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Pilot study of the Olympia oyster *Ostrea conchaphila* in the San Francisco Bay estuary: description and distribution of diseases

Carolyn S. Friedman[1,*], Heather M. Brown[2,4], Timothy W. Ewing[1], Frederick J. Griffin[2], Gary N. Cherr[2,3]

[1]School of Aquatic and Fishery Sciences, University of Washington, Box 355020, Seattle, Washington 98195, USA
[2]Bodega Marine Laboratory, PO Box 247, Bodega Bay, California 94923, USA
[3]Departments of Environmental Toxicology and Nutrition, University of California Davis, Davis, California 95616, USA

[4]*Present address:* Department of Molecular, Cellular and Developmental Biology, University of California, Santa Cruz, California 95064, USA

ABSTRACT: Olympia oysters *Ostrea conchaphila* have declined markedly during the last century and are a focus of restoration in many embayments, including the San Francisco Bay (SFB) estuary. Oysters were collected from 17 sites in this estuary and nearby Tomales Bay in an effort to characterize diseases that may impact recovery of this species and captive rearing programs. Three diseases/disease agents including a *Mikrocytos*-like protist (microcell), a haplosporidian and hemic neoplasia were observed from several sites along the western margins of the SFB estuary suggesting a geographic localization of disease presence. Based on fluoresecent *in situ* hybridization (FISH) assays, the microcell is distinct from *M. mackini* and *Bonamia* spp. These data highlight the need for further elucidation of the haplosporidian and for careful health management of a declining species destined for captive rearing and supplementation.

KEY WORDS: *Ostrea conchaphila* · Disease · San Francisco Bay · Microcell · Haplosporidian · Hemic neoplasia

INTRODUCTION

The native North American Olympia oyster *Ostrea conchaphila* (= *O. lurida*) inhabits shallow subtidal areas of many bays and estuaries from SE Alaska to California (Baker 1995). This species was commercially exploited by European settlers, beginning in the mid-1850s, particularly in San Francisco and Tomales Bays in California, Yaquina Bay in Oregon and Willapa and Samish Bays in Washington State. Unfortunately, little management of the stocks was implemented and the populations were nearly extirpated in many locations (Baker 1995). Currently, Olympia oyster populations remain reduced throughout their range. Restoration efforts, which may require the movement of animals between embayments, should not be undertaken without an understanding of their health. What little is known about the diseases and parasites affecting this species is focused on observations from Yaquina Bay, Oregon. Mix (1975) reported a fatal neoplastic disorder in Olympia oysters from Yaquina Bay, Oregon; prevalence of up to 20% was observed. A haplosporidian parasite was also reported from this embayment in the late 1960s but has not been reported since this time (Mix & Sprague 1970). In the winters of 1969 and 1970, *Mikrocytos mackini* was reported as infecting this species in Yaquina Bay (Farley et al. 1988). The flagellate *Hexamita nelsoni* was observed in association with Olympia oyster mortalities in Puget Sound, Washington, in the late 1950s (Stein et al. 1959). These reports of

*Email: carolynf@u.washington.edu

multiple diseases and potential pathogens from Oregon and Washington State, coupled with recent examples of range expansions of disease via movement of aquatic species (Burreson et al. 2000, Naylor et al. 2001, Friedman & Finley 2003) and the desire for captive rearing and re-seeding of cultured Olympia oysters in San Francisco Bay, stimulated an initial examination of the health of this species in the San Francisco Bay estuary and nearby Tomales Bay.

MATERIALS AND METHODS

Animal collection and maintenance. Oysters (n = 4 to 60 per site) were collected from intertidal margins of 16 locations within San Francisco and Richardson Bays, California, and 1 site within Tomales Bay, California, at low tides between April and September of 2001 (Fig. 1, Table 1). Seawater temperatures ranged between 16 and 28°C at the collection sites. Collected animals were isolated by collection site into plastic bags that were floated in ice chests containing ambient seawater and transported to the Bodega Marine Laboratory within 3 h of collection. Subsequently, the animals were placed in constant-flow seawater tanks with aeration at an ambient temperature of 12°C, and fed an algal paste daily consisting of *Chaetoceros* sp., *Tetraselmis* sp. and Tahitian *Isochrysis galbana* (Reed Mariculture). Individuals were sampled for histology within 1 to 3 d of collection.

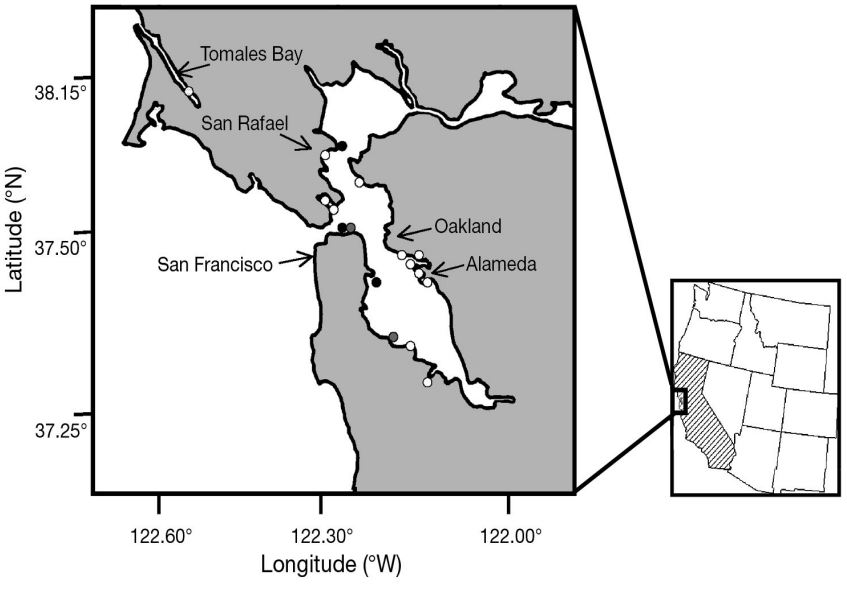

Fig. 1. San Francisco Bay estuary and Marin County, California; the 17 sites where native Olympia oysters were collected. ○: sites where no diseases were observed; ◍: sites where *Mytilicola* sp., hemic neoplasia, haplosporidiosis or microcell infections were observed; ●: sites where 2 parasites or diseases were present

Histology. One 3 to 5 mm posterior–anterior cross section was excised from each oyster just ventral to the labial palps and placed in Invertebrate Davidson's solution (Shaw & Battle 1957) for 24 h and processed for routine paraffin histology. Each section contained gill, mantle, gonad and digestive gland. Deparaffinized 5 µm sections were stained with hematoxylin and eosin (Luna 1968) and viewed by light microscopy. Specimens containing known microcells, kindly donated by Drs. Ralph Elston (*Bonamia ostreae*) and Susan Bower (*Mikrocytos mackini*), were examined for morphometric comparison of these parasites and those observed in Olympia oysters from this study.

Fluorescent *in situ* hybridization. Upon observation of a microcell in several oysters, infected samples were examined using the fluorescent *in situ* hybridization (FISH) tests of Carnegie et al. (2003a,b) to determine if *Bonamia ostreae*, *B. exitiosa* or *Mikrocytos mackini* was present. During each FISH test, the slides with the unidentified microcell from San Francisco Bay Olympia oysters were examined in conjunction with a negative control (same slide with no labeled probe) and a known positive control slide (*M. mackini*-infected *Crassostrea gigas* or *Bonamia ostreae*-infected *Ostrea edulis*). No *B. exitiosa* tissue sections were available; however, according to Diggles et al. (2003), the *B. ostreae* FISH probes of Carnegie et al. (2001) worked well in detecting *B. exitiosa* infections. In order to confirm that both *Bonamia* species could be detected with the FISH probes, we used the web-based MIT Primer3 program (www.basic.nwu.edu/biotools/Primer3.html) to determine which of the 3 *Bonamia* specific oligonucleotides (Bo-1, 2, 3) could bind with the 18s rDNA sequences of *B. exitiosa* (GenBank accession AF337563) and, as a control, *B. ostreae* (GenBank accession AF192759). Based on these data (not shown), we used probes Bo-1 and Bo-2 to detect *B. exitiosa* and all 3 probes for *B. ostreae.* Slides were examined with a Nikon E600 epifluorescence microscope using a dual FITC–Texas Red filter and photographed with a Nikon Spot camera system.

RESULTS

Four parasites or diseases were observed in Olympia oysters from San Francisco Bay, including *Mytilicola* sp., a haplosporidian-like plasmodium, a microcell and hemic neoplasia (Table 1, Figs. 1 to 3). *Mytilicola* sp. was observed in 2 oys-

Table 1. *Ostrea conchaphila.* Collection and survey sites within San Francisco Bay, 2001

Site no.	Location	n	Number diseased	Histology
1	Alameda Marina	9	–	–
2	Alameda-Willie Stargell Field	8	–	–
3	Bay Farm Island	6	–	–
4	Candlestick Point	60, 41	30 & 7, 2	Hemic neoplasia, microcell
5	China Camp	11	–	–
6	East Anchorage, Richmond/San Rafael Bridge	6	–	–
7	Loch Lomond boat ramp, San Rafael	12	–	–
8	Marin Island	6	–	–
9	Oakland Public Marina, Jack London Square	6	–	–
10	Pier 39, San Francisco	11	2	Microcell
11	Redwood Port, Redwood Creek	8	2	*Myticola*
12	Richardson Bay: Strawberry Point	22	1 each	Hemic neoplasia, microcell
13	St. Francis Yacht Harbor	5	2	Microcell, *Haplosporidium* sp.
14	San Leandro Marina	8	–	–
15	San Mateo Bridge	4	–	–
16	Sausalito	12	–	–
17	Tomales Bay	60	–	

ters from Redwood Creek; no host response was observed with these infestations. A systemic infection with a haplosporidian-like protozoan was observed within the connective tissues of a single oyster from the St. Francis Yacht Club (Fig. 2). Multinucleated plasmodia measured an average of 7.0 × 10.4 µm with a range of 4.4 to 12.7 × 5.9 to 13.7 µm (Tables 1 & 2). A mean of 6 nuclei (up to 10) that measured <2 µm were observed within an individual plasmodium. Microcells, morphologically indistinguishable from *Mikrocytos mackini*, were observed within inflammatory lesions of the intestine and adjacent connective tissues in oysters from Richardson Bay and from St. Francis Yacht Harbor, Pier 39 and Candlestick Point in San Francisco Bay (Tables 1 & 2). The parasites were typically free within an intense, abscess-like host inflammatory response within the stomach or intestinal epithelium; microcells were only rarely observed within a host hemocyte. Individual uninucleate microcells contained a central nucleus that measured 1.12 ± 0.05 µm, were irregular in shape and measured an average of 3.01 µm with a range of 2.0 to 3.9 µm (Fig. 3, Tables 1 & 2). On rare occasions (n = 3), microcells that appeared to be binucleate were observed and measured a mean of 3.6 µm. However, these microcells did not bind with the *M. mackini* or *Bonamia* spp. FISH probes; all controls reacted as expected (Fig. 4). Spherical circulating neoplastic cells were observed in numerous oysters from Candlestick and Strawberry Points with varying intensity from mild to advanced (Table 2, Fig. 5); cells measured a mean of 10.1 µm with a large nucleus measuring a mean of 6.0 µm. Hemic neoplasia was documented from 2 collections at Candlestick Point. In June 2001, 50% of 60 individuals assessed for disease/para-

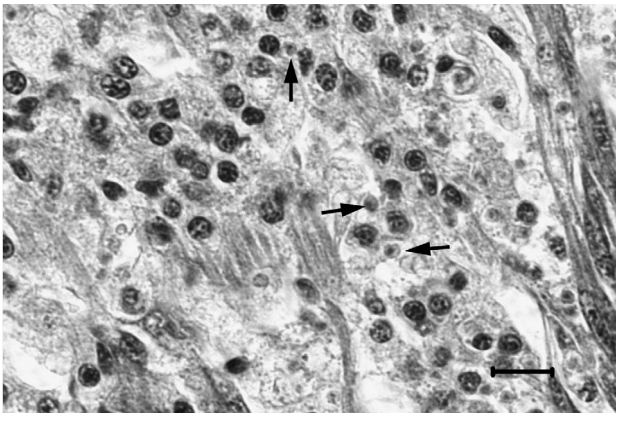

Fig. 3. Microcells resembling *Mikrocytos mackini* (arrows) within an inflammatory lesion in the intestine of an *Ostrea conchaphila* from Richardson Bay. Scale bar = 10 µm

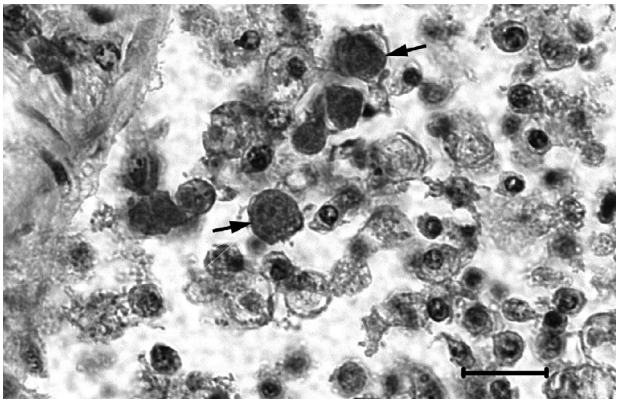

Fig. 2. Haplosporidian multinucleated plasmodia (arrows) within mild inflammation in an Olympia oyster collected from the St. Francis Yacht Club in San Francisco Bay. Scale bar = 15 µm. Hematoxylin & eosin

site infection were affected by hemic neoplasia. The percent affected by hemic neoplasia at Candlestick Point in the September 2001 collection was 17% (7 out of 41 individuals).

The 4 diseases/parasites were observed in select populations of oysters collected from the western shores of Richardson and San Francisco Bays (n = 192; Table 1). None of the disease conditions were observed in animals taken from the eastern shores of San Francisco Bay (n = 43), including the east end of the San Rafael Bridge (n = 6), Oakland Marina (n = 6), San Leandro (n = 8), Bay Farm Island (n = 6) and Alameda (n = 17), or from Tomales Bay (n = 60).

DISCUSSION

Although the parasites *Mytilicola* spp., *Haplosporidium* spp., *Bonamia ostreae* and *Mikrocytos mackini* have been observed in a number of oysters within the genera *Crassostrea* and or *Ostrea* (Farley et al. 1988, Friedman & Perkins 1994), their observation in Olympia oysters in California has not been previously documented. *Mytilicola* spp. have been observed in flat and Pacific oysters from Tomales Bay and many locations globally and are typically nonpathogenic (Friedman et al. 1989, Steele & Mulchay 2001). Several haplosporidian protists cause serious losses of the eastern oyster *C. virginica* along the east coast of the US (Andrews 1984) and have been reported in Pacific oysters in Japan (Friedman et al. 1991, Friedman 1996), in flat oysters *O. edulis* in Maine (Friedman & Perkins 1994) and in France (Bachere & Grizel 1982), and in abalone *Haliotis iris* in New Zealand (Diggles et al. 2002). The observation of a multinucleated haplosporidian in California is not unprecedented; Friedman (1996) reported *Haplosporidium nelsoni* in Pacific oysters from Drakes Estero, which is approximately 25 miles north of San Francisco Bay. Burreson et al. (2000) provided evidence suggesting that this parasite was introduced into California via imports from Japan and was subsequently introduced to the east coast of the US via movement of stock for culture purposes. The haplosporidian-like parasite observed in Olympia oysters suggests that both the host and geographic range of these parasites are wider than previously observed.

Two microcells, *Bonamia* spp. and *Mikrocytos* spp., cause moderate to severe losses of oysters in North America, western Europe, New Zealand and Australia (Pichot et al. 1979, Farley et al. 1988, Friedman et al. 1989, Hine et al. 2001). *B. ostreae* is responsible for losses of European flat oysters *Ostrea edulis* in the United States (Katkansky et al. 1974, Elston et al. 1986, Friedman et al. 1989, Friedman & Perkins 1994) and western Europe (Pichot et al. 1979), while *B. exitiosa* causes losses of the Chilean oyster *Tiostrea chilensis* in New Zealand (Hine 1991). Both species of *Bonamia* are typically found within host hemocytes and are associated with diffuse or generalized infections; no abscesses are formed. These protists cause severe losses (80 to 90%) in naïve populations with declining losses in subsequent years (Elston et al. 1987, Farley et al. 1988, Hine 1996). Although *B. ostreae* is thought to be endemic to California, no reports of bonamiasis in Olympia oysters have been published. *B. ostreae* is spherical in shape, measures 2.4 ± 0.5 µm, and contains an eccentric nucleus and large vacuole (Hine et al. 2001). In the present study, *B. ostreae* measured approximately 2 µm with an eccentric 0.89 mm nucleus (Table 2). The slightly larger *B. exitiosa* measures 3.0 + 0.3 µm, has an irregular margin and contains a centrally located nucleus (Hine et al. 2001) and thus resembles *M. mackini*, another distantly related protist (Carnegie et al. 2003), as well as the microcell reported in this present study. Although it is possible that terminal market imports from New Zealand into San Francisco and Marin County could have introduced *B. exitiosa* into these populations, it is also plausible that the protist in Olympia oysters is related to the regional parasite *M. mackini*.

Both species in the genus *Mikrocytos* are associated with abscess-like lesions and cause losses of oysters in North America and Australia (Farley et al. 1988). *M. roughleyi*, which causes Australian winter disease in *Saccostrea commercialis*, is morphologically distinct from the microcell we observed in Olympia oysters from California. Infection with this small 1 to 2 µm

Table 2. Comparison of morphology of parasites (CA microcell) and neoplastic cells from *Ostrea conchaphila* from San Francisco Bay and 2 microcell parasites (*Bonamia ostreae* and *Mikrocytos mackini*) from North America

	Bonamia ostreae		*M. mackini*		CA microcell		Haplosporidian		Neoplastic cells	
Measurement	Cell	Nucleus	Cell	Nucleus	Cell	Nucleus	Width	Length	Cell	Nucleus
Mean (µm) ± SE	1.95±0.07	0.89±0.03	3.02±0.21	1.22±0.06	3.01±0.16	1.12±0.05	7.01 ± 0.43	10.39 ± 0.58	10.11 ± 0.25	5.97 ± 0.30
Minimum (µm)	1.47	0.49	1.96	0.88	1.96	0.98	4.41	5.88	8.82	3.92
Maximum (µm)	2.45	1.08	3.92	1.47	3.92	1.47	12.74	13.72	12.74	7.84
Number	17	17	15	15	15	15	20	20	16	16

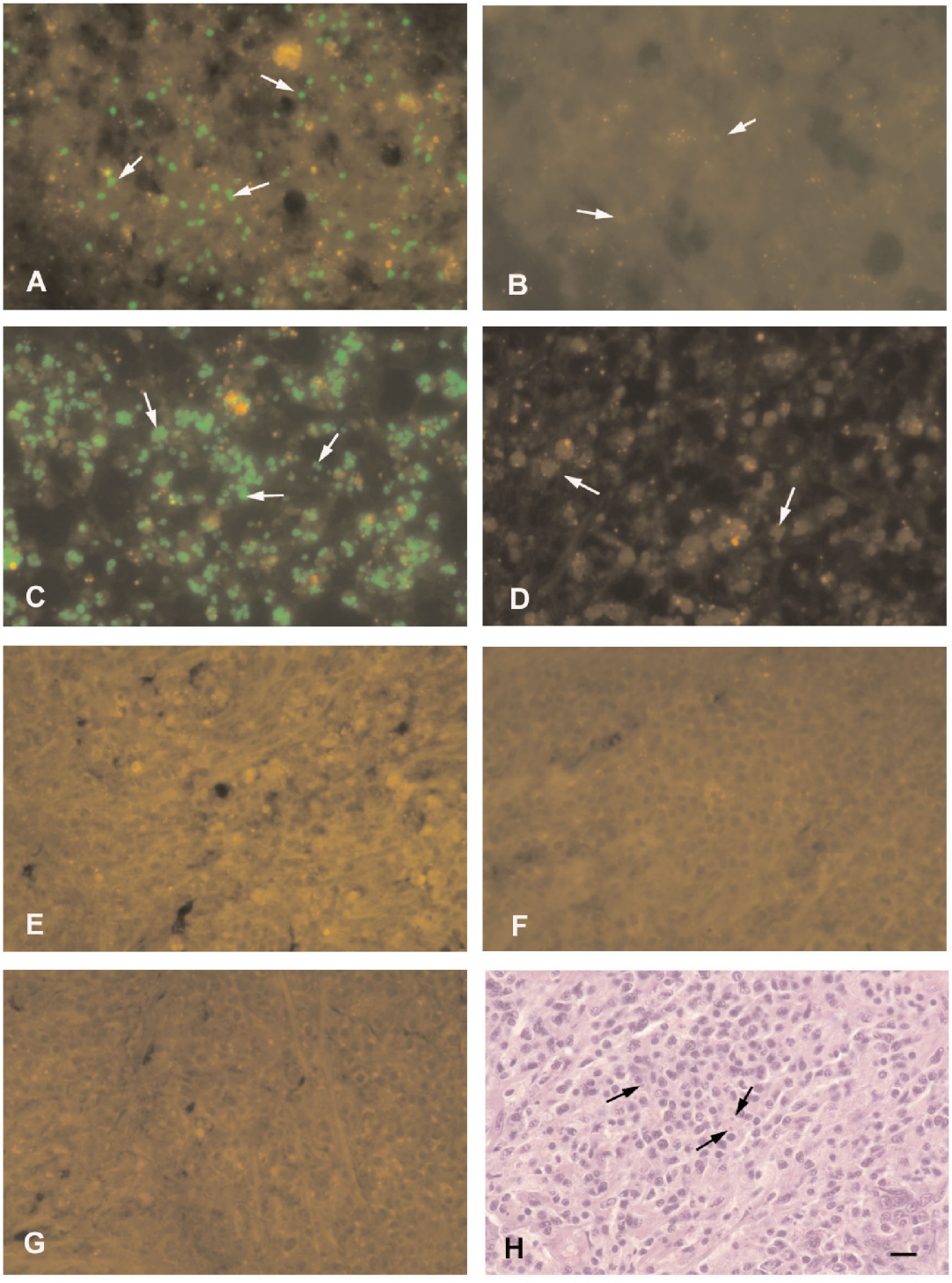

Fig. 4. Fluorescent *in situ* hybridization (FISH) of known and unidentified microcells. Arrows illustrate microcell parasites. (A) Positive control *Mikrocytos mackini* specific fluorescence (arrows) within an inflammatory lesion in the palps of *Crassostrea gigas.* (B) Negative control (no probe) lacks fluorescenct labeling of *M. mackini.* (C) Specific labeling of both free and engulfed positive control *Bonamia ostreae* cells within *Ostrea edulis* connective tissues. (D) No probe negative control lacks labeling of *B. ostreae* cells. (E) Microcells within an inflammatory lesion in the intestine of an *O. conchaphila* from Richardson Bay do not bind with the *M. mackini* probes. (F) *Bonamia* labeled oligonucleotides also did not bind with the unidentified microcells. (G) No probe control also lacks specific fluorescence. (H) Micrograph illustrates the presence of microcells (arrows) within a serial section of the same *O. conchaphila* tissue examined by FISH in (E–G). Scale bar = 10 µm. Hematoxylin & eosin and FISH hematoxylin & eosin

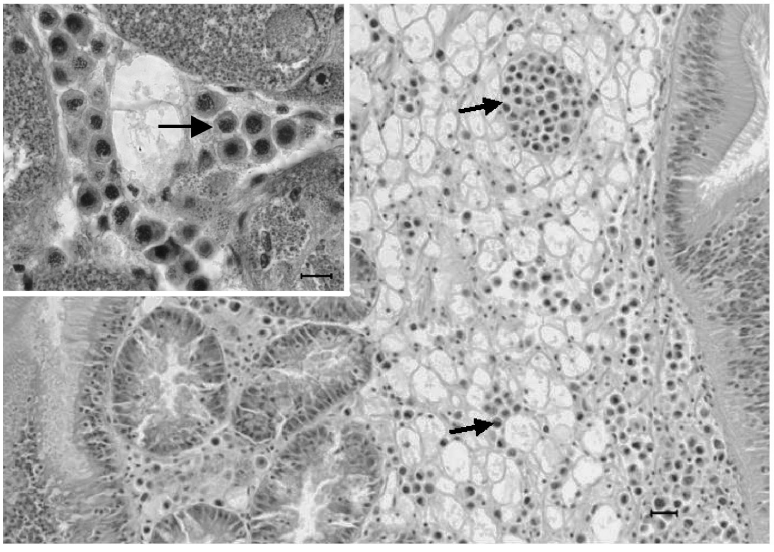

Fig. 5. *Ostrea conchaphila.* Olympia oyster from Candlestick Point, San Francisco Bay, with advanced hemic neoplasia. Note the aggregations of large circulating cells with a large nucleus to cytoplasmic ratio (arrows). Scale bars = 10 µm (insert), 20 µm. Hematoxylin & eosin

Australian microcell, which contains a large >1 µm nucleus, results in the formation of pustules in the mantle, gonad and adductor muscle (Farley et al. 1988) and clearly differs from the microcell observed herein.

Mikrocytos mackini emerged as the cause of Denman Island disease in Pacific oysters *Crassostrea gigas* in British Columbia, Canada in the 1960s (Farley et al. 1988). This pathogen is associated with serious (40%) mortality of Pacific oysters exceeding 2 yr in age, especially those in the lower intertidal (Bower 1988). *Mikrocytos* species have been observed in Olympia oysters in Yaquina Bay, Oregon (Farley et al. 1988), and were recently identified in Pacific oysters in Washington state in May 2002 (R. Elston, G. Meyers &S. Bower unpubl.). Olympia oysters are susceptible to *M. mackini* infection (Hervio et al. 1996, Bower et al. 1997) but the importance of this pathogen and its role in unexplained mortalities during the past century (Galtsoff 1929, Quayle 1988) remain unknown. However, the potential threat of this disease agent on recovery of this declining species cannot be overlooked. *M. mackini* measures 1 to 4 µm, contains a central nucleus, is irregular in shape and is found in abscess-like lesions (Farley et al. 1988). The unidentified protist measured herein was morphologically identical to *M. mackini* (Table 2, Fig. 3). However, our FISH tests suggest that the protist in San Francisco Bay oysters is distinct from known microcells. All positive control slides showed specific fluorescence as predicted, while the unknown protists did not fluoresce with any of the specific probes, suggesting that this organism is neither *Bonamia ostreae* nor *M. mackini*. As all tissues (unknowns and positive controls) were preserved in Davidson's solution (Shaw & Battle 1957) for 24 h and processed by the same histology laboratory, it seems unlikely that the tissue processing methods were responsible for the observed response. Alternatively, slight differences in the nucleic acid sequence of this microcell may have resulted in the absence of specific fluorescence, suggesting further studies on the taxonomic placement of this protist are needed.

Hemic neoplasia has been reported from many bivalve species in a wide range of locations (Balouet et al. 1982, Elston et al. 1992) including *Ostrea conchaphila* from Yaquina Bay, Oregon. Our observations expand the known range of this disease. Although mortality rates of Olympia oysters to this disease are not known, the reduced prevalence of hemic neoplasia between the summer and fall samples may reflect losses of affected oysters. Mortality in other mollusc species affected by hemic neoplasia, such as *Mya* sp. and *Mytilus* sp., can reach 100% (reviewed by Elston et al. 1992). The etiology of hemic neoplasia in molluscs is not clear; some studies suggest a viral link while others indicate physiological stress from either anthropogenic or natural environmental stressors (Elston et al. 1988, 1992, House et al. 1998, McGladdery et al. 2001, Collins & Mulcahy 2003).

Pathology screening of animals from this pilot study in San Francisco Bay suggests an east–west demarcation of infectious agents within the basin. The pathological conditions were observed only in oysters from the western margins of south and central San Francisco Bay and western Richardson Bay, while *Ostrea conchaphila* collected from the eastern margins of South and Central San Francisco Bay were not infected. This could be due to life history patterns in combination with currents in the bay and may indicate a degree of isolation of *O. conchaphila* populations, at least regionally within the bay. Alternatively, due to the small numbers of oysters we were able to collect at many sites, parasite and disease prevalences may have been below our detection limit and warrant further investigation. Collectively, these data highlight the need for careful assessment of animal health prior to moving individuals between locations as often occurs during captive breeding of declining populations, aquaculture, research or as the consequences of other potential vectors of disease transfer (Carlton 2001, Naylor et al. 2001, Friedman & Finley 2003). Although many possible vectors for transfer of exotic species including parasites and diseases exist (e.g. ballast

water exchange, seafood processing, animal vectors and fisheries, Lightner et al. 1997, Carlton 2001), it is crucial for restoration efforts to reduce any potential negative impacts to the populations being restored. Restoration efforts were recently implicated in the introduction of the etiological agent of withering syndrome, a catastrophic bacterial disease of abalone in California (Haaker et al. 1992, Friedman et al. 2000), into 2 populations of red abalone *Haliotis rufescens* in northern California (Friedman & Finley 2003). These observations further illustrate the importance of identifying parasites and diseases in a species, particularly one that is moved between geographic locations. More extensive health examinations of Olympia oysters are warranted prior to movement between sites within the San Francisco Bay estuary and nearby locations.

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