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Elucidating the life cycle of *Marteilia sydneyi*, the aetiological agent of QX disease in the Sydney rock oyster (*Saccostrea glomerata*)

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ABSTRACT

Marteilia sydneyi (Phylum Paramyxea, Class Marteiliidea, Order Marteiliida) (the causative agent of QX disease) is recognised as the most severe parasite to infect *Saccostrea glomerata*, the Sydney rock oyster, on the east coast of Australia. Despite its potential impact on industry (>95% mortality), research towards lessening these effects has been hindered by the lack of an experimental laboratory model of infection as a consequence of our incomplete understanding of the life cycle of this parasite. Here, we explored the presence of this parasite in hosts other than a bivalve mollusc from two study sites on the Hawkesbury River, New South Wales, Australia. We employed PCR-based in situ hybridisation and sequence analysis of a portion of the first internal transcribed spacer (ITS1) of rDNA in an attempt to detect *M. sydneyi* DNA in 21 species of polychaete worm. *Marteilia* DNA was detected in 6% of 1247 samples examined by PCR; the analysis of all amplicons defined one distinct sequence type for ITS1, representing *M. sydneyi.* Of the polychaete operational taxonomic units test-positive in PCR, we examined 116 samples via in situ hybridisation DNA probe staining and identified *M. sydneyi* DNA in the epithelium of the intestine of two specimens of *Nephtys australiensis*. Two differing morphological forms were identified: a 'primordial' cell that contained a well-defined nucleus but had little differentiation in the cytoplasm, and a 'plasmodial' cell that showed an apparent syncytial structure. This finding represents the first known record of the identification of *M. sydneyi* being parasitic in an organism other than an oyster, and only the third record of any species of *Marteilia* identified from non-molluscan hosts. Future work aims at determining if *N. australiensis* and *S. glomerata* are the only hosts in the life cycle of this paramyxean, and the development of experimental models to aid the production of QX disease-resistant oysters.

Key words:
Marteilia sydneyi
QX disease
Saccostrea glomerata
Sydney rock oyster
Polychaete
Nephtys australiensis
Life cycle

1. Introduction

Since the mid-1970s, the protozoan *Marteilia sydneyi* (Phylum Paramyxea), the aetiological agent of QX disease, has been recognised as the most pathogenic parasite of the Sydney rock oyster (SRO), *Saccostrea glomerata*, particularly in estuaries of southern Queensland (Qld) and northern New South Wales (NSW), Australia (Adlard and Ernst, 1995). As a consequence of the significant impact the disease may have during outbreaks (i.e. ≥ 95% mortality; (Bezemer et al., 2006)), control measures typically require quarantining entire estuaries in order to restrict the movement of infected stock. In the absence of data on transmission and the causative elements that promote these outbreaks, this has been the most conservative course of action and, until recently, the only management tool available to protect the SRO industry. However, with reports indicating that *M. sydneyi* is present in most estuaries in which major SRO culture is undertaken (Adlard and Wesche, 2005), even though many have never suffered significant disease events, research has turned to investigating the contribution of oyster immuno-competence to disease inhibition (Bezemer et al., 2006; Butt and Raftos, 2008; Green et al., 2009; Dang et al., 2011), the production of QX disease-resistant oysters (Nell et al., 2000; Nell, 2001; Green et al., 2008) and disease resistance biomarkers (Simonian et al., 2009).

One major obstacle to furthering these avenues of research is the lack of a laboratory or experimental model of infection, a consequence of our incomplete understanding of the life cycle of this parasite. The best known component(s) of the *M. sydneyi* life cycle (as with the pathogenic *Marteilia refringens* from *Ostrea edulis* in Europe) involve the definitive host (Perkins and Wolf, 1976). Similar to studies from Europe which postulate the existence of a complex life cycle for *M. refringens* (Berthe et al., 1998; Audemard et al., 2001, 2002; Berthe et al., 2004; Arzul et al., 2013; Boyer et al., 2013), the suggestion that the life cycle of *M. sydneyi* is indirect originated in the mid-1980s when cross-infection experiments failed (Lester, 1986). Additionally, the discovery in vitro that spores of *M. sydneyi* have a limited viability in the marine environment (Wesche et al., 1999), implicated the existence of one or more intermediate host(s). Adlard and Lester (1996) postulated the existence of a direct correlation between the abundance of the major component of benthic organisms (i.e. polychaete worms) and the prevalence of QX disease during outbreaks, which spurred interest in investigating this group as possible alternate hosts. However, the use of classical laboratory techniques has inhibited the reliable and unambiguous identification of unknown morphological stages of *M. sydneyi* in alternate hosts (Kleeman and Adlard, 2000), indicating the clear need to undertake a systematic molecular investigation of a range of polychaetes in affected estuarine systems.

PCR-based techniques, employing suitable gene markers, coupled with diagnostic methods such as in-situ hybridisation (ISH), have been used to investigate parasitic life cycles (Fong et al., 1993; Stokes et al., 1995). Previously, we have shown this approach is highly sensitive and specific for the accurate identification of *Marteilia* infections in oysters (Anderson et al., 1995; Kleeman and

Adlard, 2000; Kleeman et al., 2002a, b; Adlard and Worthington-Wilmer, 2003; Adlard and Wesche, 2005). To date, PCR-only based approaches have revealed the presence of *M. sydneyi* nucleic acids in polychaetes collected in QX-endemic areas; however, whether the *M. sydneyi* DNA detected in these samples is derived from developing infections or the result of accidental ingestion of the pathogen during feeding remains to be explored. Thus, in the present investigation we examined a range of polychaetes in the Hawkesbury River, NSW, Australia, employing a combined PCR/ISH-based approach in an attempt to identify and characterise previously unknown life cycle stages of *M. sydneyi* and link these genetically with those detected previously in *S. glomerata*.

2. Materials and methods

2.1. Sample timing

The current study employed an established PCR protocol (Kleeman and Adlard, 2000; Adlard and Worthington-Wilmer, 2003) to detect the presence of *M. sydneyi* DNA in benthic macrofauna. We anticipated that PCR-positive samples would fall into two categories; 'false positives' that were the product of incidental ingestion of spores which remain in the digestive tract of benthic fauna, and 'real positives' that were the result of uptake and development of the parasite within 'true' alternate hosts. To minimise the likelihood of detecting false positives we sampled benthic organisms in November (late spring). We anticipated that sampling during this temporal window would mean that the majority of infected oysters had already shed spores and died and parasite development within an alternate host would be well advanced to allow infection of oysters during the following (mid-summer) infection period (Bower et al., 1994). Consequently, the probability of detecting developing *M. sydneyi* stages in alternate hosts would be maximised.

2.2. Sample collection on the Hawkesbury River, NSW

The New South Wales Department of Primary Industries (NSW DPI), Australia provided a geographic map of the upper Hawkesbury River region. Two areas, Cobar (33°32'37"S 151°08'17"E) and Kimmerikong (33°32'51"S 151°09'10"E), associated with current and former oyster leases and unfarmed neighbouring areas, were selected for sampling (Fig. 1). Both areas were overlaid with a numbered grid; 150 computer generated random numbers were then plotted across each area and the latitude and longitude determined for these (data not shown). GPS points for each sampled site were relocated on the Hawkesbury River using a hand-held GPS unit (see Figs. 2A, B). Benthic samples were collected using a van Veen grab sampler between 7 - 20 November 2006 from 50 sites across Cobar (Fig. 2A) and 64 at Kimmerikong (Fig. 2B). One sample was taken at each site and only five samples were collected at one time to prevent deterioration of the macrobenthic fauna. Samples were placed in separate 5 L containers for transport and labelled with the site number (1 - 150 for Cobar and 151 - 300 for Kimmerikong samples) before each was reduced in volume by washing it through a

series of two stacked sieves (1 mm and 500 µm). 'Semi-clean' samples were then soaked for a further 45 min, followed by a second wash (500 µm sieve) to remove loosened sediment.

'Clean' samples were poured into Petri dishes and allowed to settle for 10 - 15 min before being scanned using a stereomicroscope. Polychaetes were removed from the sample using featherweight forceps and stored in hemagglutination trays in river water. Polychaetes were classified into operational taxonomic units (OTUs) (family; putative species) for each site. Such an approach to classification was felt appropriate because Australia has some of the highest diversity of polychaetes in soft sediments and a large number of taxa remain to be described (Beesley et al., 2000). Numbers of each OTU from each site were recorded before individual site OTUs were combined and half the specimens transferred to microcentrifuge tubes containing 95% ethanol (for DNA analysis) and half to 10% formalin (to represent an OTU or for ISH) (both at room temperate; RT). Specimens for ISH were changed from formalin to ethanol after 1 - 2 weeks. Formalin-fixed OTU specimens were photographed using a Nikon Digital Sight camera (DS-5M) attached to a stereomicroscope located at the Queensland Museum, Brisbane, Australia. Voucher specimens for all polychaete OTUs have been retained in the research collection at the Queensland Museum.

2.3. Genomic DNA extraction and PCR-coupled sequencing

Genomic DNA (gDNA) was extracted from single polychaete specimens using a DNeasy® Blood and Tissue Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. The PCR protocol employed here essentially followed that reported by Kleeman and Adlard (2000) and Adlard and Worthington-Wilmer (2003). In brief, 195 nucleotides of the first internal transcribed spacer (ITS1) of rDNA were amplified using the primers LEG1 (forward: 5'–CGATCTGTGTAGTCGGATTCCGA–3') and PRO2 (reverse: 5'–TCAAGGGACATCCAACGGTC–3') (Kleeman and Adlard, 2000). PCR was carried out in a volume of 25 µl containing 2.5 µl of 10× HotMaster™ *Taq* buffer (Qiagen) with 25 mM magnesium chloride ($MgCl_2$), 200 µM of each dNTP, 50 pmol of each primer, 1.25 µl of DMSO (5.0% final concentration) and 0.75 U of HotStar*Taq* DNA polymerase (Qiagen) utilising a cycling protocol that consisted of 95 °C for 10 min (initial denaturation), followed by 35 cycles of 95 °C for 30 s (denaturation), 55 °C for 30 s (annealing) and 65 °C for 30 s (extension), with a final extension at 65 °C for 5 min.

Following PCR, all amplicons were run on a 1% TBE (0.89 M Tris base, 0.89 M boric acid, 0.5 M EDTA buffer; Sigma Aldrich, USA) agarose gel; amplicons indicated to be of the appropriate size (i.e. 195 nucleotides) and representing each distinct OTU from Cobar and Kimmerikong were purified using the QIAquick® PCR Purification Kit (Qiagen), according to the manufacturer's instructions. Sequencing reactions for each purified amplicon were performed in 10 µl volumes containing 0.3 µl of BigDye Terminator (BDT) ready reaction mix (BigDye® Terminator v.3.1 chemistry, Applied Biosystems, USA), 2.0 µl of 5× BDT dilution buffer, 50 pmol of each primer

(separate reactions were performed for each sample using the forward or reverse primer), and 1 - 3 ng of purified PCR product, employing a protocol that consisted of one cycle of 96 °C for 1 min, followed by 25 cycles of 96 °C for 10 s, 50 °C for 5 s, 60 °C for 4 min, and a final holding temperature of 4 °C. Products were precipitated in 2.0 µl of 125 mM EDTA, 2.0 µl of 3 M sodium acetate (pH 4.6) and 50 µl of 100% ethanol, and the pellets dried at 37 °C for 30 min. Samples were then subjected to automated sequencing at the Australian Genome Research Facility, Brisbane, Australia. Sequence quality was verified by comparison with corresponding electropherograms using the software BioEdit (Hall, 1999).

The taxonomic identity of each sequence was determined by Basic Local Alignment Search Tool analyses (BLAST®: http://blast.ncbi.nlm.nih.gov/Blast.cgi). In each case, matches were sought to published *M. sydneyi* sequence data available in GenBank represented by the accession numbers **AF159248** (Kleeman and Adlard, 2000) and **AY504628** - **AY504632** (Kleeman et al., 2004). Sequences generated in the present investigation were aligned using the program ClustalX (Thompson et al., 1997) and the resultant alignments were adjusted manually using the BioEdit software.

2.4. Labelling of the ISH DNA probe and ISH

The DNA probe generated for ISH in the present investigation utilised the primers CS2 (5'–GCAAGTCTGGTGCCAGCAGC–3') and SAS1 (5'–TTCGGGTGGTCTTGAAAGGC–3'), which incorporate the 18S rRNA region reported as 'Smart 2', the most specific DNA probe employed to detect *M. refringens* in infected European flat oysters (*O. edulis*) and naturally infected mussels (*Mytilus edulis* and *Mytilus galloprovincialis*) (Le Roux et al., 1999). Despite its specificity to *M. refringens*, Kleeman et al. (2002a) determined that this probe provided greater resolution for the detection of all stages of *M. sydneyi* compared with a species-specific ITS1 probe (Kleeman and Adlard, 2000). The probe was synthesised by incorporating digoxigenin-11-dUTP (DIG) during PCR and employing a PCR DIG Probe Synthesis Kit (Roche Diagnostics Australia Pty. Ltd.), following the manufacturer's instructions. Incorporation of DIG was signalled by an increase in molecular mass as indicated on a 1% TBE agarose gel. Labelled PCR products were purified utilising the High Pure PCR Product Purification Kit (Roche Diagnostics), as per the manufacturer's instructions.

Formalin-fixed specimens were embedded in paraffin. Longitudinal sections were cut at a thickness of 6 µm, floated onto silane slides (2% (3-aminopropyl)triethoxysilane in acetone) and baked overnight (ON) at 62 °C. Tissue was deparaffinized in Histo-Clear II (100%, 2 × 10 min washes) and the solvent removed with ethanol. Air-dried sections were permeabilised with 100 µg/ml of proteinase K in 1× TE buffer (10× solution; 100 mM Tris-Cl (pH 8.0), 10 mM EDTA (pH 8.0)) at 37 °C/30 min in a humid chamber, before each section was dehydrated in a 1 min wash of 95% ethanol then 100% ethanol, and air-dried. Samples were then prehybridised with 500 µl of hybridisation buffer (3× SSC, 50% formamide, 1× Denhardt's solution, 0.5 mg ml^{-1} heat denatured herring sperm DNA and 5% dextran sulphate) at 42 °C for 60 min (20× SSC; 3 M sodium chloride,

0.3 M sodium citrate, pH 7.0). The prehybridisation buffer was then replaced with 55 µl of diluted DIG-labelled probe in hybridisation buffer (5 µl in 50 µl, respectively), cover-slipped and placed on a heating block at 95 °C for 5 min. Slides were cooled on ice for 5 min before ON hybridisation at 42 °C in a humid chamber. Post hybridisation included 2× 5 min washes in 2× SSC (RT), 1 × 10 min wash in 0.4× SSC at 42 °C, and equilibration in maleic acid buffer (100 mM maleic acid, 150 mM sodium chloride, pH 7.5) for 1 min at room temperature (RT). DIG-labelled probe detection included blocking sections with 200 µl of blocking buffer (maleic acid buffer, 1% blocking reagent) for 30 min at RT followed by incubation for 60 min at 37 °C in a humid chamber with 200 µl of dilute anti-digoxigenin-alkaline phosphatase (AP) conjugated antibody (1:500 in blocking buffer). Unbound antibody was removed with 2× 1 min washes in maleic acid buffer and slides were equilibrated in detection buffer (100 mM Tris-HCl, 100 mM sodium chloride, 50 mM $MgCl_2$, pH 9.5) for 5 min. BCIP/NBT (5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium) was diluted in detection buffer (20 µl in 1 ml, respectively) and 200 µl of the colour solution added to the tissue and incubated in the dark for 4 h at RT. The reaction was stopped by washing tissue in 1× TE buffer for15 min at RT. Slides were washed with DNase/RNase free H_2O, stained in Bismarck Brown Y (9% solution) for 1 min followed by dehydration in 95% and 100% ethanol and mounted in Depex (Adlard and Wesche, 2005).

3. Results

3.1. Polychaetes, PCR and ISH

We provisionally classified 21 species of polychaete in 13 families from more than 2700 specimens collected from Cobar (16 OTUs from 12 families) and Kimmerikong (20 OTUs from 12 families) (Table 1). The total combined numbers of each OTU were typically similar between sample areas (except for Lumbrineridae sp. 1 and Magelonidae sp. 1) although differences amongst the numbers of each OTU varied considerably; combined totals for individual OTUs from both sample areas ranged from one specimen to 1875 specimens (Table 1). In addition, more than 95% of the total polychaete abundance sampled here consisted of members from just six of the 12 families collected, namely Lumbrineridae, Magelonidae, Nephtyidae, Sabellidae, Spionidae and Trichobranchidae.

PCR-based screening for *M. sydneyi* DNA was conducted on 1247 samples from Cobar (*n* = 545/1186; 46%) and Kimmerikong (702/1566; 45%). This approach identified a proportion of each Cirratulidae sp. 1 (20%), Lumbrineridae sp. 1 (11.3%), Magelonidae sp. 1 (33.3%), Nephtyidae sp. 1 (19.2%), Sabellidae sp. 1 (17.9%) and Trichobranchidae sp. 1 (2.2%) as test-positive at Cobar and Kimmerikong, while Spionidae sp. 1 (10.0%) and 2 (30.7%) were test-positive at Kimmerikong only (Table 1). Subsequent comparisons amongst all ITS1 amplicons generated in this study, together with information available in GenBank, inferred the presence of *M. sydneyi* DNA (data not shown; contact primary author for sequence alignment) in these eight OTUs. There were no nucleotide differences among generated *M. sydneyi* ITS1 sequences from different polychaete OTUs, different collection

areas or nucleotide sequence data available in GenBank (i.e. **AF159248** (Kleeman and Adlard, 2000) and **AY504628** - **AY504632** (Kleeman et al., 2004)).

Of the PCR-positive polychaete OTUs, we were able to embed specimens of Cirratulidae sp. 1 (n = 7 worms examined in total), Lumbrineridae sp. 1 (n = 22), Magelonidae sp. 1 (n = 20), Nephtyidae sp. 1 (n = 50) and Sabellidae sp. 1 (n = 17) for ISH analysis. Of 116 samples tested, only two specimens of Nephtyidae sp. 1 from Cobar (8.3% of 24 specimens) were confirmed as positive for *M. sydneyi* DNA by ISH while no sections from 26 specimens tested from Kimmerikong were test-positive by ISH. Dr P. Hutchings, of the Australian Museum, Sydney, Australia (http://australianmuseum.net.au/) identified this nephtyid as *Nephtys australiensis*. *Marteilia sydneyi* DNA identified via in situ DNA probe staining was located in the epithelium of the intestine of *N. australiensis* (see Fig. 3). Two differing morphological forms were identified: a 'primordial' cell that contained a well-defined nucleus but had little differentiation in the cytoplasm, and a 'plasmodial' cell that showed an apparent syncytial structure. These morphological types measured 30 × 10 µm in dimension and were associated with, or adhered to, the membrane of polychaete epithelial cells (Fig. 3).

4. Discussion

The results presented here represent the first known record of the identification of *M. sydneyi* being parasitic in an organism other than an oyster and only the third record of any species of *Marteilia* identified from non-molluscan hosts collected from coastal systems. The presence of a member of the phylum Paramyxea in a polychaete worm is perhaps unsurprising. *Paramyxa paradoxa* and *Paramyxa nephtys* have both been reported from the gut epithelium of the polychaetes *Poecilochaetus serpens* (Poecilochaetidae) and *Nephtys caeca* (Nephtyidae), respectively (Desportes, 1981; Larsson and Køie, 2005), although neither has been connected via their life cycle to a bivalve mollusc. Nonetheless, the presence of polychaetes in the life cycle of these two species strengthens the proposal that polychaetes may regularly act as hosts for paramyxean parasites. Additionally, a recent investigation in the Diana lagoon, in the northeast of Corsica, France, indicated the presence of *M. refringens* DNA in polychaete larvae in zooplankton samples (Arzul et al., 2013). Given this possibility, it is intriguing that two copepods are commonly implicated in the life cycle of *M. refringens* (Audemard et al., 2002; Arzul et al., 2013; Boyer et al., 2013), another highly pathogenic parasite of oysters. Employing similar methods to those utilised here, life cycle investigations of *M. refringens* have focused on the identification of previously unknown morphological stages developing within *Paracartia grani* and more recently *Paracartia latisetosa* (Audemard et al., 2002; Carrasco et al., 2007, 2008; Arzul et al., 2013; Boyer et al., 2013). To date, *M. refringens* has been identified in CI - CV copepodid stages of *P. grani*, adult females of *P. grani* and *P. latisetosa* (CIII is the earliest stage of *P. grani* in which *M. refringens* has been detected by ISH; parasitic stages have been detected in the alimentary canal, digestive epithelium and germinal site; (Boyer et al., 2013)), CV males of *P.*

grani and in eggs following egg production experiments (Audemard et al., 2002; Arzul et al., 2013; Boyer et al., 2013). In addition, *M. refringens* has also been successfully transmitted from infected oysters (*O. edulis*) to *P. grani* where parasite development was detected (Arzul et al., 2013), but conversely, these experiments have so far failed to infect oysters from infected copepods (Carrasco et al., 2007). Audemard et al. (2001) hypothesised that either a period of maturation in the environment is required or that a second intermediate host is necessary. However, Carrasco et al. (2007) emphasised that difficulties, either in collecting sufficient infected hosts or producing them through laboratory infections, severely limits the experimental capacity to establish the true nature of infection.

Using our combined molecular approach, we confirmed two *M. sydneyi* infections by ISH-probe staining, which were situated in the epithelium of the intestine of *N. australiensis*. While PCR detection of *M. sydneyi* DNA in polychaetes was relatively common, with a prevalence of 6.2% and 5.8% from Cobar and Kimmerikong, respectively, it is difficult to quantify what proportion of these represent developing stages of *M. sydneyi* in natural polychaete hosts. Equally, it is unlikely that the two *N. australiensis* identified with ISH-probes as containing developing parasitic infections were the only 'real' positives in this study. Given that the simple but overriding objective here was to unambiguously identify developing stages of *M. sydneyi* in an alternate host(s), such a goal required a methodology that reduced sample size and detection sensitivity at each stage of the investigation. First, polychaetes had to be identified to OTUs, which were each approximately equally divided for PCR or ISH analyses, effectively reducing the sample size for each OTU by 50%. PCR amplification using highly sensitive, specific and optimised protocols offers the minimum loss of detection possible. Nonetheless, amplification of parasite DNA can be swamped by overwhelming amounts of host DNA leading to false negatives (Kleeman and Adlard, 2000). Conversely, the presence of *M. sydneyi* DNA in the intestinal lumen of worms as a result of incidental ingestion of parasite spores while feeding potentially overestimates parasite prevalence in alternate hosts (what we refer to as 'false positives'). The last level of reduced detection occurs during processing for ISH. Detection was maximised by optimising the duration of formalin fixation and by using DNA probes designed to anneal in the small subunit region of rRNA (rather than the ITS region) to maximise the ISH staining signal (Kleeman et al., 2002a). However, this technique relies on histological sectioning which, of necessity, sub-samples the target tissue. Where the distribution (and intensity of infection) of a parasite in the tissues of its host is unknown, sub-sampling may or may not affect levels of detection. Consequently, it is perhaps more surprising that we detected 'true' positives in polychaetes at all, rather than the small number that we actually identified.

Use of *N. australiensis* as an alternate host in the life cycle of *M. sydneyi* leads us to consider at least three possible scenarios before assessing the implications of this study. The first is that *N. australiensis* is the only alternate host required for the completion of the life cycle of *M. sydneyi*; the second, *N. australiensis* is one of a number of benthic species that can act as an alternate host for the

completion of a two-host life cycle of *M. sydneyi*, and finally, *N. australiensis* and *S. glomerata* are only two hosts in the three-or-more host life cycle of *M. sydneyi*. If scenario three is correct it is unlikely that any benefit(s) of continued research towards developing a laboratory model of infection would outweigh the cost of doing so. However, if scenarios one or two prove correct then the development of an experimental model is facilitated by the outcome of this project. The first phase of future studies, as an extension to the current investigation, would involve the growth of a parasite-free culture of *N. australiensis* to allow in vivo confirmation of infection by the introduction of mature spores of *M. sydneyi* in controlled experiments. This would parallel experiments reported by Audemard et al. (2002) from flat oysters in France. The next phase would then involve the infection of parasite-free oysters with parasitic stages derived from *M. sydneyi*-infected polychaetes. It is this phase of laboratory model development that has so far been unsuccessful in the attempted experimental infections of *O. edulis* from infected copepods in France (Audemard et al., 2002; Carrasco et al., 2007, 2008) and has led them to hypothesise that a third host, or period of maturation, is required in the life cycle of *M. refringens*.

One factor that will need detailed consideration if future infection experiments are to proceed is the host oysters themselves; oyster genetics and/or their level of immuno-competence will determine whether infective stages of *M. sydneyi* originating from polychaetes will establish. Studies on the mechanism of resistance developed through the SRO selective breeding program conducted by the NSW DPI (see Nell and Perkins (2006)) implicates one form of the defensive enzyme, phenyloxidase, as being negatively selected in resistant lines (Bezemer et al., 2006). Furthermore, resistant lines have now been shown to have higher phagocytic and phenyloxidase activity, a greater number of circulating haemocytes, and a higher percentage of granulocytes than wild-type oysters (Butt and Raftos, 2008; Kuchel et al., 2010; Dang et al., 2011). Consequently, compelling evidence exists that oyster genetics directly impact on their susceptibility to infection with *M. sydneyi*. Another confounding issue to future experiments is the link between environmental stressors and immuno-suppression. Butt and Raftos (2007) suggested that the presence of a transient environmental stressor in the Hawkesbury River in 2004 - 2005 may have affected phenyloxidase activity and, in turn, increased the susceptibility of oysters to infection. Such a scenario would explain the appearance of severe mortalities in an estuary that had previously been unaffected by QX disease. As such, any attempts to either confirm or further develop in vitro life cycle studies of *M. sydneyi* should involve experimental oysters of known genetic susceptibility and be undertaken with sufficient replicates to allow for experimental assessment of environmental stressors.

In conclusion, polychaete worms dominate the benthic fauna associated with sediment near oyster leases in the upper region of the Hawkesbury River. A single species, *N. australiensis*, was confirmed to harbour developing stages of *M. sydneyi* in the epithelium of the intestine of two individuals. Importantly, by detecting these previously unidentified and differing morphological parasitic forms of *M. sydneyi*, this study represents a breakthrough in QX disease research and may

correspond to the identification of the only two hosts required in the life cycle of this parasite. Such a contention would require confirmation through experimental infection of *N. australiensis* from infected oysters followed by back-cross infections from infected polychaetes to known uninfected oysters. Importantly, results from this study provide an unprecedented opportunity to (i) develop an in vivo laboratory model of infection to enhance and expedite the existing selective breeding program for disease resistance and (ii) to identify the principal effectors and the presence/existence of synergistic effects on outbreak events and the subsequent severity of this disease. Consequently, the immediate benefits of this study are directly connected to the commercial oyster industry and associated management sectors. The results presented here should clarify industry members' understanding of disease interactions in the aquatic environment and will be of broader interest to the global aquatic animal health community through the novel identification of alternate hosts required for disease transmission.

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Figure legends

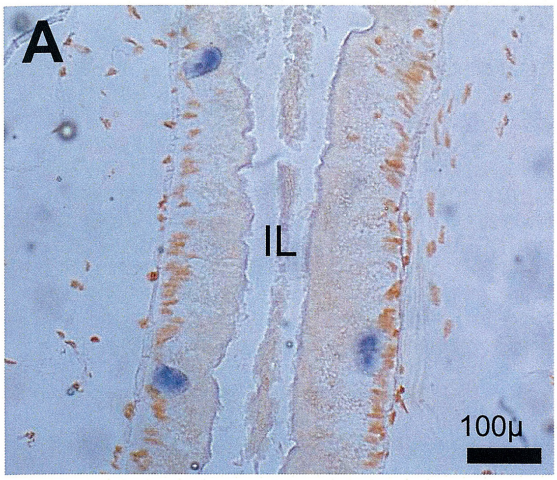
Fig. 1. The location of two areas (i.e. Cobar (red) and Kimmerikong (blue)) in the upper Hawkesbury River, Sydney, Australia, associated with current and former oyster leases and unfarmed neighbouring areas, selected for benthic sampling in 2006. Base map provided by New South Wales Department of Primary Industries, Australia.

Fig. 2. Random GPS points (red circles) plotted across two sampling areas in the upper Hawkesbury River, Sydney, Australia. (A) Fifty points (blue squares) were located across the Cobar sampling site; and (B) 64 points were sampled across the Kimmerikong site. Base map provided by New South Wales Department of Primary Industries, Australia.

Fig. 3. Photomicrographs of tissue sections from the polychaete worm, *Nephtys australiensis*, stained with (A - D) in-situ hybridisation (ISH) DNA probe specific for *Marteilia* spp. and (E – F) H & E, from the same histological preparation. (A – D) ISH staining of two distinct parasite morphologies/stages in the intestinal epithelium of *N. australiensis*; (E) both parasite morphologies stained with H & E; (F) high magnification of 'primary' and 'plasmodial' morphologies of *Marteilia* sp. IL, intestinal lumen.

Table 1. The total numbers of each polychaete operational taxonomic unit (OTU) sampled at Cobar and Kimmerikong, New South Wales, Australia together with the numbers of animals that were PCR test-positive for *Marteilia sydneyi* DNA (number of PCR test-positive samples/total number tested; percentage).

Polychaeta OTU	Putative species	Cobar	Kimmerikong	Totals
Capitellidae sp. 1		0	1 (0/0; 0.0)	1 (0/0; 0.0)
Capitellidae sp. 2		2 (0/0; 0.0)	3 (0/0; 0.0)	5 (0/0; 0.0)
Cirratulidae sp. 1		10 (2/7; 28.6)	26 (3/18; 16.7)	36 (5/25; 20.0)
Lumbrineridae sp. 1	*Augeneria verdis*	108 (4/36; 1.0)	244 (9/79; 11.4)	352 (13/115; 11.3)
Lumbrineridae sp. 2		1 (0/1; 0.0)	6 (0/4; 0.0)	7 (0/5; 0.0)
Lumbrineridae sp. 3		14 (0/7; 0.0)	20 (0/7; 0.0)	34 (0/14; 0.0)
Magelonidae sp. 1	*Magelona* sp.	6 (3/4; 75.0)	76 (9/32; 28.1)	82 (12/36; 33.3)
Nephtyidae sp. 1	*Nephtys australiensis*	86 (10/40; 25.0)	85 (5/38; 13.2)	171 (15/78; 19.2)
Nephtyidae sp. 2	*Nephtys inornata*	0	1 (0/1; 0.0)	1 (0/1; 0.0)
Opheliidae sp. 1	*Armandia intermedia*	11 (0/6; 0.0)	26 (0/12; 0.0)	37 (0/18; 0.0)
Phyllodocidae sp. 1	*Paranaitis inflata*	0	2 (0/1; 0.0)	2 (0/1; 0.0)
Polynoidae sp. 1		1 (0/0; 0.0)	0	1 (0/0; 0.0)
Sabellidae sp. 1	*Jasminiera* sp.	13 (3/7; 43.0)	40 (2/21; 9.5)	53 (5/28; 17.9)
Sabellidae sp. 2	*Laonome triangularis*	0	1 (0/0; 0.0)	1 (0/0; 0.0)
Scalibregmatidae sp. 1	*Scalibregma inflatum*	1 (0/0; 0.0)	1 (0/0; 0.0)	2 (0/0; 0.0)
Spionidae sp. 1		17 (0/7; 0.0)	9 (1/3; 33.3)	26 (1/10; 10.0)
Spionidae sp. 2		14 (0/6; 0.0)	12 (4/7; 57.1)	26 (4/13; 30.7)
Spionidae sp. 3		14 (0/0; 0.0)	3 (0/0; 0.0)	17 (0/0; 0.0)
Spionidae sp. 4		0	1 (0/0; 0.0)	1 (0/0; 0.0)
Terebelidae sp. 1	*Polycirrus rosea*	3 (0/1; 0.0)	19 (0/10; 0.0)	22 (0/11; 0.0)
Trichobranchidae sp. 1	*Terebellides stroemii*	885 (12/423; 2.8)	990 (8/469; 1.7)	1875 (20/892; 2.2)
		1186 (35/545; 6.4)	1566 (41/702; 5.8)	2752 (76/1247; 6.0)



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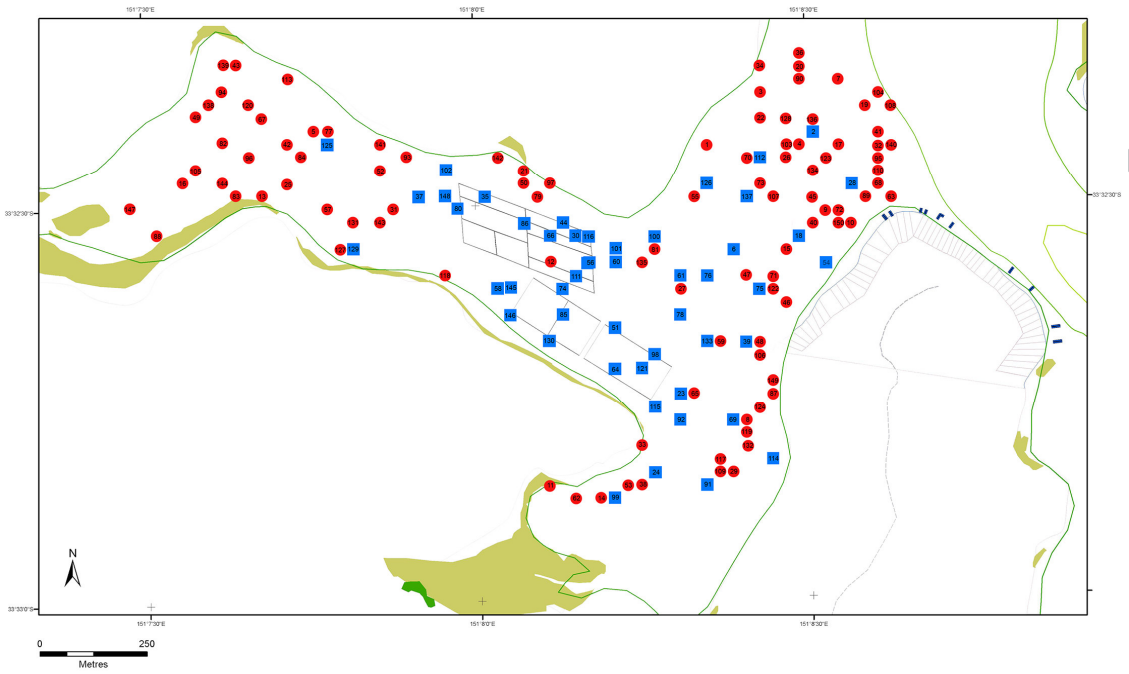

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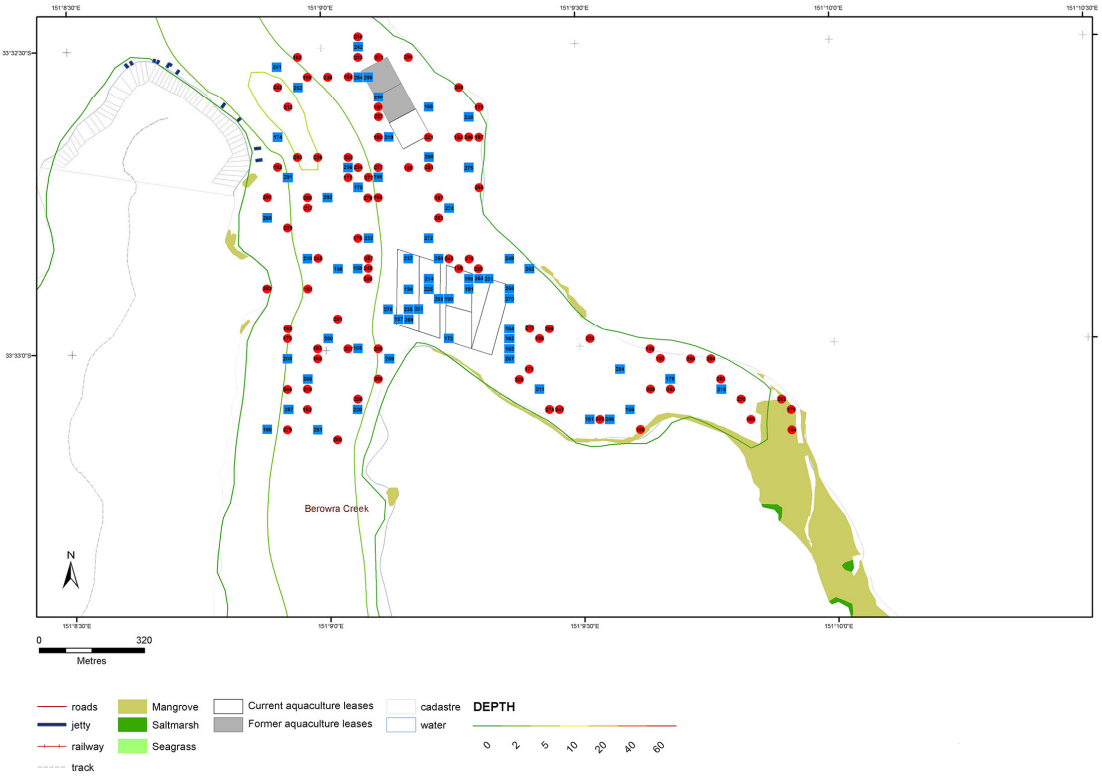

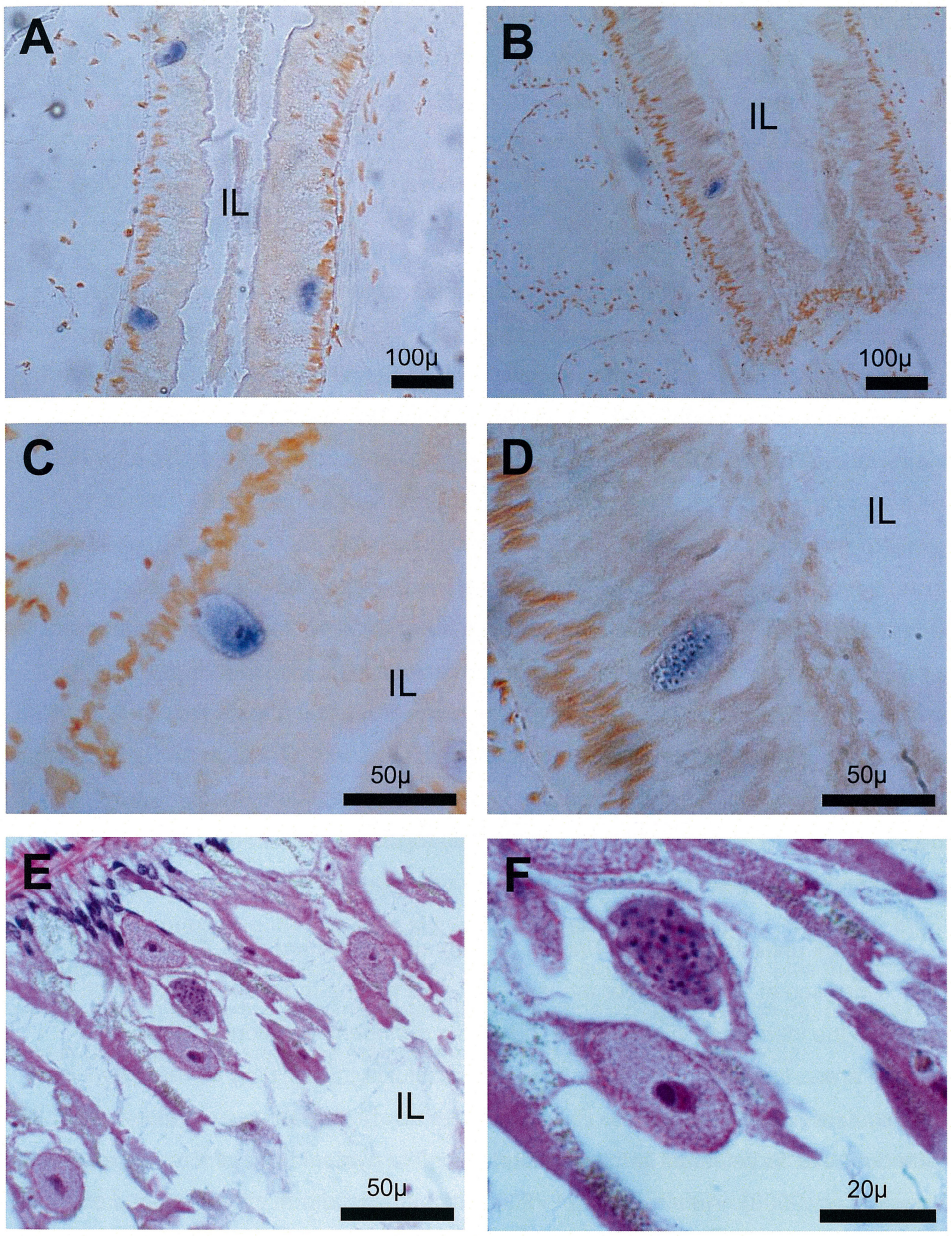

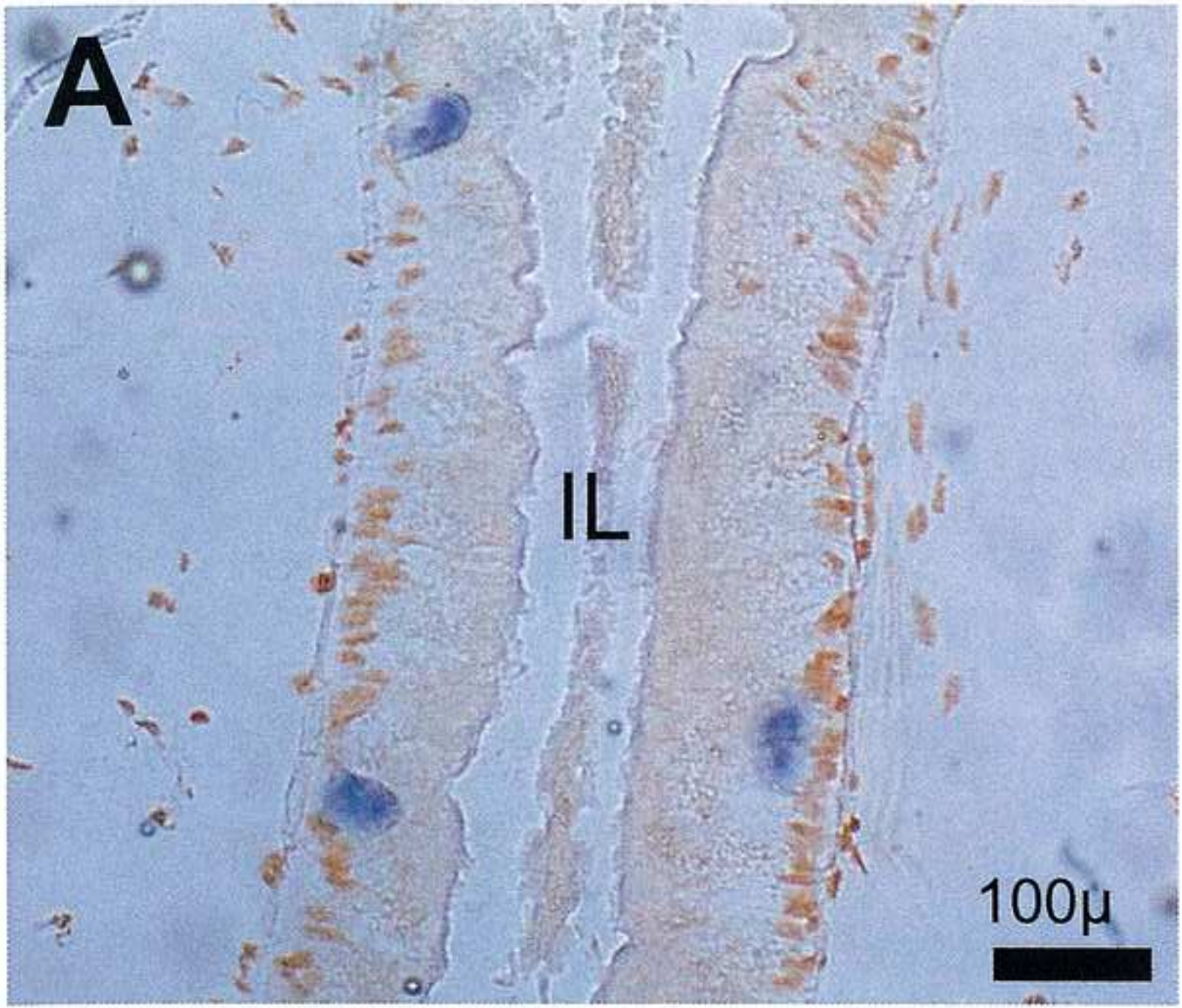

Highlights

- An intermediate host is described in the life cycle of *Marteilia sydneyi*
- A polychaete worm had life stages of *M. sydneyi* in the epithelium of the intestine
- An opportunity may now exist to develop an in vivo laboratory model of infection
- Results have direct downstream application for a selective breeding program for disease resistant oysters
- Identification of principal synergistic effectors causing outbreaks is now possible