Current trends in the study of molluscan diseases

J. BRIAN JONES

Department of Fisheries, P.O. Box 20, North Beach, WA 6920, Australia

ABSTRACT

The study of molluscan diseases has a long history. The first publication on the redial stages of a trematode appeared in the 18th century; early papers on molluscan phagocytosis appeared in the last half of the 19th century and yet much work published before about 1975 does not appear in electronic abstract databases and is effectively "lost". By contrast, a recent search of a leading abstract database for the terms "mollusc" and "disease" shows that the number of publications has exploded in the last eight years and the exponential trend looks set to continue. Much of the increase has been driven by the introduction of molecular technologies, the rediscovery that the immunology of invertebrates generally is a rich hunting ground for new biochemical defence systems and thus potential medical breakthroughs and the desire to publish multiple papers from the same project. As this publication trend continues, it will become increasingly difficult to be knowledgeable on all aspects of molluscan diseases and considerable specialisation is inevitable.

It is not only our knowledge about known mollusc diseases that has grown, since new diseases continue to be reported as: aquaculture becomes more intensive; the Asia/Pacific regional skills base develops; and international reporting becomes more accurate. Transfer of disease between jurisdictions is also becoming more rapid as products are sent live around the world both as broodstock and for human consumption. Thus, the work of the Network of Aquaculture Centres in the Asia and the Pacific and the Food and Agriculture Organization of the United Nations in awareness raising and skills development will continue to make an impact. It is inevitable that, as the initial work on mollusc diseases developed around shellfish growing areas in Europe and America, the next generation of molluscan disease experts will be based in the Asia and the Pacific region.

Key words: diagnosis, taxonomy, physiology, parasite-host relationships

Jones, J.B. 2011. Current trends in the study of molluscan diseases, pp. 75-92. *In* Bondad-Reantaso, M.G., Jones, J.B., Corsin, F. and Aoki, T. (eds.). Diseases in Asian Aquaculture VII. Fish Health Section, Asian Fisheries Society, Selangor, Malaysia. 385 pp.

Corresponding author: Brian Jones, brian.jones@agric.wa.gov.au

INTRODUCTION

Global demand for seafood, including molluscs, continues to grow (FAO, 2009). However, disease continues to be a major financial constraint to growth of mollusc culture. Losses due to *Marteilia refringens* and *Bonamia ostreae* in French oyster farms over the period 1980-1983 were estimated at US$31 million dollars (Grizel and Héral, 1991). Abalone mortalities of unknown aetiology in Taiwan cost US$11 million (Bondad-Reantaso and Subasinghe, 2005). Ongoing mortalities in abalone in Australia had, by February 2007, resulted in a loss of US$4.5 million in exports (Lannen, 2007).

The study of molluscan diseases has a long history. The first publication on the redial stages of a trematode appeared in the 18th century (Swammerdam, 1737), based on work he completed during the 17th century. Early papers on molluscan phagocytosis appeared in the last half of the 19th century (Metchnikoff, 1893; De Bruyne, 1893, 1896) as well as early papers on the histology of molluscan hosts (Grey, 1853; Peck, 1877). According to Yonge (1926), in his excellent description of the histology of the digestive diverticula in lamellibranchs, it was in 1880 that the name "hepatopancreas" for the digestive gland of crustacea was first used, a term still sometimes used for the digestive diverticula in molluscs. Unfortunately, much work published before about 1975 does not appear in electronic abstract databases and is effectively "lost".

By contrast, a recent search of CABI® abstract database for the terms "mollusc" and "disease" showed that the total number of publications in the database for the period 1900-1950 was only 16, all of which were human or veterinary health references, and clearly missed all of the pertinent aquatic mollusc disease papers. In the next 25 years (1951-1975) there were 55 references, only three of which were on aquatic molluscs and none of which included those cited above. The next 25-year period (1976-2000) revealed 1 815 references, yet in the next eight years there were 10 814 references, of which 1 174 were of aquatic relevance. A similar trend is evident when using Aquatic Sciences and Fisheries Abstracts (ASFA) or other such databases. While clearly, much work (including foreign language papers) is not being captured by such databases, it is clear that this exponential trend is set to continue. It also means that, where it was possible to have read all of the literature on the subject up to about 1990, it is now no longer the case. As this publication trend continues, it will become increasingly difficult to be knowledgeable on all aspects of molluscan diseases and considerable specialisation in a team environment is inevitable (Sparks, 2005; Whitfield, 2008).

The increasing specialisation has been driven by a number of factors including the:

- introduction of molecular, genomic and proteomic technologies; microfluidics and the development of microfluidic biochips capable of continuous sampling and real-time (and remote) testing of air/water samples for pathogens and toxins;
- rediscovery that the immunology of invertebrates generally is a rich hunting ground for new biochemical defence systems and thus potential medical breakthroughs;

- science-wide moves to publish frequently, which forces scientists to publish their research in parts, rather than waiting until the research has been completed. In this regard there is a clear trend towards papers with a multitude of authors; and
- increase in number of scientists in general.

For the purpose of this review, the current trends in the study of mollusc diseases can be divided into three categories: (i) diagnostic testing for known mollusc diseases; (ii) taxonomic and phylogenetic studies on pathogens and their host molluscs; and (iii) investigations of known diseases, their impact on the host and the environment. Of necessity, these categories are artificial – the boundaries between them tend to merge.

(i) Diagnostic testing of molluscs for disease.
Diagnostic tests fall into three broad categories:

- Screening apparently healthy animals for specific pathogens of concern. This is most commonly applied to stocks destined for live transfer or as part of a surveillance program.
- Determining the cause of poor health/mortality. This can often be a very complex process in determining the relationships between the host, pathogens (there may be more than one) and the environment (Snieszko, 1974; Berthe, 2002; Garnier *et al.*, 2007).
- Development of new test methodologies or procedures, or improvement of existing procedures. This is becoming more important as issues of sensitivity, specificity and fitness for purpose become more important.

Unfortunately, the results derived from molecular methods are sometimes at odds with more conventional methods, but too often the assumption is made that a positive polymerase chain reaction (PCR) result verifies an infection in a tested host, or that a negative PCR means that infection is not present. This assumption is valid only if the assay has been properly validated for the geographic area and for the hosts examined (Burreson, 2008). For example, based on histology, epidemiology and visualised by Transmission Electron Microscopy (TEM), Hine, Wesney and Hay (1992) and Hine, Wesney and Besant (1998) reported the presence of a herpes virus in oysters (*Ostrea chiliensis* and *Crassostrea gigas*) in New Zealand, which is certainly there (Jones, unpublished TEM obs.). A more recent study using histology and confirmatory PCR (Webb, Fidler and Renault, 2007) failed to amplify ostreid herpesvirus (OsHV-1) from New Zealand shellfish, leading the authors to discount the previous observations and question whether OsHV-1 is present in New Zealand at all. Ulrich *et al.* (2007) claim, based only on PCR results, the presence of *Haplosporidium nelsoni* infections in the Gulf of Mexico despite the results of thousands of oysters having been examined by histology from the same area for over 20 years which failed to observe a single infection (Burreson, 2008). PCR may show that parasite DNA[1] is apparently present in a sample, but in the absence of independent verification it does not confirm or refute infection in the environment (see also Kanagawa, 2003).

(ii) Taxonomic and phylogenetic studies on pathogens and their host molluscs

Work to classify the large numbers of pathogenic organisms associated with molluscs has suffered from the ongoing decline in numbers of trained taxonomists. It is becoming difficult to find people who can identify and describe new metazoan parasites, especially since all of the classical taxonomic literature is based on morphology, and relatively few species are represented in Genbank. Nevertheless, accurate identification of both the host and the parasite is of importance. For example, Nakano and Spencer (2007) used phylogenetic analysis of DNA sequences to show that a species of small intertidal limpet *Notoacmea helmsi* from New Zealand was in fact a taxon comprising 5 morphologically cryptic species. Any study of the parasitology of the group would have been confounded by this discovery.

The current taxonomic difficulties are well illustrated by studies of the molluscan parasites informally grouped as "microcells" because of their small size (about 2 microns). The genera *Marteilia, Mikrocytos*, *Bonamia* and *Haplosporidium* spp. are relatively easily detected by routine histology, but species determination is much more problematic. *Marteilia refringens* and *Marteilia maurini* are morphologically indistinguishable (Longshaw *et al*., 2001) but can be separated by molecular means (Le Roux *et al*., 2001) and both infect oysters and mussels. López-Flores *et al*. (2004) suggested that the *Marteilia* from oysters and from mussels may be two different strains of the same species that appear to readily infect both hosts while other authors accept that they are closely related species - thus their taxonomic status is still open to debate (Berthe *et al*., 2004). A similar situation occurs with the occurrence of *Bonamia* sp. in Australia that has molecular and histological differences to *B. exitiosa (*J. Handlinger, pers. comm.*)*. The distinction is important, for some species are internationally reportable, while other species of the genus are not.

While the use of sequence data from the small subunit ribosomal RNA **(**SSU r RNA[2]) gene is now commonly referred to in the description of new haplosporidians (Azevedo *et al*., 2006), sequence data alone is generally not sufficient to separate a new species. The distance between *Bonamia* sp. from New South Wales (Australia) and *B. exitiosa* from New Zealand over the 1 586 base pair sequence from the 18S gene is only 0.9% (Corbeil *et al*., 2006), yet there are differences in geography, morphology, ultrastructure and histopathology between the two microcells (B. Jones pers. obs., J. Handlinger, pers. comm.)

(iii) Investigations of known diseases of molluscs, their interaction with the host and the environment

There is a rich and growing body of literature on molluscan diseases and their associated pathogens, their interactions with each other and with the wider environment. Comparative genomics is also a source for major advances in our understanding of the regulatory systems not only in molluscs but also in their parasites.

[1] Deoxyribonucleic acid
[2] Ribonucleic acid

In order to make some sense of the voluminous literature, it is useful to adapt Figure 1 below, developed by Snieszko (1974), to show the interrelationships of the host, parasite and the environment and to categorise the types of studies undertaken, as follows:

- **Host-parasite interactions** fall into two groups: firstly, the investigation of molluscan host impact on the pathogen (including host defence mechanisms involving the detection of and subsequent neutralising of pathogen); and secondly, studies on the impact of the parasites on molluscan host (including mechanisms by which the parasite overcomes the host defences and appropriates the host to its own purposes).
- **Parasite-environment interactions** can also be divided into two groups: the investigation of the pathogens impact on environment and the investigation of the environments effect on the pathogen (as opposed to the environmental impact on the host).
- **Environment-host interactions** include investigation of the environment on the host mollusc (including the effects of stress, pollution, tumours- environmental diseases); and investigations of how the host mollusc alters or affects the environment.

Host-parasite interactions

a) Impact of host on pathogen

Host defense mechanisms now constitute a rich area of research. By the end of last century, it was thought that molluscs did not have an acquired immune system and lacked immunoglobulin antibodies (Chu, 1988). Defense was ascribed to both cellular and humoral factors with phagocytosis as the primary response to foreign matter (Feng, 1988).

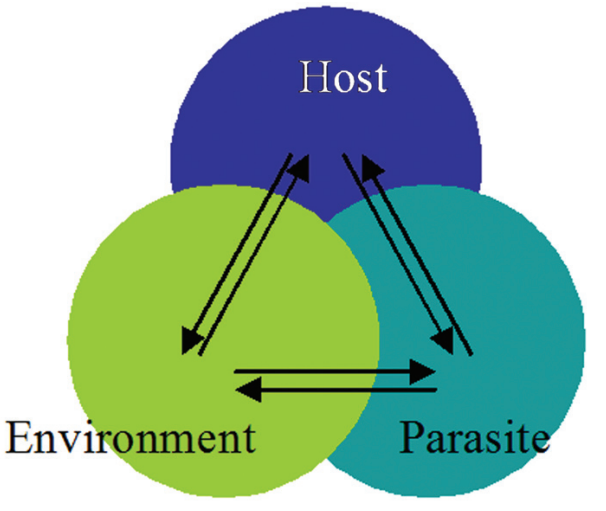

Figure 1. Interrelationship of the host, parasite and the environment (Snieszko, 1974)

Phagocytosis, in particular was studied in the 1890's and that early work was built on by Stauber (1950) who studied the effect of injected India ink particles in *Crassostrea virginica*. There is clearly variation in haemocytes among molluscs; for example, scallops and abalone do not have granulocytes yet other gastropods do (Hine, 1999; Travers *et al*., 2008; Mahilini and Rajendran, 2008). Despite over 100 years of research and numerous papers describing the various morphological forms that haemocytes display, Allam, Ashton-Alcox and Ford (2002) were still able to write "*the origin, life cycle and life span of bivalve haemocytes are still largely unknown and the role of each cell type has not been completely elucidated*". Harris, Lambkin and O'Byrne-Ring (2006) and others have been using immunohistochemistry to identify structural and functional proteins in abalone leading Travers *et al*. (2007) to recommend that resolving the controversies over the classification of haemocytes in molluscs would require the "obligatory" use of mollusc-specific antibodies and gene probes. Whether the application of these new technologies will settle the controversy remains to be seen. Part of the problem is probably an assumption that haemocyte form and function will be the same across all molluscan groups, and a review, such as that by Hine, Wain and Boustead (1987) for teleost leucocytes, is well overdue.

It could have been added that both the origin and fate of haemocytes is also unknown. Haemocytes can be seen to pass through the intact columnar epithelial layers, especially those of the gut, in a process known as diapedesis (from the Greek '*diapedan*' = to ooze through). Though in human pathology the term is confined to the passage of blood cells through unruptured vessel walls, in invertebrates, especially molluscan pathology, the term is also used for the passage of haemocytes, which may or may not contain phagocytosed material, across epithelial borders to the exterior of the body (Onstad *et al*., 2006). Cheng (1967) noted that it was unknown if haemolymph was lost during diapedesis and what factors influenced the rate of diapedesis. That is still unknown and it is also still unclear what role diapedesis plays in the response to infectious diseases.

What we do know is that haemocytes are both mediated by and also produce "humoral factors". For example, killing mechanisms associated with haemocytes involve reactive oxygen molecules, such as the phenoloxidase cascade, that are now known to be important for phagocytosis, melanisation and encapsulation. Inducible serum antimicrobial factors including lysozyme (a bacteriolytic protein) are released by degranulation when phagocytosis occurs (Cheng *et al.*, 1975; Mohandas, Cheng and Cheng, 1985; Xu-Tao Hong, Li-Xin Xiang and Jian-Zhong Shao, 2006). However, the distinction between immunomediators, hormones and neurotransmitters, has become blurred by the finding that haemocytes can synthesis neuroendocrine peptide hormones and also have receptors for these peptides (Ottaviani and Franceschi, 1998a, 1998b).

Though most work has concentrated on the haemocyte-mediated response, other defense mechanisms have been studied. Wright (1959) demonstrated species specific substances in the mucous of a number of snail species and Cheng, Shuster and Anderson (1966a, 1966b) showed that haemolymph of *C. virginica* and *C. gigas* will stimulate cercariae of *Himasthla quitessetensis* to encyst, immobilising them and preventing infection.

There has been increasing activity looking for bioactive compounds in molluscs. β-glucuronidase, phosphatases, lipases, aminopeptidase amylase and antimicrobial factors have been described from molluscs (Chu, 1988; Montes, Durfort and Garcia-Valero, 1996; Montes *et al*., 1997; Roch *et al*., 2008). Agglutinins including haemaglutinins have also been widely reported and these may increase phagocytosis by acting as opsonins (Olafsen *et al*., 1992). Faisal, Oliver and Kaattari (1999) also showed that resistance to *Perkinsus* infections by *Crassostrea* species is effected by protease inhibitors to the extracellular serine proteases secreted by the parasite.

The finding of virus particles in molluscs, when looking for causes of disease, is complicated by the ability of molluscs to sequester live viruses (such as norwalk and hepatitis viruses) from their environment. The reason why this occurs and the relationship of these sequestered viruses to the host mollusc immune systems are unknown. The virus is not simply bioaccumulated since recent work (Le Guyader *et al*., 2006; Tian *et al*., 2007) suggests that clams, mussels and oysters trap the norwalk virus (or the virus actively binds) through an intestinal type A-like histo-blood group antigen. Flegel proposed in 1998 that crustaceans accommodate live virus as a means of continually challenging the immune system in the absence of an acquired immune system (Flegel, 2006). Berthe (2002) also suggested that the invertebrate immune system response is complex and suggests that an 'ecological', or whole system approach to immunology should be considered, rather than relying on the mechanistic 'cause-effect' interpretation of host-pathogen relationships that have, to date, dominated studies on infectious diseases of invertebrates.

b) Impact of pathogen on host

It has long been hypothesised that pathogens may be able to secrete extracellular products to inhibit the host response. For example, there is a strong negative association between the presence of *Marteilia sydneyi* and phenoloxidase activity in *Saccostrea glomerata*, possibly through the release of serum proteases, as happens with *Perkinsus marinus* (Faisal *et al*., 1999; Peters and Raftos, 2003). Variations in phenoloxidase also affect the resistance of oysters to QX disease[3] (Peters and Raftos, 2003; Bezemer *et al*., 2006; Aladaileh, Nair and Raftos, 2007). Bezemer *et al*., (2006) used native-PAGE[4] to identify five discrete forms of phenoloxidase in wild oysters, of which one was associated with disease susceptibility. This raises the question, is it the lack of a specific phenoloxidase type that permits disease or can the pathogen "knock out" a specific phenoloxidase molecule?

Some pathogens are clearly able to inhibit or modify the host response. For example, *Bonamia roughleyi* microcells stimulate phagocytosis by suitable haemocytes but are not killed and instead proliferate within the host cell, eventually lysing the host to release more microcells (Da Silva *et al*., 2008). The cycle results in massive destruction of haemocytes leading to death of the host oyster.

[3] QX stands for Queensland Unknown the title given to this disease prior to the discovery of the organism that is now known to cause it.

[4] Native-Page stands for native polyacrylamide gel electrophoresis

Parasites may also affect biochemical processes other than those involved in defence. Cheng, Sullivan and Harris (1973) reported that the marine gastropod *Nassarius obsoletus* was castrated by chemicals secreted by the sporocysts of *Zoogonius rubellus* and that were specific for germinal epithelium and gametes. Studies on the freshwater snail *Lymnaea stagnalis* infected with *Trichobilharzia ocellata* have shown that substances secreted by the trematode induce changes in host gene expression to directly inhibit mitotic division in the male copulatory organ and also stimulate development of the female endocrine dorsal bodies (De Jong-Brink, Bergamin-Sassen and Solis-Soto, 2001). Likewise Rice *et al*. (2006) showed that infection of the mollusc *Haliotis asinina* by the trematode *Allopodocotyle* sp. results in parasitic castration and is accompanied by differential expression of a number of regulatory genes. Manger, Christensen and Yoshino (1996) found that *Schistosoma mansoni* appropriated, for its own use, the hosts neurotransmitters serotonin and dopamine leading to changes in the host *Biomphalaria glabra* endocrine system.

Perkinsus marinus, P. olseni and *Haplosporidium nelsoni* have all been shown to affect the growth and condition of their host molluscs which led Flye-Sainte-Marie *et al*. (2007) to demonstrate that brown ring disease of clams *(Ruditapes philippinarum*) caused by the bacteria *Vibrio tapetis* affected the energy budget of the clams and resulted in a reduction of the clearance and respiration rate, possibly due to the energy requirements associated with the immune response and tissue repair. How many published studies of the physiology of molluscs have been compromised because the disease status of the animals was not considered a factor?

Parasite-environment interactions

a) Impact of pathogen on the environment of the host

It is known that parasite mortality events can alter the host density leading to major changes in the ecology of an area. *Marteilia* sp. infections lead to the commercial extinction of *Ostrea edulis* from the Gulf of Thessalonaiki (Virvilis and Angelides, 2006). Also, Miura *et al.* (2006) showed that the mud snail *Batillaria cumingi*, when infected by the trematode *Cercaria batillariae*, develop a different morphological form, move to the lower intertidal zone and consume different resources from uninfected snails. The parasites are, thus, indirectly altering the food web of many marsh species.

b) Impact of environment on pathogen

Studies on the effect of the environment (including pollution) on the susceptibility of the host to the pathogen have been done, particularly for MSX (*Haplosporidium nelsoni*) in oysters in the United States of America (USA), but studies on other pathogens and their hosts are more limited. Hégaret *et al*. (2007) showed that infection of clams (*Rudetapes philippinarum*) with *Perkinsus olseni* had no measurable effect on haemocyte parameters measured, but when the clams were exposed to toxic algal blooms there was a measurable change in haemocyte parameters monitored, and thus immunomodulation, in heavily infected clams exposed to toxic algae when compared to heavily infected clams that were not exposed. While we know a lot about the effects of the environment on the mollusc immune system, we know very little

about the effect of the environment on the biochemistry of the pathogen when it is in the host. The optimal conditions for the host may not be those of the pathogen, such that changes in the environment (salinity, temperature) may place the pathogen at a disadvantage.

Research has tended not to disengage the effects on the external infectious stages from the impact of the environment on established infections. For example, the probability of a severe kill due to winter mortality (*B. roughleyi*) is higher after dry autumns and early winters. Growers minimise losses by relaying oysters to low salinity upstream locations during periods of potential infection. Whether these actions are mitigating the effects of subclinical infections, perhaps by reducing parasite replication in the host, or are avoiding new infections by breaking the life cycle has apparently not been studied.

Environmental pollution affects both host communities and parasite populations. Again, there has been much recent work done on the effect of pollutants on the host, and recognition in the literature that parasite communities are a potential indicator of environmental disturbance but there has been little study on the effect of pollutants on the parasite itself.

Changes in parasite abundance and prevalence have also been used as an environmental monitor. Ectoparasites tend to increase and endoparasites decrease in prevalence and abundance in fish after chronic exposure to xenobiotics and aromatic hydrocarbons (MacKenzie, 1999; Kahn, 2004), and the impact on invertebrate hosts is likely to be similar, leading to establishment of monitoring programs such as the USA's "mussel watch" programme (Kim *et al.,* 2008). Contaminants may favour the propagation of parasites by excluding predators, reducing host resistance, improving living conditions of host, or may interfere with parasite biochemistry thus reducing parasite burden or pathogenicity.

Environment-host interactions

a) Non-pathogenic diseases directly induced by environmental changes and pollution. Examples include tumours, toxins, endocrine disruptors and other non-infectious diseases. Describing tumours in molluscs has a long history starting with Ryder (1883), see also subsequent reviews by Pauley (1969), Farley and Sparks (1970), Elston, Moore and Brooks (1992), and Sparks (2005). More recently there has been a growing body of literature on the tissue effects of pollutants, particularly heavy metals, on molluscs following on from the work on tributyl tin antifouling and the imposex[5] that it causes (Smith, 1981; Tallmon and Hoferkamp, 2009). Work has also focussed on oil pollution and endocrine disruption associated with veterinary and medical drug residues (Marigomez *et al.,* 2006; Matthiessen, 2008; Morley, 2008). There has been a parallel growth in papers researching the use of mollusc diseases as a bioindicator of environmental pollution (see review by Au, 2004).

[5] Imposex is a descriptive term applied to some seasnails, marine gastropod molluscs which, under the toxic effects of pollutants, develop sex organs that are in contrast to their actual sex. It is a pathological condition where male sex characteristics, such as the development of male sex organs, (for example the penis and the vas deferens) form in female gastropods (http://en.wikipedia.org/wiki/Imposex)

b) Changes to host susceptibility driven by the environment, leading to disease
Environmental changes do not have to damage tissues and interfere with biochemical processes to affect oyster immune status. More subtle environmental changes are often classified as 'stress' and include, but are not limited to: mechanical disturbance (Lacoste *et al.,* 2002; Ballarin, Pampanin and Marin, 2003); salinity changes (Fisher, Auffret and Ballouet, 1987; Butt, Shaddick and Raftos, 2006); temperature (Soudant *et al*., 2004; Cheng *et al*., 1975; Cheng *et al*., 2004; Zhang *et al*., 2006); chemical pollution (Pipe and Coles, 1995; Oliver *et al*., 2001; Cheng, Hsiao and Chen, 2004; Cheng, Juang and Chen, 2004) and diet including starvation (Butt *et al*., 2007). For example, Hong Chen *et al*. (2005) found that both phagocytosis and phenoloxidase activity in *Haliotis discus hannai* were affected by lack of dietary pyridoxine (vitamin B6). It is also certain that the enzyme systems of molluscs will be adapted to optimally perform at the normal temperature range. Changes in temperature may change host biochemistry thus favouring pathogens.

The 1986 *Bonamia* epizootic in Foveaux Strait, New Zealand, began in areas that had been fished intensively for years and where benthic habitat was highly modified (Cranfield, Michael and Doonan, 1999). Similar escalating disease mortality in oysters in Chesapeake Bay, USA, has been attributed to modification of oyster habitat by fishing (Rothschild *et al.,* 1994), and the environmental stress caused by this modification has been directly implicated in increasing acetosporan disease levels of oysters in experiments (Lenihan *et al*., 1999).

CONCLUSIONS

It is not only our knowledge about known mollusc diseases that has grown. New diseases continue to be regularly reported as aquaculture becomes more intensive; as the Asia and the Pacific regional skills base develops and more diseases are reported; as international reporting also becomes more accurate; and as transfer of disease between jurisdictions becomes more rapid as products are sent live - both as broodstock and as products for human consumption (such as the spread of *Bonamia* spp. infected oysters throughout Europe).

There is a need for an increase in the numbers of trained diagnosticians as well as those investigating the ecology of diseased molluscs at a local level. It has been recognised by agencies such as the Network of Aquaculture Centres in Asia/Pacific and the Food and Agriculture Organization of the United Nations that the skills base needs to be developed at three levels: Level I (farm/production site observations, record-keeping and health management) is strongly emphasized throughout the *Asian Diagnostic Guide (*Bondad-Reantaso *et al*., 2000*)* as this forms the basis for triggering the other diagnostic levels (II and III). Level II includes specialisations such as parasitology, histopathology and bacteriology that, generally speaking, cannot be conducted at the farm or culture site. Level III comprises advanced diagnostic specialisation that requires significant capital and training investment, such as TEM. Immunology and biomolecular techniques are included in Level III, although field kits are now being developed for farm or pond-side use (Level I) as well as use in microbiology or histology laboratories (Level II). These efforts are good indication that technology transfer is now enhancing diagnostics and, with solid quality control and field

validation, it is certain that more Level III technology will become field accessible in the near future (Walker and Subasinghe, 2000).

It is inevitable that, as the initial work on mollusc diseases developed around shellfish growing areas in Europe and America, the next generation of molluscan disease experts will be based in the Asia Pacific region.

REFERENCES

Aladaileh, S., Nair, S. and Raftos, D.A. 2007. Induction of phenoloxidase and other immunological activities in Sydney rock oysters challenged with microbial pathogen-associated molecular patterns. *Fish and Shellfish Immunology* 23:1196-1208.

Allam, K.A., Ashton-Alcox, K.A. and Ford, S.E. 2002. Flow cytometric comparison of haemocytes from three species of bivalve molluscs. *Fish and Shellfish Immunology* 13:141-158.

Au, D.W.T. 2004. The application of histo-cytopathological biomarkers in marine pollution monitoring: a review. *Marine Pollution Bulletin* 48:817-834.

Azevedo, C., Balseiro, P., Casal, G., Gestal, C., Aranguren, R., Stokes, N.A., Carnegie, R.B., Novoa, B., Burreson, E.M. and Figueras, A. 2006. Ultrastructural and molecular characterisation of *Haplosporidium montforti* n. sp., parasite of the European abalone *Haliotis tuberculata*. *Journal of Invertebrate Pathology* 92:23-32.

Ballarin, L., Pampanin, D.M. and Marin, M.G. 2003. Mechanical disturbance affects haemocyte functionality in the Venus clam *Chamelea gallina*. *Comparative Biochemistry and Physiology. A, Molecular and Integrative Physiology* 136:631-640.

Berthe, F.C.J. 2002. Keynote: Pacem in terris pathogenibus bonae voluntatis: molluscs-pathogens relationships prospects. *Bulletin of the European Association of Fish Pathology* 22:52-57.

Berthe F.C.J., Le Roux, F., Adlard, R.D. and Figueras, A. 2004. Marteiliosis in molluscs: A review. *Aquatic Living Resources* 17:433-448.

Bezemer, B., Butt, D., Nell, J., Adlard, R. and Raftos, D. 2006. Breeding for QX disease resistance negatively selects one form of the defensive enzyme phenoloxidase in Sydney rock oysters. *Fish and Shellfish Immunology* 20:627-636.

Bondad-Reantaso, M.G., McGladdery, S.E., East, I. and Subasinghe, R.P. (eds.) 2001. Asia Diagnostic Guide to Aquatic Animal Diseases. *FAO Fisheries Technical Paper* No. 402, Supplement 2. Rome. FAO, 236p.

Bondad-Reantaso, M.G. and Subasinghe, R. 2005. Aquatic animal diseases and their economic impact. A global perspective. *Aquaculture Health International* 1:4-5.

Burreson, E.M. 2008. Misuse of PCR assay for diagnosis of mollusc protistan infections. *Disease of Aquatic Organisms* 80:81-83.

Butt, D. Shaddick, K. and Raftos, D. 2006. The effect of low salinity on phenoloxidase activity in the Sydney rock oyster, *Saccostrea glomerata*. *Aquaculture* 251:159-166.

Butt, D., Aladaileh, S., O'Connor, W.A. and Raftos, D.A. 2007. Effect of starvation on biological factors related to immunological defense in the Sydney rock oyster (*Saccostrea glomerata*). *Aquaculture* 264:82-91.

Cheng, T.C. 1967. Marine molluscs as hosts for symbiosis with a review of known parasites of commercially important species. *Advances in Marine Biology* 5:1-424.

Cheng, T.C., Shuster, C.N. and Anderson A.H. 1966a. A comparative study of the susceptibility and response of eight species of marine pelecypods to the trematode *Himasthla quissetensis*. *Transactions of the American Microscopical Society* 85:284-295.

Cheng, T.C., Shuster, C.N. and Anderson A.H. 1966b. Effects of plasma and tissue extracts of marine pelecypods on the cercaria of *Himasthla quissetensis*. *Experimental Parasitology* 19:9-14.

Cheng, T.C., Sullivan, J.T. and Harris, K.R. 1973. Parasitic castration of the marine prosobranch gastropod *Nassarius obsoletus* by sporocysts of *Zoogonus rubellus* (Trematoda): histopathology. *Journal of Invertebrate Pathology* 21:183-190.

Cheng, T.C., Rodrick, G.E., Foley, D.A. and Koehler, S.A. 1975. Release of lysozyme from hemolymph cells of *Mercenaria mercenaria* during phagocytosis. *Journal of Invertebrate Pathology* 25:261-265.

Cheng, W., Hsiao, I-S. and Chen, J-C. 2004. Effect of ammonia on the immune response of Taiwan abalone *Haliotis diversicolor supertexta* and its susceptibility to *Vibrio parahaemolyticus*. *Fish and Shellfish Immunology* 17:193-202.

Cheng, W., Hsiao, I-S., Hsu, C-H. and Chen, J-C. 2004. Change in water temperature on the immune response of Taiwan abalone *Haliotis diversicolor supertexta* and its susceptibility to *Vibrio parahaemolyticus*. *Fish and Shellfish Immunology* 17:235-243.

Cheng, W., Juang, F-M. and Chen, J-C. 2004. The immune response of Taiwan abalone *Haliotis diversicolor supertexta* and its susceptibility to *Vibrio parahaemolyticus* at different salinity levels. *Fish and Shellfish Immunology* 16:295-306.

Chu, F.L.E. 1988. Humoral defense factors in marine bivalves. *American Fisheries Society Special Publication* 18:178-188.

Corbeil, S., Azul, I., Robert, M., Berthe, F., Besnard-Cochennec, N. and Crane M. St.J. 2006. Molecular characterisation of an Australian isolate of *Bonamia exitiosa*. *Diseases in aquatic organisms* 71:81-85.

Cranfield, H.J., Michael, K.P., and Doonan, I.J. 1999. Changes in the distribution of epifaunal reefs and oysters during 130 years of dredging for oysters in Foveaux Strait, southern New Zealand. *Aquatic Conservation: Marine and Freshwater Ecosystems* 9:461-483.

Da Silva, P.M., Comesaña, P., Fuentes, J. and Villalba, A. 2008. Variability of haemocyte and haemolymph parameters in European flat oyster *Ostrea edulis* families obtained from brood stocks of different geographical origins and relation with infection by the protozoan *Bonamia ostreae*. *Fish and Shellfish Immunology* 24:551-563.

De Bruyn, C. 1893 La phagocytose observée sur la vivant, dans les branchies des mollusques lamellibranches. *Comptes rendus hebdomadaires des séances de l'académie des sciences, Paris* 116:65-68

De Bruyne, C. 1896. Sur l'intervention de la phagocytose dans le développement des invertébrés. Académie Royale, Brussels, 114 pp

De Jong-Brink, M., Bergamin-Sassen, M. and Solis Soto, M. 2001. Multiple strategies of schistosomes to meet their requirements in the intermediate snail host. *Parasitology* 123: S129-S141.

Elston, R.A., Moore, J.D., and Brooks, K. 1992. Disseminated neoplasia of bivalve molluscs. *Reviews in Aquatic sciences* 6 :405-466.

FAO 2009. The state of world fisheries and aquaculture 2008. Food and Agriculture Organization of the United Nations. Rome.

Faisal, M., Oliver, J.L. and Kaattari, S.L. 1999. Potential role of protease-antiprotease interactions in *Perkinsus marinus* infection in *Crassostrea* spp. *Bulletin of the European Association of Fish Pathologists* 19: 269-276.

Farley C.A. and Sparks A.K. 1970: Proliferative diseases of hemocytes, endothelial cells, and connective tissue cells in molluscs. *Bibliotheca haematologica* 36 :610-617.

Feng, S.Y. 1988. Cellular defense mechanisms of oysters and mussels *American Fisheries Society Special Publication 18*:153-168.

Fisher, W.S., Auffret, M. and Balouet, G. 1987. Response of European flat oyster (*Ostrea edulis*) hemocytes to acute salinity and temperature changes. *Aquaculture* 67: 179-190.

Flegel, T.W. 2006. Review: Detection of major penaeid shrimp viruses in Asia, a historical perspective with emphasis on Thailand. *Aquaculture* 258:1-33.

Flye-Sainte-Marie, J., Pouvreau, S., Paillard, C. and Jean, F. 2007. Impact of brown ring disease on the energy budget of Manilla clam *Ruditapes philippinarum*. *Journal of Experimental Marine Biology and Ecology* 349:378-389.

Garnier, M., Labreuche, Y., Garcia, C., Robert, M. and Nicolas, J. L. 2007. Evidence for the involvement of pathogenic bacteria in summer mortalities of the Pacific oyster *Crassostrea gigas. Microbial ecology* 53:187-196.

Grey, J.E. 1853. On the teeth and the tongues of Mollusca. *Quarterly journal of microscopical science* S1-1:170-176.

Grizel, H. and Héral, M. 1991. Introduction into France of the Japanese oyster *Crassostrea gigas*. *Journal du Conseil International pour l'Exploration de la Mer* 47, 399-403.

Harris, L., Lambkin, H. and O'Byrne-Ring, N. 2006. Characterisation of cell types in abalone (*Haliotis* spp.) tissues using immunohistochemical techniques. *Aquaculture* 261:1413-1421.

Hégaret, H., Da Silva, P.M., Wikfors, G.H. Lambert, C., De Bettignies, T., Shumway, S.E. and Soudant, P. 2007. Hemocyte responses of Manila clams *Rudetapes philippinarum*, with varying parasite, *Perkinsus olseni*, severity to toxic-algal exposures. *Aquatic Toxicology* 84:469-479.

Hine, P.M. 1999. Review: The interrelationships of bivalve haemocytes. Fish and Shellfish Immunology 9:367-385.

Hine, P.M., Wain, J.M. and Boustead, N.C. 1987. The leucocyte enzyme cytochemistry of fish. *New Zealand Fisheries Research Bulletin* 28:1-74.

Hine, P.M., Wesney, B. and Hay, B.E. 1992. Herpes virus associated with mortalities among hatchery reared larval pacific oysters *Crassostrea gigas*. *Disease of Aquatic Organisms* 12:135-142.

Hine, P.M., Wesney, B. and Besant, P. 1998. Replication of a herpes-like virus in larvae of the flat oyster *Tiostrea chilensis* at ambient temperatures. *Diseases of aquatic organisms* 32:161-171.

Hong Chen, Kangsen Mai, Wenbing Zhang, Zhiguo Lifu, Wei Xu and Beiping Tan. 2005. Effects of dietary pyridoxine on iimmune responses in abalone *Haliotis discus hannai* Ino. *Fish and shellfish immunology* 19:241-252.

Kanagawa, T. 2003. Bias and artifacts in multitemplate polymerase chain reactions (PCR). *Journal of Bioscience and Bioengineering* 96:317-323.

Khan, R.A. 2004. Parasites of fish as biomarkers of environmental degradation: a field study. *Bulletin of Environmental Contamination and Toxicology* 72:394-400.

Kim, Y., Powell, E.N., Wade, T.L. and Presley, B.J. 2008. Relationship of parasites and pathologies to contaminant body burden in sentinel bivalves: NOAA status and trends 'Mussel Watch" program. *Marine Environmental Research* 65:101-127.

Lacoste, A., Malham, S.K., Gelebart, F., Cueff, A. and Poulet, S.A. 2002. Stress-induced immune changes in the oyster *Crassostrea gigas*. *Developmental and Comparative Immunology* 26:1-9.

Lannen, D. 2007. Deadly virus threatens abalone stocks. Geelong Advertiser, issue of 14 Feb 2007.

Le Guyader, F.S., Loisy, F., Atmar, R.L., Hutson, A.M. Estes, M.K. Ruvoën-Clouet, N. Pommepuy, M. and Le Pendus, J. 2006. *Emerging Infectious Diseases* 12:931-936.

Lenihan, H.S., Micheli, F., Shelton, S.W., and Peterson, C.H. 1999. The influence of multiple environmental stressors on susceptibility to parasites: an experimental determination with oysters. *Limnology and Oceanography* 44:910-924.

Le Roux, F., Lorenzo, G., Peyret, P., Audemard, C., Figueras, A., Vivarès, C., Gouy, M. and Berthe, F. 2001. Molecular evidence for the existence of two species of *Marteilia* in Europe. *Journal of Eukaryotic Microbiology* 48:449-454.

Longshaw, M., Feist, S.W., Matthews, R.A. and Figueras, A. 2001. Ultrastructural characterisation of *Marteilia* species (Paramyxea) from *Ostrea edulis*, *Mytilus edulis* and *Mytilus galloprovincialis* in Europe. *Diseases of aquatic organisms* 44:137-142.

López-Flores, I., De la Herrán, R., Garrido-Ramos, M.A., Navas, J.I., Ruiz-Rejón, C. and Ruiz-Rejón, M. 2004. The molecular diagnosis of *Marteilia refringens* and differentiation between *Marteilia* strains infecting oysters and mussels based on the rDNA IGS sequence. *Parasitology* 129:411-419.

Mackenzie, K. 1999: Parasites as pollution indicators in marine ecosystems: a proposed early warning system *Marine Pollution Bulletin* 38 :955-959.

Mahilini, H.M. and Rajendran, A. 2008**.** Categorization of hemocytes of three gastropod species *Trachea vittata* (Muller), *Pila glabosa* (Swainson) and *Indoplanorbis exustus* (Dehays). *Journal of Invertebrate Pathology* 97:20-26.

Manger, P., Christensen, B.M. and Yoshino, T.P. 1996. Biogenic monoamines in the freshwater snail *Biomphalaria glabrata*: influence of infection by the human blood fluke, *Schistosoma mansoni*. *Comparative Biochemistry and Physiology* 114A:227-234.

Marigomez, I., Soto, M., Cancio, I., Orbea, A., Garmendia, L. and Cajaraville, M.P. 2006. Cell and tissue biomarkers in mussel, and histopathology in hake and anchovy from Bay of Biscay after the Prestige oil spill (Monitoring Campaign 2003). *Marine Pollution Bulletin* 53:287-304.

Matthiessen, P. 2008. An assessment of endocrine disruption in molluscs and the potential for developing internationally standardized mollusc life cycle test guidelines. *Integrated environmental assessment and management* 4:274-284.

Metchnikoff, E. 1893. Lectures on the comparative pathology of inflammation. (Reprinted in 1968). Dover, New York.

Miura, O., Kuris., A.M., Torchin, M.E., Hechinger, R.F. and Chiba, S. 2006. Parasites alter host phenotype and may create a new ecological niche for snail hosts. Proceedings of the Royal Society. *B. Biological Sciences* 273:1323-1328.

Mohandas, A., Cheng, T.C. and Cheng, J.B. 1985. Mechanism of lysosomal enzyme release from *Mercenaria mercenaria* granulocytes: a scanning electron microscope study. *Journal of invertebrate pathology* 46:189-197.

Montes, J.F., Durfort, M. and García-Valero, J. 1996. When the venerid clam *Tapes decussatus* is parasitized by the protozoan *Perkinsus* sp. it synthesizes a defensive polypeptide that is closely related to p225. *Diseases of Aquatic Organisms* 26:149–157.

Morley, N.J. 2009. Environmental risk and toxicology of human and veterinary waste pharmaceutical exposure to wild aquatic host-parasite relationships. *Environmental Toxicology and Pharmacology* 27:161-175.

Montes, J.F., Del Río, J.A., Durfort, M. and García-Valero, J. 1997. The protozoan parasite *Perkinsus atlanticus* elicits a unique defensive response in the clam *Tapes semidecussatus*. *Parasitology* 114:339–349.

Nakano, T. and Spencer, H. 2007. Simultaneous polyphenism and cryptic species in an intertidal limpet from New Zealand. *Molecular Phylogenetics and Evolution* 45:470-479.

Olafsen, J.A., Fletcher, T.C. and Grant, P.T. 1992. Agglutinin activity in Pacific oyster *Crassostrea gigas* hemolymph following *in vivo Vibrio anguillarum* challenge. *Developmental and Comparative Immunology* 16:123–138.

Oliver, L.M., Fisher, W.S., Winstead, J.T., Hemmer, B.L. and Long, E.R. 2001. Relationships between tissue contaminants and defense-related characteristics of oysters (*Crassostrea virginica*) from five Florida bays. *Aquatic Toxicology* 55:203-222

Onstad, D.W., Fuxa, J.R., Humber, R.A., Oestergaard, J., Shapiro-Ilan, D.I., Gouli, V.V., Anderson, R.S., Andreadis, T.G. and Lacey, L.A. 2006. An Abridged Glossary of Terms Used in Invertebrate Pathology, 3rd Ed. Society for Invertebrate Pathology. http://www.sipweb.org/

Ottaviani, E. and Franceschi, C. 1998a. The invertebrate phagocytic immunocyte: clues to a common evolution of immune and neuroendocrine systems. *Immunology Today* 18:169-174.

Ottaviani, E. and Franceschi, C. 1998b. A new theory on the common evolutionary origin of natural immunity, inflammation and stress response: the invertebrate phagocytic immunocyte as an eye witness. *Domestic Animal Endocrinology* 15: 291-296.

Pauley G.B. 1969. A critical review of neoplasia and tumor like lesions in Mollusks. *United States National Cancer Institute Monograph* 31:509-539.

Peck, R.H. 1877. The minute structure of the gills of Lamellibranch Mollusca. *Quarterly journal of microscopical science* s2-17:43-66.

Peters, R. and Raftos, D.A. 2003. The role of phenoloxidase suppression in QX disease outbreaks among Sydney Rock oysters (*Saccostrea glomerata*). *Aquaculture* 223:29-39.

Pipe, R.K. and Coles, J.A. 1995. Environmental contaminants influencing immunefunction in marine bivalve mollusks. *Fish and Shellfish Immunology* 5:581-595.

Rice, T., McGraw, E., O'Brien, E.K., Reverter, A., Jackson, D.J. and Degnan, B.M. 2006. Parasitic castration by the digenian trematode *Allopodocotyle* sp. alters gene expression in the brain of the host mollusc *Haliotis asinina*. *FEBS letters* 580:3769-3774.

Roch, P., Yang, Y., Toubiana, M. and Aumelas, A. 2008**.** NMR structure of mussel mytilin and antiviral and antibacterial activities of derived synthetic peptides. *Development and Comparative Immunology* 32:227-238.

Rothschild, B.J., Ault, J.S., Goulletquer, P. and He´ral, M. 1994. Decline of the Chesapeake Bay oyster population: a century of habitat destruction and overfishing. *Marine Ecology Progress Series* 111:29-39.

Ryder, J.A. 1883. The protozoan parasites of the oyster. *Science, NY* 1:567-568.

Smith, B.S. 1981. Male characteristics on female mud snails caused by antifouling paints. Journal of applied toxicology 1:22-25.

Sniezko, S.F. 1974. The effects of environmental stress on outbreaks of infectious diseases in fishes. *Journal of Fish Biology* 6:197-208.

Soudant, P., Paillard, C., Choquet, G., Lambert, C., Reid, H.I., Marhic, A., Donaghy, L. and Birkbeck, T.H. 2004. Impact of season and rearing site on the physiological and immunological parameters of the Manila clam *Venerupis*. *Aquaculture* 229:401-418.

Sparks, A.K. 2005. Observations on the history of non-insect invertebrate pathology from the perspective of a participant, *Journal of Invertebrate Pathology, Volume 89, Special SIP Symposium Issue*:67-77.

Stauber, L. 1950. The fate of India ink injected intracardially into the oyster, *Ostrea virginica* Gmelin. *Biological Bulletin* 98:227-241.

Swammerdam, J. 1737. Biblia Naturae. - Amsterdam, 137 pp.

Tallmon, D.A. and Hoferkamp, L. 2009. Long-term changes in imposex frequency in file dogwinkles, *Nucella lima* G., and tributyltin concentrations in bay mussels, *Mytilus trossulus* G. *Bulletin of environmental contamination and toxicology* 83:235-238.

Tian, P., Engelbrektson, A.L., Jiang, X., Zhong, W. and Mandrell, R.E. 2007. Norovirus recognizes histo-blood group antigens on gastrointestinal cells of clams, mussels, and oysters: a possible mechanism of bioaccumulation. *Journal of Food Protection* 70:2140-2147.

Travers, M.A., Da Silva, P.M., Le Goic, N., Marie, D., Donval, A., Huchette, S., Koken, M. and Paillard, C. 2008. Morpologic, cytometric and functional characterisation of abalone (*Haliotis tuberculata*) haemocytes. *Fish and Shellfish Immunology* 24:400-411.

Ulrich, P.N., Colton, C.M., Hoover, C.A., Gaffney, P.M. and Marsh, A.G. 2007. *Haplosporidium nelsoni* (MSX) rDNA detected in oysters from the Gulf of Mexico and the Caribbean Sea. *Journal of Shellfish Research* 26:195-199.

Virvilis, C. and Angelidis, P. 2006. Presence of the parasite *Marteilia* sp. in the flat oyster (*Ostrea edulis* L) in Greece. *Aquaculture* 259:1-5.

Walker, P. and Subasinghe, R. 2000. DNA-based Molecular diagnostic techniques: Research needs for standardization and validation of the detection of aquatic animal pathogens and diseases. *FAO Fisheries Technical Paper* 395:1-100.

Webb, S.C., Fidler, A. and Renault, T. 2007. Primers for PCR-based detection of ostreid herpes virus-1 (OsHV-1): Application in a survey of New Zealand mollusks. *Aquaculture* 272: 126-139.

Whitfield, J. 2008. Group Theory. *Nature* 455 (7214):720-723.

Wright, C.A. 1959. The application of paper chromatography to a taxonomic study in the molluscan genus *Lymnaea*. *Journal of the Linnean Society (Zoology)* 44:222-237.

Yonge, C.M. 1926. Structure and physiology of the organs of feeding and digestion in *Ostrea edulis*. *Journal of the Marine Biological Association* 12:295-386.

Xu-Tao Hong, Li-Xin Xiang and Jian-Zhong Shao 2006. The immunostimulating effect of bacterial genomic DNA on the innate immune responses of bivalve mussel *Hyriopsis cumingii* Lea. *Fish and Shellfish immunology* 21:357-364.

Zhang, Z., Li X., Vandepeer, M. and Zhao W. 2006. Effects of water temperature and air exposure on the lysosomal membrane stability of hemocytes in pacific oysters, *Crassostrea gigas* (Thunberg). *Aquaculture* 256:502-509.