. Introduction especially of *B. ostreae* and *B. exitiosa* to naïve host populations has been shown to cause mass mortalities in the past and has had a dramatic impact on oyster production. Both *B. ostreae* and *B. exitiosa* are pathogens notifiable to the World Organisation for Animal Health (OIE) and the European Union. Effective management of the disease caused by these pathogens is complicated by the extensive nature of the oyster production process and limited options for disease control of the cultured stocks in open water. This review focuses on the recent advances in research on genetic relationships between *Bonamia* isolates, geographical distribution, susceptible host species, diagnostics, epizootiology, host–parasite interactions, and disease resistance and control of this globally important genus of oyster pathogens.

KEY WORDS: *Bonamia ostreae* · *Bonamia exitiosa* · Oyster disease · Phylogenetics · Geographical spread · Diagnostics · Epizootiology · Host–parasite interaction · Bonamiosis

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INTRODUCTION

Since their first detection and description, intracellular protistan parasites of the genus *Bonamia* have been recognized as serious pathogens of oysters, severely impairing the health of oyster populations and the production of these economically important molluscs. The appearance of *B. ostreae* in the 1970s in Europe caused mass mortality in its host *Ostrea edulis* and hastened the decline of this species (Grizel 1985, Montes 1990, Hudson & Hill 1991, McArldle et al. 1991, Van Banning 1991). This was a dramatic impact that may have reflected the encounter between an introduced pathogen (Elston et al. 1986) and a host with no natural resistance to it. The effects of *B. exitiosa* infection of *O. chilensis* in New Zealand

have been similarly severe, reducing the population to 9% of the pre-disease level, although this host–parasite relationship may be long-standing (Doonan et al. 1994, Hine & Jones 1994). The impact of *B. exitiosa* on European oyster populations since its discovery in Europe is less clear. The extensive nature of the oyster production process and limited options for disease control in cultured stocks in open water surrounded by wild oyster populations hampers effective management of disease outbreaks. Both *B. ostreae* and *B. exitiosa* are pathogens listed by and notifiable to the World Organisation for Animal Health (OIE) and the European Union (EU). With the advances in research on *Bonamia* species over the years, knowledge on their global spread has been extended, as additional species have been discovered and assigned

to the genus *Bonamia* (Carnegie et al. 2006). Hosts are now known to include not only ostreid oysters but also *Crassostrea* species. Recently, mechanisms of interaction between *Bonamia* spp. and their hosts, including mechanisms involved in the resistance to the disease, have been investigated in more depth at cellular and molecular levels (Morga et al. 2009, 2011, 2012). Furthermore, it seems that in some cases, after their introduction, the parasites are only present at a low prevalence and with presumably limited effects on the population (Culloty & Mulcahy 2007). In other populations exposed to the parasites over a long period of time, there are indications that a certain level of resistance is acquired (Culloty et al. 2004).

In this review, we focus on the advances in research on *Bonamia* over the last decade in phylogenetics, geographical distribution, susceptible host species, diagnostics, epizootiology, host–parasite interactions, and disease resistance and control.

PHYLOGENETICS AND TAXONOMY

The genus *Bonamia* represents a derived clade within the phylum Haplosporidia whose members have generally adopted (1) life cycles based on direct oyster to oyster transmission of uninucleate amoeboid cell forms, and (2) intracellular infection of oyster haemocytes by these cell forms (Carnegie et al. 2000, Carnegie & Cochenne-Laureau 2004). The 4 described species are *B. ostreae*, described from *Ostrea edulis* in France (Pichot et al. 1980); *B. exitiosa*, from *O. chilensis* in New Zealand (Hine et al. 2001, Berthe & Hine 2003); *B. roughleyi*, originally identified as *Mikrocytos roughleyi*, from *Saccostrea glomerata* in southeastern Australia (Farley et al. 1988, Cochenne-Laureau et al. 2003b); and *B. perspora*, from *O. stentina* (= *Ostreola equestris*; Shilts et al. 2007) in the southeastern USA (Carnegie et al. 2006; Fig. 1). Among these species, *B. perspora* is unique in retaining the basal haplosporidian characteristics of spore formation (Fig. 2) and extracellular infection. Expression of conventional haplosporidian spores by a *Bonamia* species, the possession of haplosporosomes by members of the genus (Pichot et al. 1980, Dinamani et al. 1987, Carnegie et al. 2006) and compelling phylogenetic evidence (Carnegie et al. 2000, 2006, Reece et al. 2004) indicate that placement of *Bonamia* within the Haplosporidia is correct. Small subunit ribosomal DNA (SSU rDNA)-based analyses typically support a sister relationship of *Bonamia* to the genus *Minchinia* (Carnegie & Cochenne-Laureau 2004, Reece et al. 2004, Carnegie et al. 2006, Abollo et al. 2008; Fig. 3

and see the Supplement at www.int-res.com/articles/suppl/d110p005_supp.pdf), although the single analysis performed to date using actin gene and amino acid sequences has been more ambiguous (López-Flores et al. 2007).

Morphology at the light microscope level provides little perspective with regard to the inter-specific relationships among *Bonamia* parasites, except to indi-

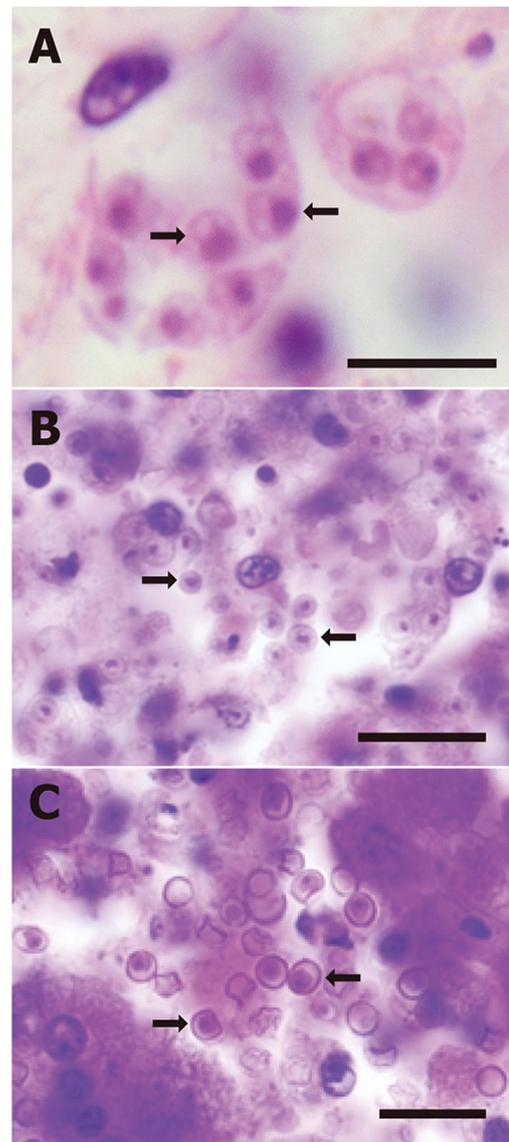


Fig. 1. Valid described species in the genus *Bonamia* (histopathological haematoxylin and eosin staining). (A) *B. ostreae* in *Ostrea edulis* from Maine, USA. Scale bar = 5 μ m. (B) *B. exitiosa* in *Crassostrea ariakensis* from North Carolina, USA. Scale bar = 10 μ m. (C) *B. perspora* in *O. stentina* from North Carolina, USA. Representative parasite cells in each image are indicated by arrows. Scale bar = 10 μ m. Specimens are from the Virginia Institute of Marine Science Shellfish Pathology Laboratory collections

asite of *Dendostrea sandvicensis* as being a distinct *Bonamia* species (Hill et al. 2014). Interestingly, although its histological presentation suggests a typical 'microcell haplosporidian' *Bonamia* parasite (Fig. 4A), the SSU rDNA-based analyses place the Hawaiian *Bonamia* sp. at the base of the *Bonamia* clade (Fig. 3). The placement on an interior branch of *B. perspora*, with its retention of the presumably ancestral characteristics of spore formation and extracellular infection, suggests either that the other *Bonamia* lineages giving rise to *B. ostreae*, *B. exitiosa* and the Hawaiian *Bonamia*

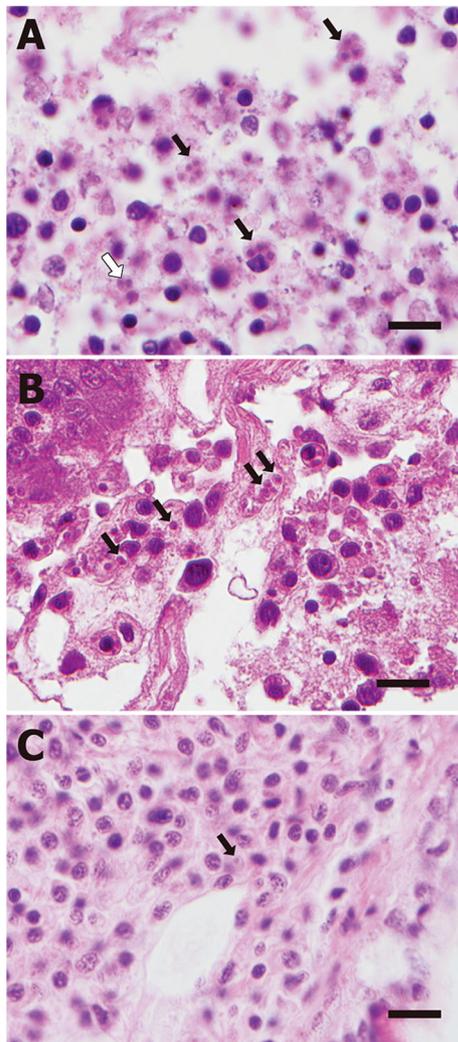


Fig. 4. Undescribed *Bonamia* parasites (histopathological haematoxylin and eosin staining). (A) *Bonamia* sp. from *Dendostrea sandvicensis* in Hawaii, with numerous intra-haemocytic and free cells (arrows) including a binucleate stage at lower left (white arrow). (B) *Bonamia* sp. from *Ostrea chilensis* in Chile, with numerous parasite cells present (arrows). (C) *Bonamia* sp. from *O. edulis* in California, USA, with a single cell displayed (arrow). All scale bars = 10 µm. Specimens are from the Virginia Institute of Marine Science Shellfish Pathology Laboratory collections

sp. adopted direct transmission by uninucleate amoeboid forms and intracellular infection multiple times over the evolutionary history of the genus, or, conversely, that the SSU rDNA gene trees do not reflect the species tree for *Bonamia* (Hill et al. 2014). Future analyses using additional genetic loci may shed further light on this. It should be noted that *B. perspora* has appeared in 1 analysis to be a sister to *B. ostreae* (Abollo et al. 2008); however, this relationship has not been strongly supported elsewhere (Hill et al. 2014).

What of the Chilean *Bonamia* sp. (Fig. 4B)? While SSU rDNA-based phylogenetic analyses suggest an affinity to *B. exitiosa* (Abollo et al. 2008, Hill et al. 2010b), higher-resolution ITS region-based analyses reveal a distinct lineage reciprocally monophyletic both with *B. exitiosa* and with another distinct lineage from *O. edulis* from California, USA (Hill et al. 2014; our Fig. 4C). These should not be considered *B. exitiosa*, based on their novel ITS sequences, and may represent fifth and sixth novel *Bonamia* species that, like the Hawaiian *Bonamia* sp., also await description.

In summary, the genus *Bonamia* as we currently appreciate it appears to comprise a possible basal parasite from *D. sandvicensis* in Hawaii, and then 3 lineages among which sister relationships cannot clearly be resolved. One is represented by *B. perspora*, and another by *B. ostreae*. *B. exitiosa* occupies the third lineage, along with the undescribed parasites from *O. chilensis* in Chile and *O. edulis* in California (see Hill et al. 2014).

GEOGRAPHICAL DISTRIBUTION

Until a decade ago, the geographical distribution of *Bonamia* species appeared to be straightforward. *B. ostreae* was the *Bonamia* species present in the Northern Hemisphere, infecting the European flat oyster *Ostrea edulis* in Europe and in Maine and California in the USA. The introduction and spread of *B. ostreae* within Europe has foremost been linked to transfers of shellfish (Peeler et al. 2010), either directly or, for example, via other anthropogenic routes such as hull fouling (Howard 1994).

In the Southern Hemisphere, the *Bonamia* species infecting the Chilean oyster *O. chilensis* in New Zealand was designated *B. exitiosa* (Hine et al. 2001, Berthe & Hine 2003). Flat oyster species from Australia, Chile and Argentina were infected with *Bonamia* species similar or identical to *B. exitiosa* (Corbeil et al. 2006b, Lohrmann et al. 2009, Hill et al. 2014). In recent years, however, the known geographical distribution of *B. exitiosa* and related parasites has ex-

panded, possibly as a result of further spread of the parasite, increased scrutiny of oyster populations that were previously ignored, and wider application of molecular methods including DNA sequencing. A *Bonamia* species similar to *B. exitiosa* was first observed in the Northern Hemisphere in *Crassostrea ariakensis* in North Carolina, USA (Burrenson et al. 2004). It was subsequently found in Florida (Dungan et al. 2012), and the novel species *B. perspora* was detected in *O. stentina* in the same region (Carnegie et al. 2006). In 2008, a *B. exitiosa*-like parasite was reported from *O. edulis* in Galicia, Spain (Abollo et al. 2008), and subsequent years brought reports of *B. exitiosa*-like parasites from the Mediterranean coastlines of Tunisia (Hill et al. 2010b) and Spain (Carrasco et al. 2012), the Adriatic Sea shore of Italy (Narcisi et al. 2010, WAHID-Interface 2010), and a single location in the UK (WAHID-Interface 2011). As we now recognize that most of the *B. exitiosa*-like parasites are indeed *B. exitiosa*, we must view *B. exitiosa* as having a wide host (see next section) and geographic range. Its distributions in Asia and Africa and along the tropical and sub-tropical coasts of all the continents are important remaining questions. ITS region characterization of the parasite from the Atlantic coast of Europe remains to be performed for definitive assignment to *B. exitiosa*, but it is widely assumed to be *B. exitiosa* (e.g.

Abollo et al. 2008). ITS sequencing of the *Bonamia* sp. from the Mediterranean coast of Spain confirmed that parasite to be *B. exitiosa* (N. Carrasco & R. B. Carnegie unpubl. data).

Hill et al. (2010a) summarised the known geographical distributions of *B. ostreae* and *B. exitiosa* in a recent literature study for the European Food Safety Authority (EFSA). Fig. 5 represents an update from this study, with the current global distribution of all known *Bonamia* species (more precise location details are given in Table S1 in the Supplement).

SUSCEPTIBLE HOST SPECIES

Primarily oysters of the genus *Ostrea* have been described as host species of the different *Bonamia* parasites. However, *Bonamia* species have also been observed in *Crassostrea* species including *C. ariakensis* and *C. angulata* and in the lophine oyster *Dendostrea sandvicensis*. Based on our present knowledge, therefore, *Bonamia* species seem to have an affinity more generally for members of the family Ostreidae. However, which hosts are susceptible to which parasite species is not perfectly clear.

A susceptible host species can be defined as a species that can support replication of an agent or an

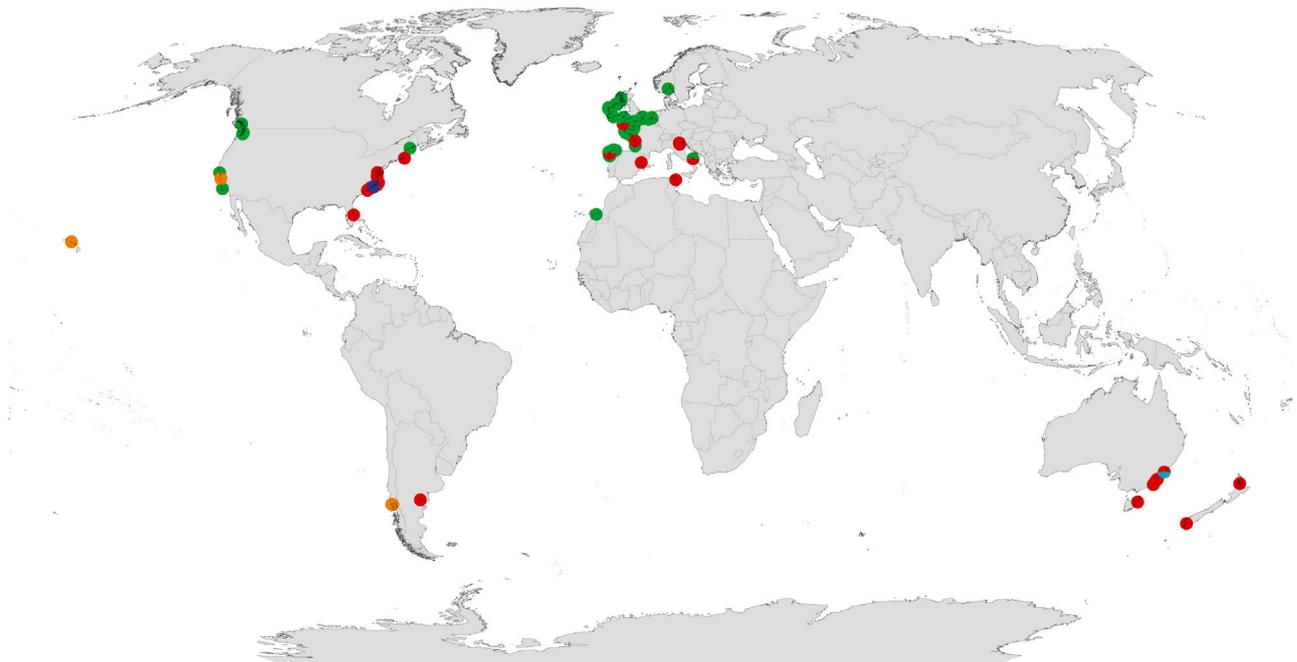


Fig. 5. Geographical distribution of *Bonamia* species based on published literature and disease reports of the OIE World Animal Health Information Database (see Table S1 in the Supplement at www.int-res.com/articles/suppl/d110p005_supp.pdf): *B. ostreae* (green), *B. exitiosa* and species of the *B. exitiosa* clade (red), *Bonamia* sp. from *Ostrea chilensis* in Chile, *O. lurida* in California and *O. sandvicensis* in Hawaii (orange), *B. perspora* (dark blue) and the microcell in *Saccostrea glomerata* (light blue)

infection, which may lead to the development of disease (Stentiford et al. 2009). EFSA expanded this definition to identify 4 criteria for use in identifying a susceptible host, as distinct from a mechanical carrier: (A) evidence of replication or growth of the organism; (B) presence of a viable organism; (C) presence of specific clinicopathological changes; and (D) specific location of the pathogen within the host (EFSA 2008). For the *Bonamia* species, characteristics and techniques supporting the different criteria are, for example, binucleate plasmodia in TEM or impression smears as evidence of replication (satisfying Criterion A); purification and cell viability tests and cohabitation with passage to a specific pathogen-free susceptible host as presence of a viable organism (B); focal to disseminated haemocytic infiltration of the connective tissues and intracellular parasite present in haemocytes as indication of clinicopathological changes (C); and in the case of *Bonamia* species, the location of the pathogen within the host (D) is often a systemic infection.

Tables 1 & 2 show the results of application of these criteria to species described in the literature as hosts for *B. ostreae* and *B. exitiosa*, respectively. The data represent an updated version of the tables in the EFSA report on aquatic animal species susceptible to diseases listed in Directive 2006/88/EC (EFSA 2008). Based on the results, hosts can be assigned to 2 main groups: Group I includes host species for which the quality of the data provides clear support for susceptibility, and Group II includes host species for which incomplete or unclear data prevent a clear conclusion. A primary limitation prevents inclusion of some of the potential host species for *Bonamia* into the first group: the *Bonamia* species involved in a case has frequently not been typed to the species level. This is especially the case in early publications but also in some of the more recent work. For example, a *Bonamia* sp. was detected in early field trials with *O. chilensis* (Grizel et al. 1983), *O. angasi* (Bougrier et al. 1986) and *O. puelchana* (Pascual et al. 1991) in French waters. As the oysters were reared in *B. ostreae*-enzootic waters, this parasite may well have been observed. However, in the light of our present knowledge of the occurrence of *B. exitiosa* in Europe and other cryptic lineages elsewhere (Hill et al. 2014), genetic identification of the parasite is essential.

As a result for *B. ostreae*, current scientific data support susceptibility of *O. edulis* and *C. ariakensis* to infection with this species (Table 1). Some scientific data suggest susceptibility of *O. angasi*, *O. puelchana* and *O. chilensis*; however, uncertainty on parasite identification and taxonomic affiliation limits conclu-

sions concerning susceptibility of these species to *B. ostreae*. In addition, available data do not support susceptibility of *O. lurida* (= *O. conchaphila*, Polson et al. 2009). Information on *C. angulata* was considered insufficient to scientifically assess susceptibility. A recent publication by Lynch et al. (2010) considers the role of *C. gigas* as a potential carrier of *B. ostreae* and *B. exitiosa*. Under field and laboratory conditions, microcells were incidentally observed in connective tissue of *C. gigas* kept in a *B. ostreae*-enzootic area or co-habitated with infected *O. edulis*. This might represent an actual infection or the uptake of the parasite released from dying *O. edulis*. Additional support of the findings would be helpful to fully assess the actual susceptibility of *C. gigas* to *B. ostreae*.

For *B. exitiosa*, the picture with respect to host specificity is resolved more clearly, though likely incompletely. Unambiguous genetic data combined with visual observations support susceptibility of *O. chilensis*, *O. angasi*, *O. lurida*, *O. edulis*, *O. puelchana*, *O. stentina* and *C. ariakensis* (Table 2). Based on recent observations in the eastern USA, *C. virginica* also seems to be a susceptible host for *B. exitiosa* (WAHID-Interface 2012). In *C. gigas*, *B. exitiosa* was only detected by PCR (Lynch et al. 2010), so actual infection of this species remains an open question. While *B. exitiosa* was unambiguously detected using molecular methods in *S. glomerata* from southeastern Australia, visual evidence of infection remains elusive. The parasite observed in this oyster by Farley et al. (1988) does not seem to have been a *Bonamia* sp. (Carnegie et al. 2014, Spiers et al. 2014), and while a *Bonamia* sp. was reported to have been observed using TEM by Cochennec-Laureau et al. (2003b), it is unclear whether these authors actually visualized a *Bonamia* sp. The potential susceptibility of *S. glomerata*, like *C. gigas*, remains uncertain.

As noted above, *O. chilensis* in Chile and *O. edulis* in California host sister *Bonamia* sp. lineages to *B. exitiosa*. For the species *B. perspora*, the only described host is *O. stentina* (= *Ostreola equestris*, Carnegie et al. 2006). Similarly, the *Bonamia* sp. from Hawaii is described with *D. sandvicensis* as the host species (Hill et al. 2014).

Oyster beds are naturally associated with a wide range of benthic and fouling organisms. Similar to non-susceptible bivalve species, these species are not considered susceptible hosts for *Bonamia*, but they could conceivably act as mechanical vectors in the spread of the parasite. Lynch et al. (2007) detected the presence of *B. ostreae* DNA by PCR in 8 benthic macro-invertebrates and 19 zooplankton samples. In subsequent laboratory trials, *B. ostreae*

Table 1. Host species susceptible to *Bonamia ostreae*. Criteria A–D are as follows: (A) evidence of replication or growth of the organism; (B) presence of a viable organism; (C) presence of specific clinicopathological changes; and (D) specific location of the pathogen within the host (EFSA 2008). An 'X' indicates that a criterion has been met, as indicated in the 'Assessment' column. Hosts can be assigned to 2 main groups: Group I includes host species for which the quality of the data provides clear support for susceptibility, and Group II includes host species for which incomplete or unclear data prevent a clear conclusion. EM: electron microscopy; nd: no data; SSU: small subunit

Host species	Natural or experimental	Criterion			Assessment	Pathogen ID	Host group
		A	B	C D			
<i>Ostrea edulis</i> (type host)	Natural	X	X	X	(A) EM observation of uninucleated and binucleated stages of <i>B. ostreae</i> in naturally infected <i>O. edulis</i> (Pichot et al. 1980) (B) Viable parasites can be purified from infected oysters (Mialhe et al. 1988a) (C & D) Systemic infiltration of infected haemocytes observed in naturally infected <i>O. edulis</i> (Balouet et al. 1983)	SSU sequence available (Cochenne et al. 2000)	I
<i>Ostrea angasi</i>	Natural (exposure of first generation from introduced <i>O. angasi</i> by rearing in <i>B. ostreae</i> -endemic waters)	nd	nd	X	Insufficient data. <i>Bonamia</i> was detected in histological sections and tissue imprints at low prevalence (Bougnier et al. 1986)	No identification	II
<i>Ostrea chilensis</i>	Natural (direct exposure of <i>O. chilensis</i> by rearing in <i>B. ostreae</i> -endemic waters)	X	nd	X	(A) Various stages of the parasite in histological sections (Grizel et al. 1983) (C) <i>Bonamia</i> gross lesions were observed (Grizel et al. 1983) (D) <i>Bonamia</i> was detected by histology and EM (Grizel et al. 1983, Bucke & Hepper 1987)	No thorough characterization of the parasite	II
<i>Ostrea lurida</i> (= <i>O. conchaphila</i>)	Natural and experimental non-invasive	nd	nd	?	Katkansky et al. (1969) and Farley et al. (1988) described a microcell parasite in <i>O. lurida</i> from Oregon, USA. Experimentally, <i>O. lurida</i> could not be infected by cohabitation with infected <i>O. edulis</i> (I. Arzul pers. obs.)	Microcell species ID uncertain	II
<i>Ostrea puelchana</i>	Natural (direct exposure of first generation from introduced animals by rearing in <i>B. ostreae</i> -endemic waters)	nd	nd	X	(D) <i>Bonamia</i> was detected in histological sections and tissue imprints (Pascual et al. 1991)	No identification	II
<i>Crassostrea angulata</i>	Natural	nd	nd	nd	Microcells observed in <i>C. angulata</i> after exposure to infected <i>O. edulis</i> (Katkansky et al. 1969)	No identification	II
<i>Crassostrea ariakensis</i>	Natural	nd	nd	X	(C & D) Detection of <i>Bonamia</i> sp. by histology and EM in <i>C. ariakensis</i> from a quarantine facility with inlet water from a <i>B. ostreae</i> -endemic area (Cochenne et al. 1998)	<i>Bonamia</i> species later confirmed by DNA sequencing (I. Arzul pers. obs.).	I
<i>Crassostrea gigas</i>	Natural and experimental non-invasive and invasive	?	nd	?	(A) Bi-nucleated cells in connective tissue (C & D) <i>B. ostreae</i> -like cells observed in connective tissue: field <i>C. gigas</i> , no associated haemocytic infiltration, parasites extracellular; lab co-habitation <i>C. gigas</i> , parasites intracellular and extracellular (Lynch et al. 2010)	Species ID confirmed by DNA sequencing	II

Table 2. Host species susceptible to *Bonamia exitiosa*. For definitions of Criteria A–D and Groups I and II, see Table 1. SSU rDNA: small subunit ribosomal DNA; ITS: internal transcribed spacer

Host species	Natural or experimental	Criterion				Assessment	Pathogen ID	Host group
		A	B	C	D			
<i>Ostrea chilensis</i> (type host)	Natural	X	nd	X	X	(A) EM observation of uninucleated and binucleated stages of <i>B. exitiosa</i> in naturally infected <i>O. chilensis</i> (Dinamani et al. 1987, Hine et al. 2001) (C) Observation of <i>Bonamia</i> within the haemocytes (Dinamani et al. 1987) (D) Systemic infiltration of <i>Bonamia</i> infected haemocytes (Dinamani et al. 1987)	SSU rDNA and ITS region-based phylogenetics (Hill et al. 2014)	I
<i>Ostrea angasi</i>	Natural	nd	nd	X	X	(C & D) Intracellular and extracellular parasites in focal areas of haemocytosis located in connective tissue and gills (Heasman et al. 2004)	SSU rDNA and ITS region-based phylogenetics (Hill et al. 2014)	I
<i>Ostrea edulis</i>	Natural	nd	nd	X	X	(C & D) Intracellular and extracellular parasites in focal areas of haemocytosis located in connective tissues (Carrasco et al. 2012)	SSU rDNA and ITS region-based phylogenetics (Carrasco et al. 2012, N. Carrasco & R. B. Carnegie unpubl.)	I
<i>Ostrea lurida</i> (= <i>O. conchaphila</i>)	Natural	nd	nd	X	X	(C & D) Intracellular and extracellular parasites in focal areas of haemocytosis located in connective tissues (Hill et al. 2014)	SSU rDNA and ITS region-based phylogenetics (Hill et al. 2014)	I
<i>Ostrea puelchana</i>	Natural	nd	nd	X	X	(C & D) <i>Bonamia</i> sp. detected by histology within haemocytes of <i>O. puelchana</i> (Kroeck & Montes 2005, Kroeck 2010)	SSU rDNA and ITS region-based phylogenetics (Hill et al. 2014)	I
<i>Ostrea stentina</i> (= <i>O. equestris</i>)	Natural	X	nd	nd	nd	(A) Dividing stages present (Hill et al. 2010b) (C & D) Parasites usually intrahaemocytic, no haemocytosis observed in the organs available	SSU rDNA and ITS region-based phylogenetics (Hill et al. 2010b, 2014)	I
<i>Crassostrea ariakensis</i>	Natural	X	nd	X	X	(A) Dividing stages present (C & D) <i>Bonamia</i> sp. detected by histology within haemocytes in different tissues of <i>C. ariakensis</i> (Burreson et al. 2004)	SSU rDNA and ITS region-based phylogenetics (Hill et al. 2010b, 2014)	I
<i>Crassostrea virginica</i>	Natural	nd	nd	?	?	(C & D) Detection by histology, no details given (WAHID-Interface 2013)	PCR and sequencing, no details on identification given (WAHID-Interface 2013)	II

could be transmitted by co-habitation to 2 naïve oysters held with a brittle star *Ophiothrix fragilis* from a *B. ostreae*-endemic area.

DIAGNOSTICS

Since the first description of *Bonamia ostreae*, a range of detection techniques for *Bonamia* species has been developed. The first descriptions of *B. ostreae* (Pichot et al. 1980) and *B. exitiosa* (Dinamani et al. 1987) were based on histology and TEM. Together with cytology (stained ventricular heart imprints), histology and TEM were the main methodologies used for detection and identification of *Bonamia* species until the more recent development of molecular techniques. Tissue imprints and histopathology are still widely used for detection today, and are recommended methods for targeted surveillance according to the OIE manual of diagnostic tests (OIE 2012). TEM has its limitations in routine surveillance but is recommended for confirmatory diagnosis. Its primary value lies in allowing visual discrimination of *Bonamia* spp., which possess haplosporosomes (Pichot et al. 1980, Dinamani et al. 1987, Carnegie et al. 2006), from *Mikrocytos* spp., which do not (Hine et al. 2001).

Several papers have compared the detection of *Bonamia* by histology with detection by cytology, but the results are not conclusive. In some studies, cytology was found to be more sensitive than histology (Diggles et al. 2003, Lynch et al. 2008). In others, histology was more sensitive (Zabaleta & Barber 1996, Balseiro et al. 2006) or the methods were comparable (da Silva & Villalba 2004). For both assays, skilled and trained personnel are needed, and differences between the assays might reflect the background and particular capabilities of the laboratories. Results of interlaboratory comparison tests organised by the EU Reference Laboratory for Molluscan Diseases (Ifremer, La Tremblade) in 2007, 2009 and 2012 (with 20, 18 and 21 participating laboratories, respectively) revealed general competency in both techniques but with more infections detected cytologically (I. Arzul unpubl. data). In general, cytology is more economical, while histology facilitates the detection of other lesions and pathogens. However, both of these methods as well as TEM lack the specificity to type the observed *Bonamia* to species level.

The first molecular assays for *B. ostreae* were antibody-based, but these assays were applied only occasionally (Mialhe et al. 1988b, Boulo et al. 1989, Cochenne et al. 1992, Zabaleta & Barber 1996) and

were abandoned with the development of genetic methods. After the elucidation of the first genetic sequences of *B. ostreae* in the late 1990s, a range of molecular detection methods became available. Conventional PCR assays described by Carnegie et al. (2000) and Cochenne et al. (2000) are based on sequences of the SSU rDNA of *B. ostreae* and will amplify all (Cochenne et al. 2000) or most (Carnegie et al. 2000) of the identified *Bonamia* spp., including *B. exitiosa*. Primer mismatches to the Hawaiian *Bonamia* sp. and *B. perspora* may limit amplification of those species using the assay of Carnegie et al. (2000). Under certain circumstances, the primers of Cochenne et al. (2000) generate a 295 bp non-specific product of approximately the same size as the expected product of 300 bp (Carrasco et al. 2012, M. Y. Engelsma et al. unpubl. results). TaqMan real-time PCR assays have been developed to target the same gene, and are likely to amplify a number of *Bonamia* lineages, including *B. ostreae* and *B. exitiosa* (Corbeil et al. 2006a, Marty et al. 2006). A new conventional PCR assay (319F/524R) was designed to amplify a ~205 bp region with the widest possible *Bonamia* spp. specificity, and will amplify all known lineages (Hill et al. 2010b). A SYBR Green assay developed by Robert et al. (2009) specifically targets the actin-1 gene sequence of *B. ostreae*. The different assays have been applied in a variety of studies and are recommended by the OIE (OIE 2012) for targeted surveillance and presumptive diagnostics. For confirmatory diagnostics, sequencing of the PCR products is recommended. In general, PCR and especially real-time PCR assays have the potential to be highly sensitive and specific compared to the classical detection methods of histology and cytology. Comparisons of the performance of PCR assays with histology and cytology techniques have been made in several studies. Although Lynch et al. (2008) found heart imprints to be the most sensitive technique followed by PCR, in other studies the molecular detection methods surpassed the sensitivity of the more conventional methods (Diggles et al. 2003, Balseiro et al. 2006). In all studies, the application of a combination of techniques resulted in increased parasite detection. Use of multiple assays provides a greater likelihood that very light and local infections will be detected by one or another of the tests, thus reducing rates of false-negative results. In addition, Flannery et al. (2014, this DAO Special) found that low intensities of infection could go undetected due to the fact that the parasite could be localised within a small area of the oyster tissue which may not neces-

sarily be screened when only very small pieces of tissue are used.

With a positive result in a PCR assay, it is necessary to bear in mind that detection of *Bonamia* DNA does not automatically indicate a viable parasite or an actual infection. Another drawback of most currently available PCR assays for detection of *Bonamia* species is the lack of specificity. Most PCR assays are generic for the genus *Bonamia*. A method applied to improve the specificity of the assay developed by Cochenne et al. (2000) is PCR-restriction fragment length polymorphism (PCR-RFLP) to resolve the different *Bonamia* species (Hine et al. 2001, Cochenne-Laureau et al. 2003b). In practice, it will distinguish *B. ostreae* from *B. exitiosa* when these are the only 2 possible *Bonamia* spp. present. One conventional PCR assay was designed for specific amplification of *B. exitiosa*, and has been in use for several years in the USA (Carnegie et al. 2008). We now know that it will also amplify members of the *Bonamia* sp. lineages sister to *B. exitiosa*, from *O. chilensis* in Chile and *O. edulis* in California. The PCR assays specific for *B. ostreae* and *B. exitiosa*, designed by Ramilo et al. (2013), represent a promising development. These species-specific primers can be used in a conventional PCR setting as well as a SYBR Green real-time PCR setting and even in combination with each other in the conventional PCR format as a dual-parasite multiplex assay. These assays will require some scrutiny before they are widely accepted, as their reverse primers bind to ITS regions that are more likely to vary intra-specifically, increasing theoretical risks of false-negative results.

The generation of false-positive results is a problem for all diagnostic assays. This becomes more critical with increasing sensitivity of the assay used. The potential for detection of false positives when using real-time PCR for detection of *Bonamia* has been raised by Ramilo et al. (2013). This can be an issue in *Bonamia*-enzootic areas, if no specific precautions are taken to prevent cross-contamination during sampling and processing of the samples (M. Y. Engelsma et al. unpubl. data). On the other hand, high sensitivity facilitates early detection of parasite, so such assays are useful tools for eradication programs. In other words, the applied sampling strategy and diagnostic method should always be fit-for-purpose (for example, demonstration of freedom or eradication, or determination of prevalence).

For localisation of the parasite in host tissues, 2 first-generation *in situ* hybridization (ISH) assays remain in use. One of these is fluorescence-based and likely to hybridize to most *Bonamia* spp. (Carnegie et al.

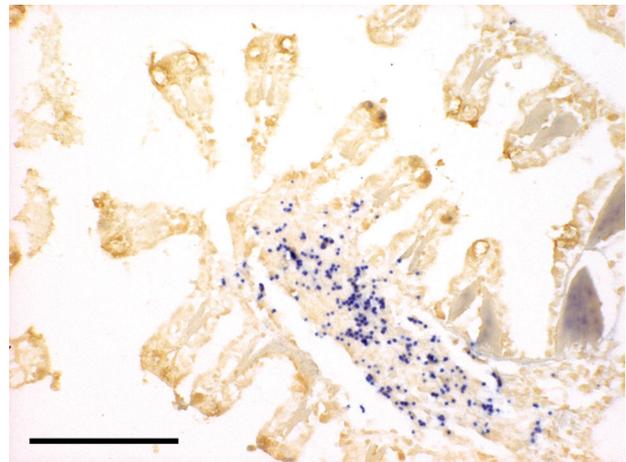


Fig. 6. *In situ* hybridization to *Bonamia ostreae* cells in the gills of *Ostrea edulis*. Image is from the Central Veterinary Institute collections. Scale bar = 100 μ m

2003), and the other is chromogenic and may be more broadly specific to haplosporidians in general (Cochenne et al. 2000; Fig. 6). A newer chromogenic ISH assay detects only *B. exitiosa* and members of its sister lineages from Chilean *O. chilensis* and *O. edulis* from California (Hill et al. 2010b).

EPIZOOTIOLOGY

Bonamia ostreae can be detected in *Ostrea edulis* larvae (Arzul et al. 2011), and *O. edulis* spat can demonstrate a high prevalence of infection (Lynch et al. 2005). However, older or larger oysters frequently appear to display the heaviest levels of infection, particularly in populations that have recently been infected with the parasite and where stressors such as dredging may exacerbate infection and mortality rates (Cáceres-Martínez et al. 1995, Culloty & Mulcahy 1996). *B. exitiosa* also affects larger and older oysters in a population (e.g. Dinamani et al. 1987, Kroeck & Montes 2005), but it is not clear that this is where infection and disease effects are most severe. In general, younger oysters, especially young-of-the-year recruits, receive less attention in pathology surveys than market-sized individuals, so part of the story with regard to size-specific infection patterns may be missed. In fact, there is clear evidence that *B. exitiosa* effects are most prevalent primarily in seed oysters of some species, like *Crassostrea ariakensis* (Burreson et al. 2004). Infection primarily of younger animals can reflect resistance dynamics in a population subjected to highly virulent pathogens, which appears to be the situation in *C. virginica* populations

long exposed to *Haplosporidium nelsoni*. *H. nelsoni* is an agent more of juvenile than adult oyster disease in such populations (Carnegie & Burreson 2011). Given the similarly high pathogenicity of *B. ostreae* and *B. exitiosa* in some naïve hosts (Pichot et al. 1980, Burreson et al. 2004), we might ask whether infections by these parasites are most prevalent among younger oysters in other systems where the epizootiology among various age classes has yet to be studied. Limited evidence from the Mediterranean coast of Spain (Carrasco et al. 2012) suggests that *B. exitiosa*–*O. edulis* does not represent one of these systems.

Environmental factors and site-related conditions may influence disease development. Arzul et al. (2009) noted that physical characteristics such as water temperature and salinity can affect the infective capacity and spread of *B. ostreae*. Engelsma et al. (2010) observed a positive relationship between *B. ostreae* prevalence and salinity in the Netherlands, which might indicate that lower salinities are less favourable for *B. ostreae* survival. Similar results have been observed in Ireland (G. Flannery et al. unpubl. data). Arzul et al. (2009) showed that, experimentally, higher salinity (≥ 35 g l⁻¹) supports better *B. ostreae* survival in seawater, thus *B. ostreae* may not be able to survive in populations in low-salinity regions such as estuaries. In the well-studied *B. exitiosa*–*C. ariakensis* system, infection and disease pressure has been found to be strongly associated with euhalinity (Bishop et al. 2006, Audemard et al. 2008a,b). This more likely reflects reduced parasite activity at lower salinities than reduced host immunocompetence at higher salinities.

B. ostreae infections occur throughout the year, yet many studies have documented a seasonal variation in infection, with temperature driving this variation (Grizel & Tigé 1982, Cáceres-Martínez et al. 1995, Culloty & Mulcahy 1996, Engelsma et al. 2010). Seasonal peaks in prevalence have been observed particularly in the spring (Engelsma et al. 2010) or in early autumn following spawning (Culloty & Mulcahy 1996). A study of *B. ostreae* survival with respect to temperature showed significantly higher survival at 15°C compared to 25°C (Arzul et al. 2009). This suggests that the ability of *B. ostreae* to survive may be reduced in waters where average temperatures are above 20°C. While potential low-temperature limitation of *B. ostreae* has not been well studied, Arzul et al. (2009) confirmed survival of the parasite at 4°C. In addition, the parasite was confirmed for the first time in a population in Norway (WAHID-Interface 2009), suggesting that low tem-

peratures might not be a limiting factor in the spread of this parasite.

Like *B. ostreae*, *B. exitiosa* can be observed throughout the year, displaying only a modest seasonal cycle, as in *O. chilensis* in New Zealand where it has best been characterized (Hine 1991). Cranfield et al. (2005) described the wave of infection with *B. exitiosa* spreading through Foveaux Strait, New Zealand, followed by a wave of mortality in *O. chilensis*. *B. exitiosa* activity in New Zealand fits a simple epizootic model suggesting that diffusion and turbulent processes are important in transmission of the infection. While natural transmission processes of *B. exitiosa* in the southeastern USA are less well understood, the parasite does display a stronger seasonal cycle there than it does elsewhere. In *C. ariakensis* in North Carolina, USA, infection and resulting mortality peaked in the summer months (Carnegie et al. 2008), with the parasite much less prevalent in winter (e.g. Burreson et al. 2004). In *O. stentina*, which is endemic in the *B. exitiosa*-enzootic North Carolina systems, a similar temporal trend in *B. exitiosa* infection is observed, although prevalence of the parasite is typically low (<5%) year-round (R. B. Carnegie unpubl. data).

HOST-PARASITE INTERACTIONS

The *Bonamia ostreae*–*Ostrea edulis* system is an interesting model to investigate interactions between molluscs and their intracellular parasites. *B. ostreae* mainly infects haemocytes, cells with a pivotal role in the oyster defences. Phagocytosis by haemocytes is the main cellular immune response against pathogens in molluscs. However, after internalization, *B. ostreae* is able to survive and multiply in the phagocytes, escaping post-phagocytosis mechanisms. Although methods for *in vitro* cultivation of *B. ostreae* are lacking, a protocol for parasite isolation from infected oysters (Mialhe et al. 1988a) and short-term haemocyte cell culture methods (Mourton et al. 1992) have enabled *in vitro* studies on interactions between haemocytes of flat oysters and the parasite.

Contact experiments between haemocytes and parasites have facilitated a better description of the host cell responses to infection with *B. ostreae*. As soon as 30 min after contact, the parasite is internalized within the haemocytes. Chagot et al. (1992) showed that exposure of haemocytes or parasites to cytochalasin B, an inhibitor of microfilament function, before the contact experiment decreased parasite internalization, suggesting that not only the host cell,

but also the parasite, contributes to its own internalization. A gene encoding *B. ostreae* heat shock protein 90 (HSP90) has recently been characterized, and the involvement of this protein in haemocyte invasion was investigated by pre-exposing parasites to radicicol, an HSP90-inhibitor. Haemocytes in contact with radicicol-exposed parasites appeared significantly less infected than haemocytes in contact with non-treated parasites (Prado-Alvarez et al. 2013). Flow cytometry was used to demonstrate that live parasites are phagocytosed at higher rates than fluorescent beads (Cochennec 2001). Numbers of infected haemocytes were similar after the contact with live and heat-inactivated parasites; however, the mean number of parasites per infected haemocyte was higher in the first treatment (Morga et al. 2009). Moreover, examination of naturally infected flat oysters revealed that the number of tissue-infiltrating haemocytes increased with infection intensity, suggesting a recruitment process at the site of infection whereas circulating haemocyte densities were not statistically different between infected and non-infected oysters (Cochennec-Laureau et al. 2003a). The parasite might activate the recruitment of host cells before actively contributing to its own internalization.

Studies have also been carried out in order to understand recognition mechanisms between haemocytes and *B. ostreae*. Incubation of parasites with sugars (glucose, mannose, fucose, N-acetyl glucosamine and galactosamine) before contact experiments induced a decrease in haemocyte infection. *B. ostreae* seems to possess lectin-like molecules involved in their recognition by the haemocyte (Chagot et al. 1992). A gene encoding a galectin (Oe-Gal) has been completely characterized using suppression subtractive hybridization, carried out between infected and uninfected haemocytes. This gene appeared to be over-expressed in haemocytes within 1 h of contact with the parasites, suggesting that the Oe-Gal is involved in the recognition of the parasite (Morga et al. 2011). Moreover, the cytoskeleton of the host cell seems to be involved in the internalization of the parasite as suggested by the over-expression of a gene coding a filamin in infected haemocytes (Morga et al. 2011) and the abundance of some genes related to the cytoskeleton in the libraries obtained from infected flat oysters (Martín-Gómez et al. 2012).

Post-internalization interactions have notably been investigated by flow cytometry: contrary to heat-inactivated parasites, live *B. ostreae* induced a decrease of reactive oxygen species (ROS) production and esterase activities between 1 and 4 h post infection and phagocytosis activity decreased at 2 h

(Morga et al. 2009, 2011). In addition, the expression of an extracellular superoxide dismutase (Oe-EcSOD) decreased after 1 h of contact. Several genes encoding proteins involved in detoxication and degradation including cytosolic SOD, omega glutathione S-transferase, tissue inhibitors of metalloproteinase and an interferon regulatory like factor were over-expressed in haemocytes in contact with live parasites (Morga et al. 2011). The energetic reserves of the host appear mobilized to obtain the energy to counter the parasite as demonstrated by the up-regulation of genes involved in metabolism, such as immunoresponsive gene 1 (Martín-Gómez et al. 2012). A significant reduction of phenoloxidase activity in the haemolymph of flat oysters infected with the parasite was also reported in comparison with uninfected oysters (da Silva et al. 2008). All of these results demonstrate that infected oysters and more particularly haemocytes develop mechanisms to degrade the parasite, but the parasite is partly able to turn off the haemocytes' metabolic destructive capacity. Mechanisms allowing intracellular survival of *B. ostreae* have mainly been investigated by measuring the impact of the parasite on haemocytes. However, few studies have directly considered parasite attributes. Hervio et al. (1991) detected acid phosphatase activity in membrane-bound organelles known as 'dense bodies' of *B. ostreae* that may be used to coat and protect the plasma membrane and in this way may contribute to the intracellular survival of the parasite. An ultracytochemical study carried out on *Bonamia* sp. in *O. chilensis* suggested that lipid bodies might contribute to intracellular parasite survival by notably preventing lysosome-phagosome fusion through modification of the host phagosome membrane (Hine & Wesney 1994). After internalization, the parasite seems to escape destruction and is even able to multiply after at least 2 h of contact. For example, some binucleate parasites were observed in haemocytes after 2 and 4 h of contact with *B. ostreae* (Morga et al. 2011).

The availability of oysters selected for their resistance to bonamiosis has allowed investigation of cellular and molecular mechanisms involved in the resistance of the oysters to the disease. Flow cytometry has shown that resistant oysters display higher esterase activities and higher ROS production after stimulation with phorbol myristate acetate than wild oysters (Cochennec 2001). Comparing infected and non-infected status in both groups of oysters revealed that wild infected oysters produced fewer ROS between 3 and 5 d after injection of the parasite while resistant infected oysters produced fewer

ROS, especially at 8 and 30 d after injection (B. Morga et al. unpubl. data). The decrease in ROS production in resistant oysters might consequently lead to reduced host cellular damage compared to infected wild oysters. Concurrently, the capacity for phagocytosis appeared lower in infected resistant oysters for both cohabitation and injection infection experiments, suggesting that resistant oysters internalize fewer parasites than wild oysters and by this mechanism limit the distribution of the parasite in the different organs of the oyster (B. Morga et al. unpubl. data). Real-time quantitative reverse-transcription PCR revealed an over-expression of Oe-EcSOD *in vitro* in haemocytes from resistant infected oysters compared to haemocytes from wild infected oysters (Morga et al. 2012). Resistant oysters might display better capacity to limit the negative impact of ROS, produced to degrade *B. ostreae*, on host cellular components. In contrast, this gene was down-regulated *in vivo* in resistant oysters challenged by injection and cohabitation. Differences observed between *in vitro* and *in vivo* conditions highlight that interactions between host and parasites are complex. While *in vitro* experiments help to illuminate some interactions at the cellular level, *in vivo* conditions reveal them at the oyster level. In addition, 3 genes involved in the modulation of apoptosis (inhibitor of apoptosis, Fas ligand and cathepsin B) were found to be over-expressed in resistant infected oysters, suggesting that induction of programmed cell death in infected haemocytes may play an important role in the resistance mechanisms (Morga et al. 2012) while the parasite might inhibit this phenomenon in order to survive in haemocytes.

In contrast to the European flat oyster *O. edulis*, at the cellular level the Pacific cupped oyster *C. gigas* is highly resistant to infection with *B. ostreae* (Renault et al. 1995). At the cellular level, *C. gigas* haemocytes are able to phagocytose and destroy the parasite (Chagot et al. 1992, Mourton et al. 1992). A comparison between both oyster species revealed significant differences in total and differential haemocyte counts (THC and DHC, respectively) and respiratory burst: *C. gigas* showed lower THC and higher numbers of granulocytes, and appeared less susceptible to interference of *B. ostreae* in the respiratory burst than *O. edulis* (Comesaña et al. 2012). Haemolymph protein profiles have also been compared by 2-dimensional electrophoresis between flat and cupped oysters and also between *O. edulis* stocks with different susceptibility to bonamiosis. The highest number of proteins was observed in *C. gigas*, whereas the lowest num-

ber corresponded to the most susceptible *O. edulis* stock (Cao et al. 2009). Earlier work showed that enzyme activities were higher in the haemocytes of *C. gigas* compared to *O. edulis*, suggesting that this difference could contribute to the natural resistance of the cupped oyster to infection with *B. ostreae* (Xue & Renault 2000).

Although many questions remain unsolved, and despite the lack of haemocyte and parasite culture systems, our understanding of interactions between oysters and the intracellular parasite *B. ostreae* and of mechanisms involved in the resistance of oysters to the disease continues to grow.

DISEASE RESISTANCE AND CONTROL

Translocation of oysters is thought to have contributed to the historical dispersal of *Bonamia* parasites (e.g. Elston et al. 1986), and it is still viewed as a primary cause of contemporary parasite dispersal. Given the limited options for management of the disease (see below), prevention of spread is a key component in handling the disease. The OIE International Aquatic Animal Health Code has recommended clear measures to prevent any further spread of the disease (OIE 2012). *B. ostreae* and *B. exitiosa* are classified as notifiable pathogens by the OIE and the EU, and under legislation, declaration of an occurrence of the disease is compulsory for authorities in affected countries. As a result of this, all countries within the EU are required to undertake national screening programmes to determine and monitor the disease status of their *Ostrea edulis* populations. Although *B. exitiosa* has been shown to be present at several locations in *O. edulis* stocks within Europe, under the current EU legislation, the parasite is still considered exotic to Europe. If an area is determined to be infected with bonamiosis, exportation of flat oysters to areas which have an equal zoosanitary status may continue, but exportation to disease-free areas is heavily limited, with any exported stock to remain in complete isolation from the native disease-free populations. Upon certification of their status, disease-free stocks can be exported without any such limitations. To prevent the further spread of the disease within a country once an infection has occurred, a zoning system is implemented around that area, with at-risk *Bonamia*-free areas declared protected zones, in which increased measures must be taken to prevent spread of infection. These measures may include movement control and an intensified degree of surveillance. Countries with areas with previously

infected stocks can declare themselves *B. ostreae* free, once the aforementioned zones have been established, all infected specimens have been destroyed, a biosecurity assessment has been performed, and disease surveillance has been conducted by the relevant state authority for at least 2 yr without any further detection of *B. ostreae*.

With the possible exception of a single isolated site with no natural population of *O. edulis* in Loch Sunart, Scotland (D. Fraser pers. comm.), eradication has not been documented from any area where *B. ostreae* has been detected. Van Banning (1987) found that even after the removal of all flat oysters from the area and a fallowing period of several years, oysters reintroduced into Lake Grevelingen, the Netherlands, developed bonamiosis. As such, prevention of the spread of bonamiosis and management of the disease in areas where it is already present are the only mechanisms available to reduce the impact of this pathogen. The near ubiquitous presence of *B. ostreae* in Europe today is due, in part, to past failures to implement such preventive measures (FAO 2004).

In terms of management, a selection of husbandry techniques have been introduced and modified into the farming of flat oysters in different areas to help minimise mortalities from the disease (Culloty et al. 2004). These include reducing stocking densities, fallowing beds, on-growing in deeper waters, using suspended culture, cleansing sites and fishing gear, culling oyster beds in which infection levels exceed 10% and marketing oysters after 15 to 18 mo (Grizel et al. 1988, Hugh-Jones 1994, Montes et al. 2003). The success of these strategies has been variable due to local conditions, but the reduction of stressors in the growth cycle can minimise the severity of the disease.

A similar observation has been made for *B. exitiosa* in *O. chilensis* in New Zealand: intense dredging of the oysters appeared to be a major source of stress, increasing the susceptibility of the oysters to *B. exitiosa* (Cranfield et al. 2005). With reduction of disturbances by use of lighter dredges and less damaging towing strategies, the probability of disease outbreaks may be reduced. At most locations in Europe where *B. exitiosa* has been observed, *B. ostreae* is also endemic. This makes it difficult to get a clear picture of the effect of *B. exitiosa* on *O. edulis* stocks. Hence, no specific management to reduce the impact of *B. exitiosa* is employed.

Several studies, incorporating laboratory and field trials at small and large scales, have been conducted over the past 30 yr to investigate the ability of flat

oyster stocks to develop resistance to *B. ostreae* (Elston et al. 1987, Martin et al. 1993, Boudry et al. 1996, Baud et al. 1997, Naciri-Graven et al. 1998, Culloty et al. 2001, 2004, Lapègue et al. 2003, Lynch et al. 2005). Studies have focused on breeding for resistance after either the selection of families by genetic analysis or the large-scale breeding from survivors in the field.

An early study by Elston et al. (1987) indicated that some difference in susceptibility of various stocks could be observed in the laboratory. Since then, a range of studies have been carried out throughout Europe. Several field trials have been carried out at Cork Harbour, Ireland, a *B. ostreae*-enzootic site since the late 1980s, to investigate the performance of the local Rossmore oyster stock (see Lynch et al. 2014, this DAO Special). These oysters have been selectively bred from survivors of disease outbreaks and mortality events on the oyster beds since the 1980s, when the parasite was first detected in this stock. When Rossmore oysters were compared to other Irish stocks that had not been selectively bred for resistance, the Rossmore oysters performed better than the 3 other Irish stocks in terms of survival and prevalence of infection, indicating that some level of resistance had been established in the Rossmore stock (Culloty et al. 2001). In a follow-up study at several European sites, Rossmore oysters and another exposed European stock from the Netherlands performed better than naïve Irish and Scottish stocks (Culloty et al. 2004). The study also indicated that very young oysters could become infected. Lynch et al. (2005) found that this difference in susceptibility could even be observed in very young oysters, i.e. spat, only several months old.

Some work on oyster breeding programmes to develop stocks resistant to *B. ostreae* has also been carried out in France, albeit with limited success (Martin et al. 1993, Boudry et al. 1996, Baud et al. 1997), as inbreeding and population bottlenecks remain an issue (Launey et al. 2001, Culloty et al. 2004). The selective breeding programme was first initiated in 1985 in France, with the production of 2 oyster populations by mass spawning (Naciri-Graven et al. 1998). Individual selection was carried out by inoculation tests and field-testing, with survivors being used as broodstock to produce 3 generations of offspring; however, the progeny exhibited low genetic diversity and were considered unsuitable for a sustainable population (Launey et al. 2001). To counteract inbreeding, oyster families were selectively bred to produce progeny with a greater genetic diversity in 1998. These families showed enhanced survival and

a lower prevalence of infection compared to control oysters in *B. ostreae*-infected areas (Lapègue et al. 2003).

FUTURE DIRECTIONS

The remarkable discovery of *Bonamia exitiosa* in so many new hosts and locations over the last decade has already opened 2 major areas of new study. The first area involves understanding the ecology and epizootiology of this parasite in the myriad systems in which it occurs, including its interactions with *B. ostreae* where the 2 species occur together (e.g. Abollo et al. 2008). The second area involves understanding the factors influencing the dispersal of *B. exitiosa* to such locales. We may hope that genetic analyses will shed light on which component of this dispersal is natural and which is anthropogenic, either one of which may include co-dispersal with the oyster *Ostrea stentina* (Hill et al. 2010b). Both of these *B. exitiosa*-related research areas should receive much attention in the coming years.

A second major area of focus on bonamiosis has also begun, and will involve a biotechnological and functional focus on host–parasite interactions. Recent studies have focused on gaining a greater understanding of the response of oysters to *B. ostreae* infection (Morga et al. 2009, 2011, 2012, Comesaña et al. 2012, Martín-Gómez et al. 2012). Functional studies combining more sophisticated genetic methodology, proteomics and cellular and ultrastructural approaches will be required to better characterize how these mechanisms allow an oyster host to resist *Bonamia* infection and the parasite to escape host responses.

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