Pathogens of marine bivalves in Maine (USA): a historical perspective

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ABSTRACT

Shellfish aquaculture (in particular oyster cultivation) has the potential to play a significant role in refreshing the coastal economy in the state of Maine (USA). Although ocean warming and acidification are often listed as the primary manifestations of climate change in the marine environment, the issue of expanding geographic ranges of parasites and pathogens is an equally serious threat to shellfish populations. Protozoan parasites of the genera *Bonamia*, *Haplosporidium*, and *Perkinsus*, the bacterial pathogen responsible for Roseovarius oyster disease, and the disease condition is known as disseminated neoplasia are currently recognized as some of the significant threats to natural and farmed bivalve populations. We have analyzed the peer-reviewed literature for reports of these pathogens/conditions in Maine. Most reports focus on oysters from the Damariscotta River Estuary and are the result of directed studies into the biology of a particular pathogen rather than the result of intensive monitoring programs. The sampling effort could be interpreted in several ways including; the pathogens/conditions not impacting the annual harvest to any great extent due to limited distribution of the causative agent, the oysters developing a tolerance to the diseases, or just a lack of resources directed at studying this topic. With the shellfish aquaculture industry expected to grow in the next several decades, we recommend that a rigorous and sustained survey of parasitic diseases and believe that such an effort is fundamental to the success, resilience, and well-being of Maine's shellfish aquaculture industry.

Keywords

Bivalves; Dermo; DN; MSX; ROD; pathogen.

Abbreviations

DN, disseminated neoplasia; MSX, multinucleated sphere X; ROD, Roseovarius oyster disease.

1. Introduction

Over the past several decades, the fishery-based economy of coastal Maine has seen a decline in economic and ecological diversity as wild stocks of harvested fish have collapsed (e.g., cod) (Meng, Oremus, Gaines, 2016; Pershing, Alexander, Hernandez, Kerr, Le Bris, Mills, Nye, Record, Scannell, Scott, Sherwood, Thomas, 2015). This decrease in fishery stocks has placed the industry in the vulnerable position of a monoculture economy, depending primarily on lobsters (Steneck, Hughes, Cinner, Adger, Arnold, Berkes, Boudreau, Brown, Folke, Gunderson, Olsson, Scheffer, Stephenson, Walker, Wilson, Worm, 2011). One critical component to revitalizing the coastal economy while preserving Maine's maritime culture is to complement the rebuilding of wild fish stocks with the development of a robust aquaculture industry. Shellfish aquaculture – of oysters in particular – has been increasing in recent years and has the potential to play a significant role in revitalizing Maine's coastal economy. In addition to being a valuable resource for coastal economies, oysters are considered ecosystem engineers; they influence estuarine water quality, provide protection and habitat for other species, and provide a significant food resource for humans (Dumbauld, Ruesink, Rumrill, 2009; Humphries, La Peyre, Decossas, 2011; Rick, Reeder-Myers, Hofman, Breitburg, Lockwood, Henkes, Kellogg, Lowery, Luckenbach, Mann, Ogburn, Southworth, Wah, Wesson, Hines, 2016). The historical record indicates that natural populations of bivalves were once an abundant source of food for the coastal inhabitants, however traditional local harvesting of the natural populations is being substituted worldwide by semi-intensive aquaculture operations (Larsen, Wilson, Morse, 2013).

Given the rapid developments of coastal aquaculture in recent times, ensuring the survival of cultured bivalves to marketable sizes has become a top priority for producers, resource managers, and other stakeholders. Bivalve researchers have therefore focused their efforts on understanding the threats to shellfish populations due to biological agents such as parasitic protozoa and bacterial pathogens (Coen, Bishop, 2015; Dégremont, Garcia, Allen, 2015; Shinn, Pratoomyot, Bron, Paladini, Brooker, Brooker, 2014). The coast of Maine has long been viewed as an ideal location for bivalve aquaculture due in part to its relatively pristine environment, miles of shoreline, and historically low occurrence of shellfish die-offs due to disease-causing agents. However, recent studies (Marquis,

Record, Fernández Robledo, 2015; Messerman, Bowden, 2016; Messerman, Johndrow, Bowden, 2014; Miles, 2016) have suggested that there are many biological threats to shellfish in coastal Maine, and that risk levels may be increasing due to northward advances of previously undetected threats. Environmental risks often become the focus of study only after damage has been done; in coastal Maine, we have the opportunity to establish a baseline in the early phase of bivalve aquaculture growth. Herein, we focus on summarizing the state of knowledge regarding some of the major biological threats to shellfish populations along the coast of Maine, USA. These threats include those from the parasitic protozoan genera *Perkinsus*, *Haplosporidium*, *Marteilia*, and *Bonamia*, as well as from the bacterial pathogen Roseovarius oyster disease (ROD; formerly JOD, juvenile oyster disease), and the disease condition known as disseminated neoplasia (DN) (Barber, 2004; Boettcher, Geaghan, Maloy, Barber, 2005). It must be recognized that global warming and ocean acidification are other key factors affecting the health and well-being of marine mollusks (Boulais, Chenevert, Demey, Darrow, Robison, Roberts, Volety, 2017; Dineshram, Chandramouli, Ko, Zhang, Qian, Ravasi, Thiyagarajan, 2016; Goncalves, Thompson, Raftos, 2017; Goncalves, Anderson, Thompson, Melwani, Parker, Ross, Raftos, 2016; Wang, Cao, Ning, You, Mu, Wang, Wei, Cong, Wu, Zhao, 2016). It is quite likely that these climate-driven changes to the marine environment will interact in complex ways with threats from the biological agents to exacerbate the risk to shellfish populations, especially in regions like the Gulf of Maine that are experiencing enhanced rates of change relative to other locations (Balch, Drapeau, Bowler, Huntington, 2012; Pershing, Alexander, Hernandez, Kerr, Le Bris, Mills, Nye, Record, Scannell, Scott, Sherwood, Thomas, 2015). However, direct linkages between climate change and both the prevalence and spread of shellfish diseases remain unclear and will require major research efforts to understand. This review, in the context of programs like the Sustainable Ecological Aquaculture Network (SEANET; https://umaine.edu/epscor/seanet), should help to develop a better understanding of the challenges that are faced by the Maine shellfish industry to ensure its continued growth and sustainability. We also discuss future perspectives and our vision for shellfish aquaculture and related research in light of the current threats.

2. Materials and methods

The SCOPUS database (http://www.info.sciverse.com/scopus/) contains over 20,500 titles from 5,000 publishers worldwide with more than 60 million records, and it goes back to 1823. We searched for peer–reviewed articles published starting from 1950 until 2016 with titles or abstracts containing particular protozoan parasite taxonomic or disease name strings and references to Maine (Table 1) as reported elsewhere (Fernández Robledo, Vasta, Record, 2014; Ward, Lafferty, 2004); no records of meeting proceedings were included. References were imported to EndNote (Thompson-Reuters), and titles and abstracts were manually curated by eliminating duplicities, searched for each of the parasites/diseases and sorted by year, and the resulting number of publications was then used to build an Excel spreadsheet to generate the plots.

3. Results and discussion

The number of papers in the literature (SCOPUS Database) reporting on the genera *Bonamia*, *Haplosporidium*, and *Perkinsus*, and the conditions disseminated neoplasia (DN) and Roseovarius oyster disease (ROD) were plotted over time (Fig. 1). Overall, the number of papers focusing on these major parasite genera and bivalve conditions was low; the highest and lowest number of papers corresponded to ROD (12 papers) and *Bonamia ostreae* (6 papers), respectively. Here we describe the conditions and pathogens in chronological order reported for the state of Maine.

3.1. Disseminated neoplasia (DN)

DN is a form of lethal cancer that affects soft shell clams (*Mya arenaria*) locally known as "steamers." Heavily affected specimens are recognized by very dense hemolymph when withdrawn from the adductor muscle (Fig. 2A) and easily identified under the microscope by round cells that do not spread when deposited on a glass slide (Fig. 2B). The first reference to DN in *M. arenaria* from Maine corresponds to a survey of clams from Portland, Goose Cove, and Searsport in 1978 by Brown et al. (Brown, Wolke, Saila, Brown, 1978). Only clams from Searsport were diseased with a prevalence of 19%. Since the first report, there have been eight references to DN in Maine, with one study focusing on discerning the molecular basis for naturally occurring diseases (Walker, Bottger, 2008) and another focused on the *in vitro* culture of the neoplastic cells (Walker, Bottger, Mulkern, Jerszyk, Litvaitis, Lesser, 2009). DN represents the first case of horizontal transmission of clonal cancer cells between individuals in marine bivalves and the third known case in the animal kingdom

140 (Metzger, Reinisch, Sherry, Goff, 2015) (Fig. 2C). The genotypes of neoplastic cells from clams
141 sampled in locations on the East Coast of North America (New York and Maine in the USA, and
142 Prince Edward Island in Canada) are nearly identical and differ from those of the host animal
143 (Metzger, Reinisch, Sherry, Goff, 2015). Similarly, analysis of DN in bivalves elsewhere indicates that
144 neoplasias are attributable to independent transmissible cancer lineages and that clonal cancer cells
145 can cross species boundaries (Metzger, Villalba, Carballal, Iglesias, Sherry, Reinisch, Muttray,
146 Baldwin, Goff, 2016). Cancer cells in the golden carpet shell clam *Polititapes aureus* are all derived
147 from the clam *Venerupis corrugata*, a different species living in the same geographical area. DN has
148 been described in *C. virginica* from the Chesapeake Bay [reviewed in (Barber, 2004)]. Demonstrated
149 horizontal transmission and the high prevalence of DN in *M. arenaria* from Maine (Nault, 2016),
150 highlights the need for a close monitoring for this condition in *O. edulis* and *C. virginica*.

151 **3.2. *Bonamia ostreae***

152 *Bonamia ostreae* (Fig. 3), a parasite of the European flat oyster *Ostrea edulis*, was described
153 after mass mortalities of *O. edulis* in Brittany (France) in 1979 (Pichot, Comps, Tigé, Grizel, Rabouin,
154 1979). There is still active debate on where *B. ostreae* evolved and how it reached areas beyond its
155 initial description area in France, but some data suggest that it may have originated from the West
156 Coast of the U.S.A. from where *O. edulis* seed was transferred to France years previous to the
157 detection of the disease there (Elston, Farley, Kent, 1986). Interestingly, some of the stocks where *B.*
158 *ostreae* was detected in the West Coast were used in hatcheries in Maine (Elston, Farley, Kent,
159 1986). The first report of *B. ostreae* in Maine corresponds to *O. edulis* from sites in the Damariscotta
160 River Estuary (DRE), where the parasite was identified in 1991-1992 with prevalence 34-45%
161 respectively (Friedman, Perkins, 1994a). Oyster samples in the middle 1990's presented a lower
162 prevalence (5%) (Zabaleta, Barber, 1996). The diagnosis was based on histological preparations,
163 stained blood smears (Fig. 3A), and immunofluorescence (Friedman, Perkins, 1994b; Zabaleta,
164 Barber, 1996). The transmission of the disease can occur between individuals (Elston, Kent,
165 Wilkinson, 1987), although the presence of an intermediate host has not been ruled out (Burreson,
166 Ford, 2004; Lynch, Armitage, Coughlan, Mulcahy, Culloty, 2007) and spores have been described for
167 *Bonamia perspora* (Carnegie, Burreson, Hine, Stokes, Audemard, Bishop, Peterson, 2006). The

parasite infects/resides inside the oyster hemocytes or extracellularly when the hemocytes dies and the parasites are released (Fig. 3A-3C). Altogether, there are only four references to *B. ostreae* in Maine with the latest reference dating back to 1998 with a prevalence below 1% of tested specimens (Carnegie, Barber, 2001). The highest prevalence reported corresponded to the DRE in 1992 (45%) (Fig. 1). There is a growing interest in bringing back the large-scale production of *O. edulis* to Maine (noting that there are still some wild populations derived from previous attempts to introduce it in Maine). 'Extreme ice' winters appear to be responsible for preventing the spread of *B. ostreae* from imported flat oysters to native flat oyster populations in the Limfjord (Denmark) (Madsen, Kamp, Mellergaard, 2013). The extreme winters in Maine could favor the expansion of flat oyster aquaculture by keeping the incidence of *B. ostreae* low. However, as environmental conditions change in the Gulf of Maine, it is entirely possible that the prevalence of *B. ostreae* will also change.

3.3. *Haplosporidium nelsoni* (Multiple Sphere X)

Arguably, *H. nelsoni* (Fig. 4) is the most pertinent protozoan parasite to the oyster farmers and oyster gardeners in Maine. Two major die-offs of oysters in the 1990's and 2010 were blamed on this parasite, and reports of these events even reached local newspapers (Deese, Schmitt, 2011). Based on histology, the MSX parasite appears in the gills during in the early stages of infection (Burreson, Ford, 2004). The parasite is also observed in the digestive gland (Fig. 4A) and hemolymph (Burreson, Robinson, Villalba, 1988). The infective stage of *H. nelsoni* remains to be identified (Fig. 4B), but sporulation has been reported in spat (Barber, Kanaley, Ford, 1991) and transmission can occur between oysters separated by a 1 mm filter (Sunila, Karolus, Lang, Mroczka, Volk, 2000). The first major epizootic event involving MSX was reported from the Piscataqua River Estuary (near the river mouth the river separates the states of New Hampshire and Maine) with prevalence ranging from 15% to 81% of sampled oysters. Unusually warm and dry weather conditions in the estuary appear to have favored the parasite (Barber, Langan, Howell, 1997). The report following the 2010 die-off indicated the presence of oysters positive for MSX (via PCR and histology) in the DRE, upper Sheepscot River, and Mill Cove in the New Meadows River. The previous survey included 15 sites between Casco Bay and Taunton Bay in Franklin, ME (Bouchard, 2012). Sites that were positive for MSX in 2010 were subsequently sampled in June/July 2011, but MSX was not detected at either of

196 those locations (Bouchard, 2012). Given the absence of oyster die-offs since 2010 and the use of
197 MSX-resistant oyster strains it is thought to have brought the threat of MSX is under control in Maine
198 (Giray, 2016). Recent PCR-based studies have reported medium to high prevalence of *H. nelsoni* in
199 farmed oysters in multiple areas in Maine, which supports a broader distribution of the etiological
200 agent, whether this high prevalence would develop into disease and mortalities remains to be seen.
201 The reported prevalence of MSX in oysters from the DRE during 2013-2014 varied between 26%
202 and 60% (Marquis, Record, Fernández Robledo, 2015; Messerman, Bowden, 2016; Messerman,
203 Johndrow, Bowden, 2014; Miles, 2016) (MMS, SEK unpub. results) and in the 2017 summer we
204 have recorded prevalence of MSX above 80% in DRE (JAFR, NDM, PDC, RRR, TB, unpub. results).
205 The latest survey expands the presence of MSX to the Bagaduce River, Basket Island, Maquoit Bay,
206 Webhannet River with low prevalence (9-17%) across all sites (Marquis, Record, Fernández
207 Robledo, 2015). These PCR-based findings indicate a range for *H. nelsoni* that is broader than
208 earlier thought and increases the complexity for understanding the epizootiology of the disease.
209 Switching from an MSX susceptible strain of oysters to an MSX resistant strain appears to have
210 reduced the prevalence of the parasite when comparing surveys two years apart (2012-2014)
211 (Dickey, Messerman, Bowden, In Press; Messerman, Johndrow, Bowden, 2014). Management of
212 oyster resources around MSX is quite challenging since the full *H. nelsoni* life cycle remains
213 unknown; and at this point, we do not know if an intermediate host is required to complete the life
214 cycle or how *H. nelsoni* enters the oyster (Arzul, Carnegie, 2015; Ford, Ashton-Alcox, 1998). In a
215 survey for potential reservoir species for MSX in the DRE during the summer of 2012, up to 70% of
216 the tunicate *Styela* sp. and about 30% of plankton sampled for were positive for MSX DNA using
217 qPCR (Messerman, Bowden, 2016). Recently, we have also identified DNA sequences matching *H.*
218 *nelsoni* in a survey from three sites in the DRE [J.A.F. et al., unpublished data]. These studies
219 (Marquis, Record, Fernández Robledo, 2015; Messerman, Bowden, 2016; Messerman, Johndrow,
220 Bowden, 2014; Miles, 2016) reporting the prevalence of *H. nelsoni* are PCR based with no
221 histopathological study associated nor do not have information on the genotype of the oysters
222 sampled (diploid vs. triploid, oyster strains origin), which adds a layer of complexity to interpreting the
223 prevalence in the absence of reported die-offs. Finally, the contribution of wild oyster populations to

224 the parasite load in the water is unknown based on the above-mentioned gaps in the life cycle but
225 also because any die-offs affecting wild oyster populations are likely to pass unnoticed, and most
226 mollusk pathogens are not associated with gross signs of disease (Carnegie, Arzul, Bushek, 2016).
227 **3.4. *Perkinsus* spp. (Dermo)**
228 *Perkinsus marinus* is a protozoan parasite that has been studied in more detail than most other
229 oyster parasites (Fernández Robledo, Vasta, Record, 2014). Trophozoites of the parasite can be
230 easily distinguished in histology samples by their characteristic 'signet ring' cell shape (Fig. 5A); with
231 a direct life cycle, most of the life cycle stages have been characterized (Fig. 5B). *Perkinsus marinus*
232 was first reported in Maine in the DRE; the protozoan was identified in *C. virginica* hemolymph using
233 Ray's Fluid Thioglycollate Medium (RFTM) (Kleinschuster, Parent, 1995) (Fig. 1). At the time, a
234 positive diagnostic by RFTM was associated to *P. marinus*, however it is now known that in the
235 absence of molecular assays the RFTM assay alone does not have the ability to differentiate *P.*
236 *marinus* from *P. chesapeaki* (Berthe, Choi, Figueras, Soudant, Villalba, 2004; Coss, Robledo, Vasta,
237 2001; Coss, Robledo, Ruiz, Vasta, 2001; Robledo, Gauthier, Coss, Wright, Vasta, 1998) and indeed
238 the same specimen can be co-infected by both host species (Arzul, Chollet, Michel, Robert, Garcia,
239 Joly, François, Miossec, 2012; Coss, Robledo, Vasta, 2001; Coss, Robledo, Ruiz, Vasta, 2001;
240 Marquis, Record, Fernández Robledo, 2015; McLaughlin, Faisal, 1998; Reece, Dungan, Burreson,
241 2008). Since the first report of *Perkinsus*, there have been 11 references to *Perkinsus* spp. in Maine.
242 In the 1990's the prevalence of *P. marinus* was qualified as "sub-clinical" (Kleinschuster, Parent,
243 1995); in the 2000's, the prevalence` for both *P. marinus* and *P. chesapeaki* was still low (Pecher,
244 Alavi, Schott, Fernandez-Robledo, Roth, Berg, Vasta, 2008). However, since the 2000's survey, we
245 have reported using PCR-based assays a 15–65-fold increase of the *P. marinus* and *P. chesapeaki*
246 prevalence over a period of 12 years in the DRE (Marquis, Record, Fernández Robledo, 2015). In
247 the same study, we reported that an oyster positive for both *Perkinsus* spp. is 3.2 times as likely to
248 be positive for a non-*Perkinsus* protozoan parasite. Early studies indicate that *P. chesapeaki* has a
249 preference for infecting clams (Arzul, Chollet, Michel, Robert, Garcia, Joly, François, Miossec, 2012;
250 McLaughlin, Faisal, 1998; Reece, Dungan, Burreson, 2008); however, the latest survey in Maine
251 shows an increase in the PCR-based prevalence of *P. chesapeaki* in oysters (Marquis, Record,

252 Fernández Robledo, 2015). The PCR-based diagnostic was run on DNA obtained from samples
253 including oyster‘s rectum, gill, and mantle. Hence, it would have included trophozoites inside
254 hemocytes circulating in the tissues, trophozoites propagating extracellularly, and trophozoites on the
255 surface of those organs. Like any other intracellular protozoan, the adherence to host tissues is part
256 of the infection cycle (Singh, Walia, Kanwar, 2016; von Itzstein, Plebanski, Cooke, Coppel, 2008). In
257 the case of *Perkinsus* spp., this adherence may even be part of the strategy of infection since
258 trophozoites lack of mechanisms for active entry into host cells (*e.g.*, gliding, apical complex).
259 Indeed, it has been shown that the galectin (CvGal1) mediates the entry in the hemocytes (Tasumi,
260 Vasta, 2007; Vasta, Feng, Bianchet, Bachvaroff, Tasumi, 2015), which can be found in the pallial
261 cavity (Lau, Gambino, Santos, Espinosa, Allam, 2018). Supporting the point that the oyster is
262 infected once trophozoites reaches the pallial cavity is that the parasite is already actively expressing
263 putative virulence genes in response to the contact with mucus (Pales Espinosa, Corre, Allam,
264 2014), translocation throughout the host body via the host's circulatory system occurs 12 days post
265 exposure to zoospores (Wang, Yoshinaga, Itoh, 2018), and *P. marinus* does not survive more than 3
266 days in the water outside the host (Chu, Lund, 2006). Recently, the presence of hemocytes
267 associated with mucus covering the pallial organs (mantle, gills, and body wall) have been implicated
268 on the pathogenesis of *P. marinus* (Lau, Gambino, Santos, Espinosa, Allam, 2018). Finally, all the
269 *Perkinsus* spp. described so far are parasites of mollusks; the Perkinsozoa group split from the
270 common ancestor circa 391 Million years ago (Okamoto, McFadden, 2008); they have evolved to
271 adhere, enter the oyster, and survive inside the defense cells of mollusks (Vasta, Feng, Bianchet,
272 Bachvaroff, Tasumi, 2015). Trophozoites reside both within the oyster hemocytes, which are
273 responsible for immune defense and transport among other functions (Comesana, Casas, Cao,
274 Abollo, Arzul, Morga, Villalba, 2012; Morga, Renault, Faury, Arzul, 2012; Morga, Renault, Faury,
275 Chollet, Arzul, 2011) and extracellularly exposing the tissues other than blood to the activity of
276 secreted proteases (Casas, Reece, Li, Moss, Villalba, La Peyre, 2008; Garreis, La Peyre, Faisal,
277 1996a; b; La Peyre, Schafhauser, Rizkalla, Faisal, 1995; Ottinger, Lewis, Shapiro, Faisal, Kaattari,
278 2001). In the absence of histopathology studies that would also help to define of the extend of the
279 damage and reaction of the hosts, hypothesis to explain these findings include new strains of *P.*

280 *chesapeaki* adapting to host/environmental conditions in Maine, an increase in the aquaculture
281 activities with more host getting infected and releasing trophozoites, and overall warmer
282 temperatures as well (Saba, Griffies, Anderson, Winton, Alexander, Delworth, Hare, Harrison, Rosati,
283 Vecchi, Zhang, 2016). So far, no oyster die-offs have been ascribed to *Perkinsus* spp. in Maine even
284 with the high PCR-based prevalence of the parasite. The lack of reported oyster mortalities in Maine
285 due to *Perkinsus* may derive from oysters reaching the commercial size and being harvested before
286 visible signs and mortalities; a situation already described in flat oysters infected with *B. ostreae*
287 (Cáceres-Martínez, Robledo, Figueras, 1995). *Perkinsus* spp. thrives at higher temperature and
288 salinity (Chu, Burreson, Volety, Constantin, 1993; Ford, Smolowitz, 2007; Queiroga, Marques-
289 Santos, De Medeiros, Da Silva, 2016); *in vitro* studies indicate that incubation at 4ºC of *Perkinsus*
290 spp. results in decreased viability with limited metabolic activity and no proliferation, but only partially
291 explains reduced parasite infection intensities in the colder months of the year (La Peyre, Casas,
292 Villalba, La Peyre, 2008). Most models for climate change predict an increase in the runoff from
293 terrestrial ecosystems in the estuaries, linked to higher variance between wet and dry conditions
294 (Balch, Drapeau, Bowler, Huntington, 2012), and an increase in the temperature in the Gulf of Maine
295 (Saba, Griffies, Anderson, Winton, Alexander, Delworth, Hare, Harrison, Rosati, Vecchi, Zhang,
296 2016). As an example, a buoy deployed in Penobscot Bay (www.neracoos.org) since 2001 has
297 shown a salinity range of 17-35 psu. Taking a coastal buoy deployment in Penobscot Bay as a
298 temperature baseline, there is currently <1 day per year with a mean temperature over the critical
299 Dermo threshold of 20 °C, whereas under 2 °C of warming, this increases to ~6 days per year, and
300 under 4 °C of warming, to ~20 days per year. With the high prevalence of *P. marinus* in Maine
301 oysters, unusually warm and dry weather conditions, a situation also associated to the die-off
302 attributed to MSX (Barber, Langan, Howell, 1997), might result in a perfect storm positioned to hit the
303 nascent oyster aquaculture industry.

304 **3.5. Roseovarius oyster disease (ROD)**

305 Caused by the α-proteobacterium *Aliiroseovarius crassostreae* (Maloy, Ford, Karney,
306 Boettcher, 2007), ROD can be tracked to 1988 (Boettcher, Barber, Singer, 1999; Bricelj, Ford,
307 Borrero, Perkins, Rivara, Hillman, Elston, Chang, 1992) (Fig. 6). The bacterium was recovered from

ROD-affected animals in numerous Northeast states in 1997 (Maloy, Barber, Boettcher, 2007). Altogether there have been 14 papers mentioning ROD in Maine including PCR diagnostic development (Maloy, Barber, Boettcher, 2005), epizootiological studies (Maloy, Barber, Boettcher, 2007), experimental challenges and survival (Dégremont, 2013; Gomez-Leon, Villamill, Salger, Sallum, Remacha-Trivino, Leavitt, Gomez-Chiarri, 2008), and localization on juvenile oyster tissues (Boardman, Maloy, Boettcher, 2008). Recently, in a study of the performance of selectively bred lines of eastern oyster, the available oyster strains performed very poorly in Maine waters with accumulated mortalities of 50% within three months of the deployment and reaching accumulated mortalities of 70% 12 months later (Proestou, Vinyard, Corbett, Piesz, Allen Jr, Small, Li, Liu, DeBrosse, Guo, Rawson, Gómez-Chiarri, 2016). The study mentioned that the mortality was coincident with increased ROD prevalence in that time of the year although samples were not examined for MSX, Dermo, or ROD (Proestou, Vinyard, Corbett, Piesz, Allen Jr, Small, Li, Liu, DeBrosse, Guo, Rawson, Gómez-Chiarri, 2016) making it more difficult to assign the mortality to any of those pathogens.

4. Future Perspectives

Maine's growing aquaculture industry is attracting capital from private equity investors. Recently, FocusMaine, a non-profit business operating under the umbrella of the Maine State Chamber of Commerce, identified aquaculture as one of the sectors in Maine with high potential to create thousands of jobs over the next decade. However, the Gulf of Maine is a rapidly changing system, with some of the fastest rates of temperature change on the planet (Pershing, Alexander, Hernandez, Kerr, Le Bris, Mills, Nye, Record, Scannell, Scott, Sherwood, Thomas, 2015), major declines in primary productivity, and increases in dissolved organic material entering from its adjacent watersheds (Huntington, Balch, Aiken, Sheffield, Luo, Roesler, Camill, 2016). These changes are rapidly altering the ecosystem, with consequences looming for fisheries (Katherine, 2013), biodiversity (Record, O'Brien, Stamieszkin, Runge, 2016) and key native species (Neckles, 2015). Managing the growth of the aquaculture industry in this rapidly changing system will be a challenge, and careful monitoring of pathogens will be critical. Expanding to the marine field (Record, 2017) 'People's science' using highly-portable technology to perform DNA-based identification (Marx,

2015) can contribute to the growing knowledge base of the distribution and diversity of microbial pathogens in the marine and complement other well establish techniques (e.g., histopathology) that inform about the presence of the pathogen and the health status of the oysters.

We previously addressed the analysis of the peer-reviewed literature on protozoan parasites of bivalve mollusks (Fernández Robledo, Vasta, Record, 2014). Here, the limited number of papers precluded us from identifying trends in the body of published work other than providing a side– by–side comparison of the publication record for threats affecting mollusks in Maine. The low number of reports may be explained by the limited size of the shellfish aquaculture compared to the lobster industry in both landings and economic impact in the state and being low on the priority list compared to surveys for fecal contamination or harmful algal blooms. Monitoring the prevalence of pathogens and limiting the movement of stocks between water bodies has been the leading strategy for intervention. Increased monitoring effort has resulted in reports of the pathogens in new areas (Marquis, Record, Fernández Robledo, 2015); however, no reports of abnormal die-offs is a reminder the complexity for understanding the epizootiology of the diseases. In the cases of oyster aquaculture relying on seed from hatcheries, another intervention strategy is to use strains selected for resistance/tolerance to protozoan parasites, with the handicap that strain performance varies significantly across sites (Frank-Lawale, Allen, Dégremont, 2014; Proestou, Vinyard, Corbett, Piesz, Allen Jr, Small, Li, Liu, DeBrosse, Guo, Rawson, Gómez-Chiarri, 2016). A second approach involving selective breeding for oysters have resulted in dozens of families with 3.4 traits selected on average (Gjedrem, Robinson, Rye, 2012). In general, it seems that breeding for higher resistance to one disease does not appear to confer greater resistance or susceptibility to another disease (Dégremont, Garcia, Allen, 2015). In light of these challenges, a consortium of researchers is in the process of sequencing, assembling, and annotating the first reference genome for the eastern oyster (Gómez-Chiarri, Warren, Guo, Proestou, 2015; Gómez-Chiarri, Guo, Tanguy, He, Proestou, 2015) to accelerate and determine the genetic basis for resistance to diseases or other highly desired traits for aquaculture. With this initiative underway, it would be of great utility to develop an oyster genetic system (OGS) for interrogating the genome for identifying genes involved in different aspects of the oyster's biology (e.g., immune defense, shell formation, adaptations to global warming and ocean

364 acidification) and highly desirable commercial traits (Fig. 7). Confirmation of the role of particular
365 candidate genes in determining a particular trait will allow for more targeted and faster selection of
366 broodstocks to improve overall breeding efforts.
367 The development of a genetic research system for commercially important species of shellfish
368 has been pursued for more than 30 years with mixed results. Gene delivery approaches that have
369 been used in an attempt to introduce desirable traits into shellfish have included electroporation,
370 lipofection, and microinjection (Boulo, Cadoret, Le Marrec, Dorange, Miahle, 1996; Buchanan,
371 Nickens, Cooper, Tiersch, 2001; Cadoret, Gendreau, Delecheneau, Rousseau, Mialhe, 1997) but
372 experiments were often short-lived and not maintained in subsequent generations. Genetic systems
373 and the capabilities they enable (e.g., knock out specific genes) have been essential for assigning
374 functions to new genes and describing new physiological processes, aspects especially relevant in
375 less studied organisms compared to mammals and medical model systems. Also, recent
376 developments in techniques for gene editing (e.g., Clustered regularly interspaced short palindromic
377 repeats, CRISPR/Cas9) have revolutionized the way researchers interrogate genomes for gene
378 function and to identify desirable genetic traits (Hille, Charpentier, 2016; Mojica, Montoliu, 2016).
379 These approaches are quickly being adopted in fish and mollusk aquaculture research (Chakrapani,
380 Patra, Panda, Rasal, Jayasankar, Barman, 2016; Perry, Henry, 2015; Zhong, Niu, Wang, Huang, Xu,
381 Sun, Xu, Hou, Sun, Yan, Wang, 2016). Moving the basic research to applied aquaculture is a step
382 that is taking place slowly and under rigorous evaluation not without a high degree of controversy
383 (Clausen, Longo, 2012; Tonelli, Lacerda, Tonelli, Costa, de Franca, Resende, 2017; Ye, Zhu, Sun,
384 2015).
385 Our review is a close-up historical snapshot of the record relating to protozoan parasites and
386 bivalve diseases in bivalves from Maine. With more and more seafood reaching the table coming
387 from aquaculture (FAO, 2012), it is important to understand pathogen baselines to maintain
388 biosecurity at state, national, and international levels (Carnegie, Arzul, Bushek, 2016). It is also
389 crucial to understand the parasite's biology, the interaction with the host, and the response to
390 changing environmental conditions to have well-informed approaches for risk management. A
391 baseline of waterborne parasites in commercial shellfisheries and aquaculture species is necessary

to ensure responsible harvesting and production. This knowledge is particularly necessary to resolve any significant die-off of commercial shellfish species in the future and to contribute to the development of a sustainable and safe aquaculture.

Conflict of interest

We declare no conflicts of interest.

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Figure Captions

Fig. 1. Number of papers in the literature (SCOPUS Database) reporting in the genera *Bonamia*, *Haplosporidium*, and *Perkinsus*, and the conditions DN and ROD.

Fig. 2. Disseminated neoplasia (DN). A. Hemolymph withdrawn from clams with advanced DN is characterized by large number of cells giving the hemolymph a milky appearance. B. DN cells are rounded and have lost the ability to readily spread on to a glass surface and project pseudopodia. C. Transmission of DN occurs when diseased clams release into the environment DN cells, which are taken up by healthy clams.

Fig. 3. *Bonamia ostreae*. A. Blood smear stained with hematoxylin and eosin. Note the groups of cells (arrowheads) inside the hemocytes. B. Transmission of *B. ostreae* remains unknown. Once inside the oyster, the parasite resides inside the hemocytes where it propagates, the parasite can also be found free-floating in the hemolymph after the rupture of the haemocytes.

Fig. 4. *Haplosporidium nelsoni*. A. Digestive gland of *Crassostrea virginica* infected with *Haplosporidium nelsoni*; dash line indicates the presence of large number of *H. nelsoni* cells. B. Transmission of *H. nelsoni* is thought to be through spores. Recent studies have identified *H. nelsoni* DNA in the tunicate *Stylea* sp., but whether or not the tunicate is an intermediate host remains to be determined.

Fig. 5. *Perkinsus marinus*. A. Trophozoites (arrowheads) reside both intracellularly inside the hemocytes and extracellularly. B. Trophozoites in the water column are drawn in the oyster during filter-feeding. Although in some cases they may be released into the water with the pseudofeces, most of them are recognized and phagocytized by the hemocytes. Inside the phagosome-like vesicles trophozoites propagate and, eventually, the hemocyte disintegrates the released

728 trophozoites that can be phagocytozed by neighboring hemocytes.

729 Fig. 6. *Aliiroseovarius crassostreae*. A. *Crassostrea virginica* affected with ROD. B. Inner shell from
730 *Crassostrea virginica* containing bacteria stained with nucleic acid viability dye (Boardman, Maloy,
731 Boettcher, 2008). C. *Aliiroseovarius* spp. are components of the estuarine microbiome.
732 *Aliiroseovarius crassostreae* affects mostly *Crassostrea virginica* spat causing heavy mortalities.
733 (Panels A and B reproduced with permission from Elsevier).

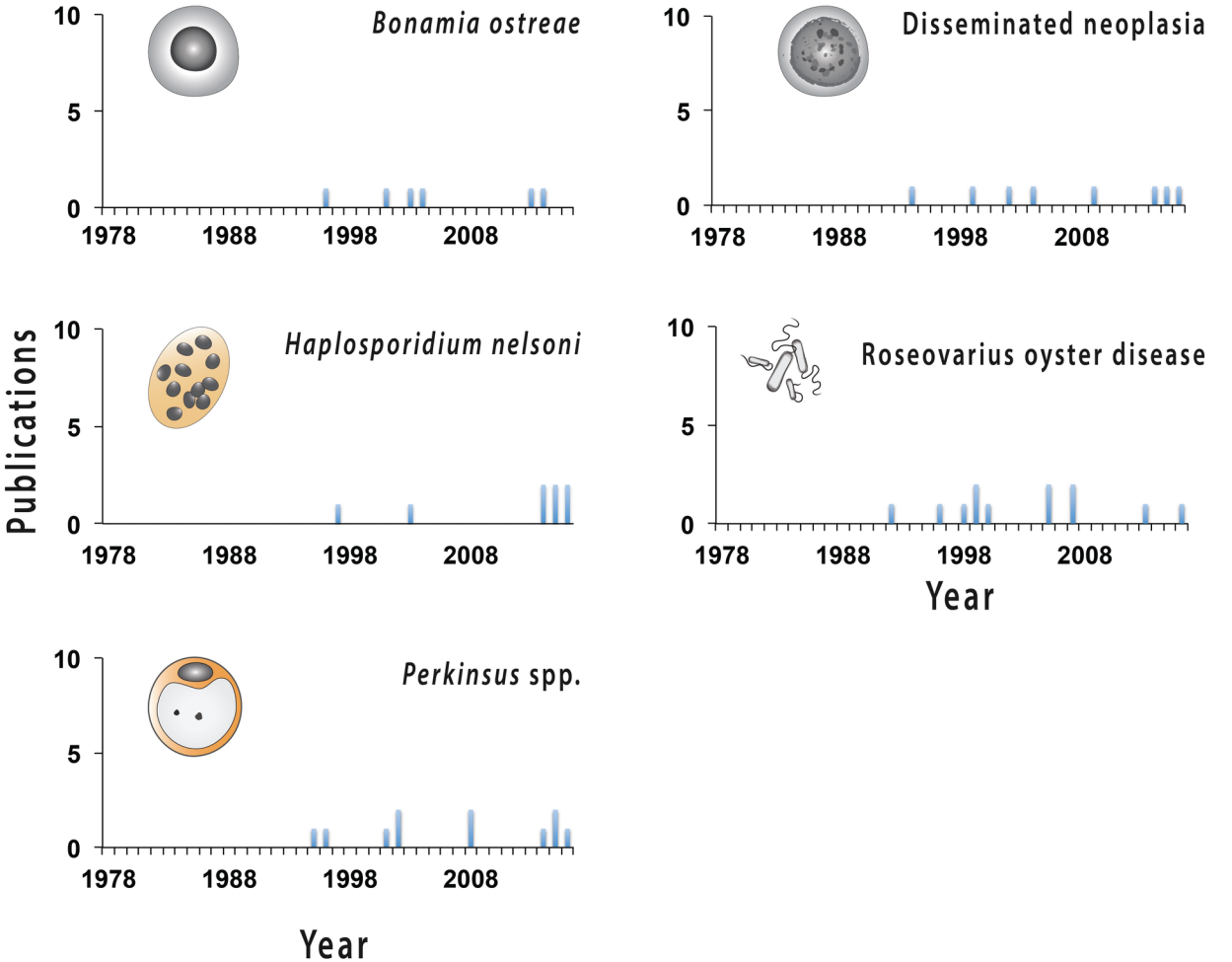
734 Fig. 7. Flow chart for developing the Oyster Genetic System. Transcriptome studies will be used to
735 identify both highly expressed constitutive and inducible genes. These genes would be mapped to
736 the genome to identify the promoters driving their transcription. Plasmid vectors based on these
737 genes would be tested on oyster oocytes/zygotes and primary cell cultures using transfection by
738 electroporation and lipofection. Once the plasmid and transfection conditions are optimized, other
739 tools (e.g., Clustered regularly interspaced short palindromic repeats, CRISPR/Cas9) will be also
740 developed. The oyster genetic system will be used for interrogating the oyster genome.

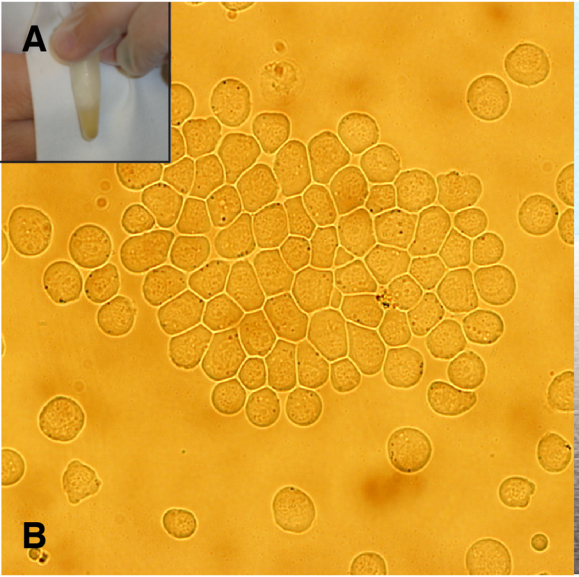
741

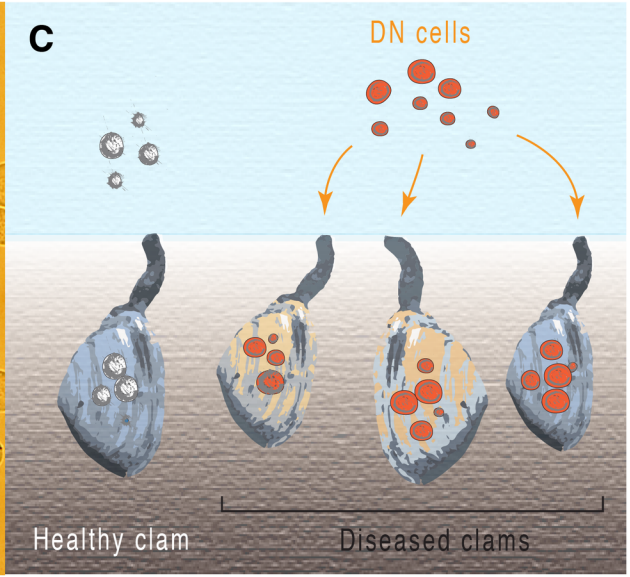
742 Table 1. Search strings for pathogens and bivalve groups.

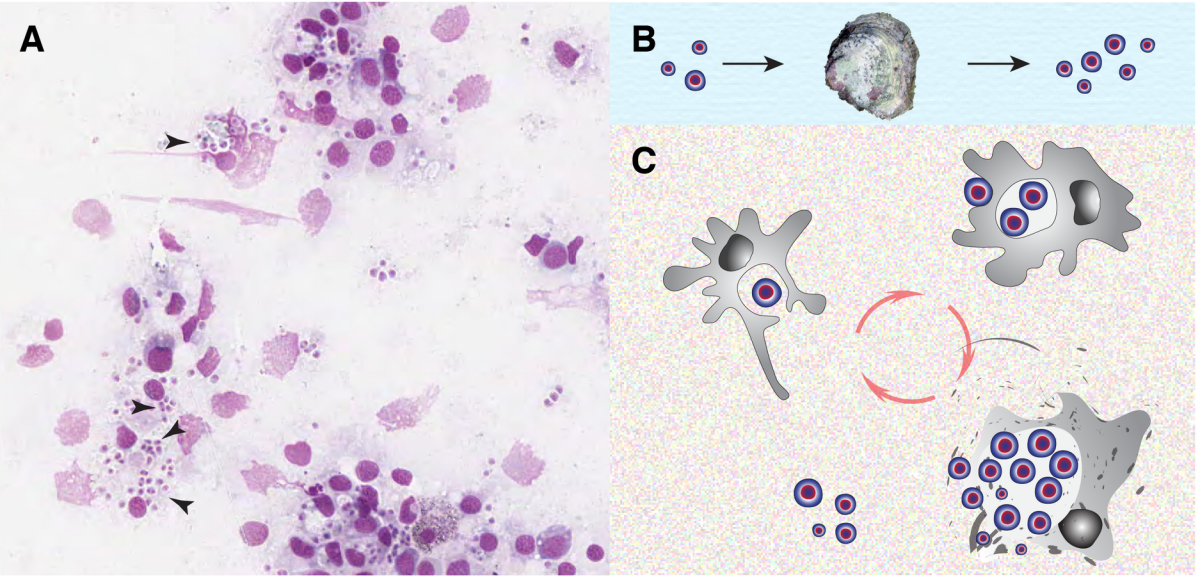
Parasite/Condition	**Search String**
Disseminated neoplasia	DN, Disseminated neoplasia
Bonamia/Bonamiosis	*Bonamia*, Bonamiosis, *Microcytos*
Haplosporidium/MSX	*Haplosporidium*, MSX
Perkinsus spp./Dermo	Dermo, *Dermocystidium*, Perkinsosis
Aliiroseovarius crassostreae/ROD	ROD, JOD, Roseovarius
Host	
Clam	Clam, *Macoma*, *Mya*
Mussel	Mussel, *Mytilus*
Oyster	Oyster, *Crassostrea*, *Ostrea*

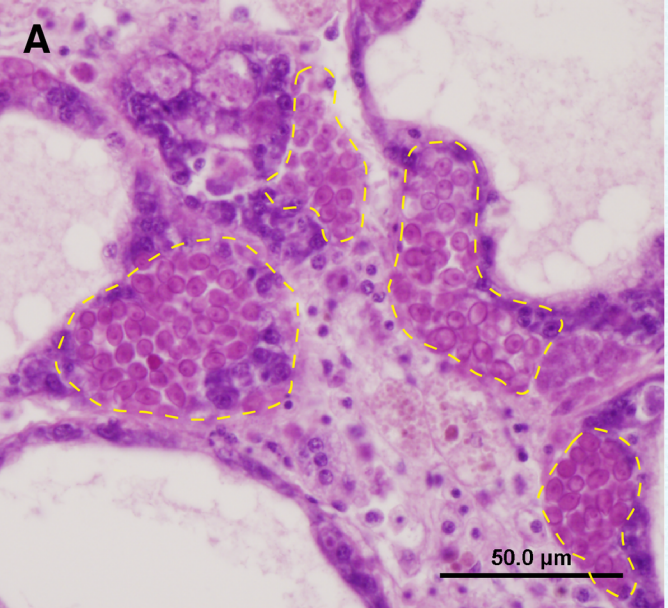
743

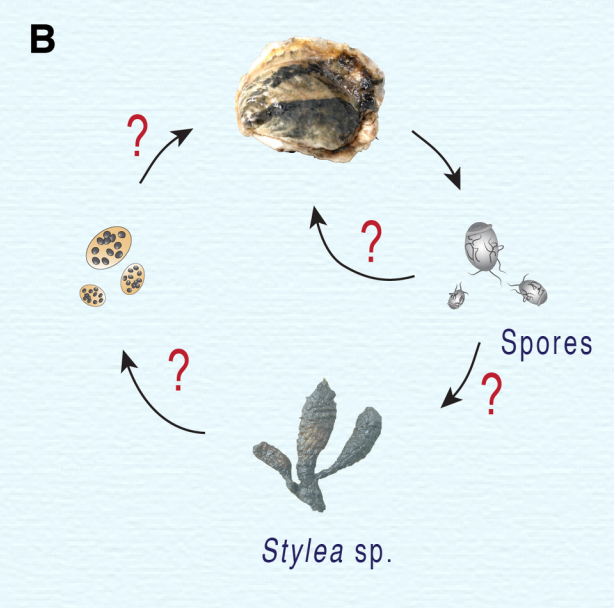

Bonamia ostreae
Disseminated neoplasia
Haplosporidium nelsoni
Roseovarius oyster disease
Perkinsus spp.
Publications
Year
10
5
0
1978
1988
1998
2008

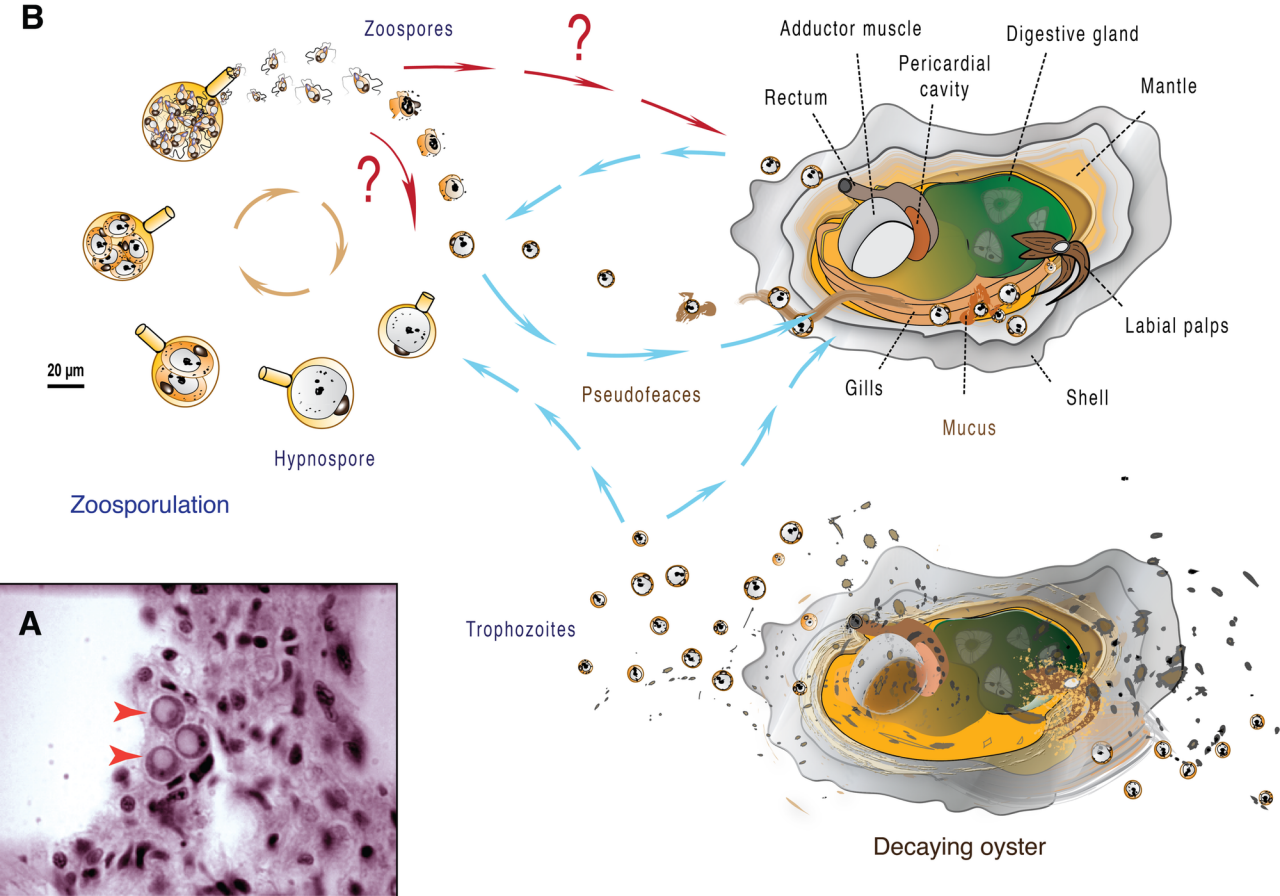

A
B

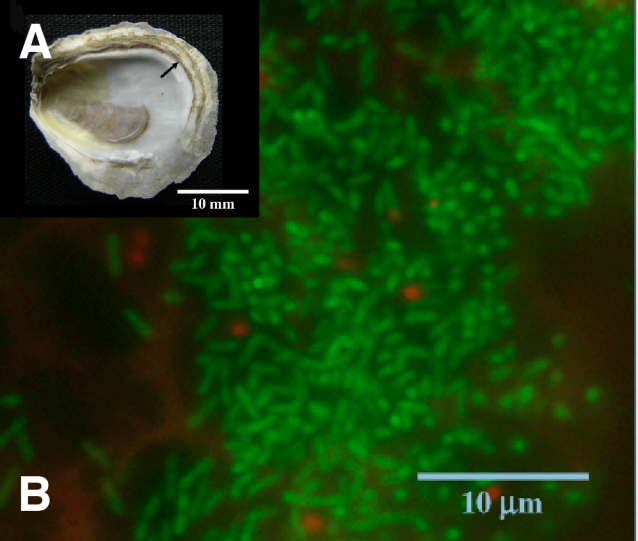

C
DN cells
Healthy clam
Diseased clams

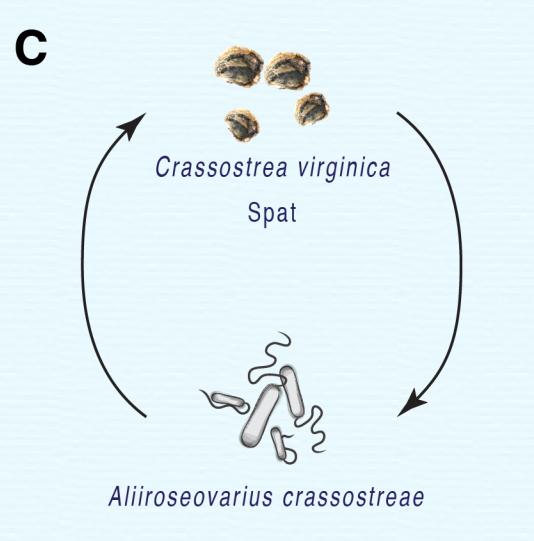

A
B
C


A
50.0 µm


B
?
?
?
?
Spores
Stylea sp.


B
Zoospores
?
?
Adductor muscle
Digestive gland
Pericardial cavity
Rectum
Mantle
Labial palps
Gills
Shell
Mucus
20 µm
Pseudofeaces
Hypnospore
Zoosporulation
A
Trophozoites
Decaying oyster


A
10 mm
B
10 µm

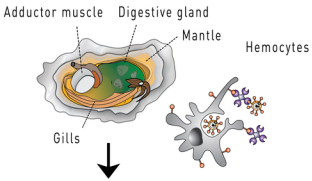

C
Crassostrea virginica
Spat
Aliiroseovarius crassostreae

Oyster Genetic System

Crassostrea virginica

Transcriptomes

Genome



CrassostreaDB

Genome mapping

illumina HighSeq

Highly expressed genes

Inducible genes

mRNA mRNA

mRNA

mRNA mRNA

mRNA

Plasmid vector construction

Electroporation Lipofection

Tool development CRISPR/Cas9

Plasmids an tools validation and distribution

Genome interrogation

Aquaculture threats
Shell formation
Osmoregulation
Immune defense
Selective breeding
Basic research