

Section I: Keynotes

Pacem in terris pathogenibus bonae voluntatis : molluscs-pathogens relationships prospects.

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Introduction

In 1999, the world aquaculture production of molluscs was estimated at 9.4 million tonnes which represents approximately a 23% contribution to the global aquaculture production. Aquaculture of molluscs has been growing at an average annual rate of 11% over the past decades, although some activities - such as aquaculture of pectens or abalones - were growing even more rapidly. In many countries, there is a significant mollusc aquaculture, traditionally based on wild stocks. However, these natural populations frequently do not fulfil market demand, because of low value of the product, over-fishing of the resource and/or mass mortality outbreaks caused by diseases. A frequent answer to this situation has been the introduction of new stocks or new species in these areas (Grizel, 1996). Furthermore, species diversification for aquaculture, as well as hatchery production improvements do increase the demand for movements and transfers of live molluscs.

Pathogen transfers via transfers of live molluscs is currently recognised as a major cause of spreading diseases (Berthe & Boudry, 1999). The detrimental effect of pathogens is also to be considered as affecting both quantity and quality of the production. There are numerous examples of crisis resulting from mass mortality outbreaks and diseases are a significant constraint to aquaculture production and trade. In this prospect, it is of a central importance to establish effective programs to prevent the transfer of infected stocks in disease free areas. For this purpose, five significant diseases are listed by the Office International des Epizooties (OIE, World Organisation for Animal Health). Molluscs originating from a zone infected by one of these diseases should not be transferred into an other zone; *a fortiori* when this late is free of the disease under consideration.

In endemic zones, there is very few ways to reduce the detrimental effect of pathogens on molluscs (Mialhe *et al.*, 1995). Molluscs are usually reared in the open sea which strongly limits the potential use of chemotherapy, because of the quantity of product to be used, its impact on the environment and the obvious risk of re-infection. On the other hand, vaccination also appears helpless due to the fact that molluscs do not possess lymphocytes B/T type and do not produce antibodies. Bearing this in mind, in endemic zones, reducing the impact of pathogens is likely to rely on the biology of pathogens (temperature and salinity preferenda, life-cycles, intermediate hosts of parasites, reservoirs and routes of transmission,...) and genetic improvements of host species towards resistance. Regarding this last question, more is awaited from defence mechanisms knowledge as an intimate prospect of host-pathogen relationships.

With regards to control of molluscs diseases, access to diagnostic tests that are rapid, reliable and sensitive is of a central importance. Techniques applicable to molluscs pathogens are limited, and recent efforts to overcome these problems have led to the development of nucleic acid based diagnostic methods (Berthe *et al.*, 1999). To date, gene probes used in the diagnosis of diseases of cultured molluscs have been for detection of the most economically important pathogens. Molecular data, sequences, have clarified taxonomy and phylogeny of these pathogens. They are now likely to shed new light on host-pathogen relationships. This is the focus of this paper.

When tolerance becomes a key word of host-pathogens relationships

Historical cases of mass mortality outbreaks resulting from pathogen transfers via transfers of live molluscs are numerous. For example, the haplosporidian parasite *Haplosporidium nelsoni* is known to dramatically affect culture of the native Eastern oyster, *Crassostrea virginica*, on the east coast of North America. This parasite was later described and identified in the Pacific oyster, *Crassostrea gigas*. A current thought is that *H. nelsoni* have been introduced onto the west coast of the United States in *C. gigas* imported from Japan, and from there moved to the east coast, where it has infected and affected *C. virginica* (Burreson *et al.*, 2000). This example demonstrates the possible dramatic consequences of transferring infected stocks. It illustrates meeting between a pathogen and a host species as *meeting with death*, if paraphrasing the Agatha Christie's famous novel. As stated above, the OIE has listed diseases of molluscs currently recognised as major concerns, among which stands haplosporidiosis

caused by *H. nelsoni*. However, listing may equivocally lead to a black and white, manichean, view of pathogens. Examples below should modulate this view.

In France, two parasites have significantly affected the production of the European flat oyster, *Ostrea edulis*, in the 70ies-80ies. One of the pathogens responsible for this is the intrahaemocytic parasite *Bonamia ostreae*. It is now recognised that *Bonamia ostreae* was introduced to Europe by means of imports of infected flat oysters from California (Cigarria & Elston 1997). The pathogen rapidly spread to almost all of the oyster farming areas in Europe. However, once mass mortality outbreaks occurred in France, it became evident that populations of flat oysters were potentially less susceptible to the parasite. This characteristic of *O. edulis*-*B. ostreae* relationships was used in the view of selecting flat oysters for resistance to bonamiosis (Martin *et al.*, 1993). In selected oysters, the parasite may develop but the disease is absent or delayed. Here, meeting between the pathogen and its host species is not an absolute equivalent of *meeting with death*.

The case of *Perkinsus marinus* is also interesting from this point of view. This dinoflagellate-like organism is a severe pathogen of the eastern oyster, *Crassostrea virginica*, on the east coast of North and Central America, from Maine to Venezuela. *Perkinsus marinus* strains composition is distinct among the north east, south east and gulf coast regions (Bushek & Allen, 1996). Based on different genotyping methods it was demonstrated that most of the gulf isolates exhibit a genotype which is not observed on Atlantic coast and vice versa. Differences in virulence of *P. marinus* isolates

were established. More interesting is susceptibility of oysters with resistance roughly linked to the parental exposure. This example, as the previous one, shows that results from coexistence of a host and a pathogen should not be systematically interpreted in terms of disease.

A counter example is given by the paramyxean parasite *Marteilia maurini*. For years, a controversy was to know if a unique species of *Marteilia* was occurring in Europe, until ITS1 sequences assess the existence of two taxa with strong links to the host species (Le Roux *et al.*, 2001). In Europe, *Marteilia refringens* is a major pathogen of the flat oyster, *Ostrea edulis*, and listed by the OIE. Mussels, *Mytilus edulis* and *M. galloprovincialis*, are known to be infected by but not affected by *M. maurini*. Consequently, current opinion is to consider mussels as a species group, tolerant to *M. maurini* infection. However, naive populations of blue mussels, *Mytilus edulis*, originating from a zone free of *Marteilia* spp., experience mass mortality when transferred in an endemic zone, in which local mussel populations are still healthy, although infected (unpublished data). Here we see a tolerant species suffering mass mortality as a result of meeting with a pathogen.

Bearing these examples in mind, diseases cannot be reduced to the only presence/absence of pathogens. There is a complex interaction between host, pathogen and their environment to be considered as proposed by Sniezko. Further than that, in this conceptual model of disease, host and pathogen - similarly - should not be reduced to their only species affiliation. Later in this paper, complexity of interactions will be considered not only

at ecosystem level but also cellular and molecular levels.

Because of possible coexistence between molluscs and potential pathogen, as demonstrated above, we consider tolerance rather than resistance as a key word describing this relationship. Here comes the title of this paper "peace in the Earth to pathogens of good willingness"; But also the two afferent questions which are what means *peace?* and what means *good willingness?*

Accepting complexity of interactions

Given the question of host pathogen relationships, the importance of immunity can hardly be over emphasised. Immune function is one of the essential physiological systems at the interface of the organism and the environment, of which pathogens are part of. Very little is known about mollusc immunity. Molluscs immunity relies upon innate immune mechanisms to ward off pathogens, which is often seen as primitive. Given the long-standing existence of molluscs species and existence of molluscs populations which appear to be less susceptible to a given pathogen, they obviously possess an efficient defence system. Given this paradox and the factual evidence of tolerance as dominating host-pathogen relationships, the current battling vision of the immune system could be positively revisited.

When a pathogen invades a mollusc, multiple reactions occur, initiated by the pathogen in an attempt to survive and develop in the host and by the host in an attempt to eliminate or segregate the pathogen. A review of defence mechanisms of molluscs (Roch, 1999) shows that we know enough to realise how little we know. Phagocytic capacity of circulating cells referred to macrophages is a part

of a complex defence system aiming at neutralisation and elimination of foreign, non self, materials. In molluscs, circulating cells can also mount phagocytic, cytotoxic or inflammatory responses. More recently, molluscs were found to possess an innate, non adaptive immune system employing a large variety of circulating molecules which broaden the number of reactions the invertebrates can employ in response to microbiological invasion.

The knowledge we have is based on a relatively high number of studies and papers. Reviewing these papers, it appears that our approach of molluscs immunity has typically focused on a single effector or even a small group of effectors. Once we have taken these effectors off their interactive context, the knowledge that can be deduced is probably as relevant to understanding an organism's overall immune system as the study of a single species is to understanding an ecosystem. They are component parts but hardly the whole picture of the immune system. Exploring the immune system, again, complexity of interactions is to be considered. This complexity we wish to illustrate here through a couple of selected examples.

Investigating oyster immunology, at a molecular level, a cDNA was cloned and characterised as *Crassostrea gigas*-TIMP, the first tissue inhibitor of metalloproteinase identified in molluscs (Montagnani *et al.*, 2000). The isolated cDNA encodes a protein of 221 residues that has a domain organisation similar to that of vertebrates TIMPs. Analysing *Crassostrea gigas*-TIMP expression in adult oyster tissues by Northern blot and *in situ* hybridisation indicates that *Crassostrea gigas*-TIMP is only ex-

pressed in haemocytes which are considered as key components of defence mechanisms. It is well established that in vertebrates, TIMPs are secreted multifunctional proteins that play pivotal roles in the regulation of extra cellular matrix metabolism. They are involved in a flurry of physiological processes including embryonic development, connective tissue remodelling, wound healing, angiogenesis, and glandular morphogenesis. In our study, it was observed experimentally that *Crassostrea gigas*-TIMP mRNA was accumulated during shell damage and bacterial challenge. This pattern of expression suggests that *Crassostrea gigas*-TIMP may be a factor in wound healing and defence mechanisms. A similar involvement of TIMPs in defence mechanisms was described in the wax moth larva, *Galleria mellonella* (Wedde *et al.*, 1998).

This dual involvement of *Crassostrea gigas*-TIMPs is an obvious example of non linearity introduced in the system. Complexity of interactions at molecular levels is inherently non linear and thus cannot be reduced to linear models (Chapman, 2001). Today, one accepts that about 35 000 human genes can encode up to 500 000 proteins approximately, once considered splice variants phosphorylation, glycosylation and lipid conjugation. This means that a strong and deep dogma we have in mind "one gene, one protein, one function" is now extremely weakened if not to say dead. Linear models have contributed to our understanding of biological systems. However they do not cope with the non linear dynamics of complex interacting systems.

Going back to the host-pathogen relationships, our approach should not be cut from attempts of the pathogen to survive and de-

velop within its host. Sticking to the example of *Crassostrea gigas* may be interesting from this point of view. We conducted a preliminary study to investigate the implication and potential role of *Vibrio* strains in summer mortality of *C. gigas* juveniles (Le Roux *et al.*, 2002). Some of the isolated strains demonstrated to be pathogenic by fulfilling Koch's postulates. These strains, belonging to the group *V. splendidus* type II, were shown to produce variable patterns of proteinases, presumably metalloproteinases (MPs). These metalloproteinases are currently recognised as virulence factors of bacteria, playing a major role in invasion processes. This was particularly well established for *V. anguillarum* (Norqvist *et al.*, 1990).

It is probably a smart speculation to consider that *V. splendidus* MPs and *C. gigas* TIMPs may be constitutive of an intimate host-pathogen dialogue. Obviously, this is ahead from the trivial presence/reaction relation we are usually restricted in, given the lack of knowledge we have. However, we must recognise that deduced knowledge from this MP/TIMP system would be as relevant to understanding an ecosystem from study of a single species. At best, it could be one functional segment of a complex system. But we can hardly believe it would provide a relevant picture of the system itself.

Going further, non linearity, when demonstrated for TIMPs (cf. supra), could be envisioned for MPs or any gene support of virulence factor. An easy mistake would be to over-interpret our data : presence of a gene, known as support of virulence factor, and/or detection of its expression does not necessarily mean the given organism is virulent. Isn't

it a current mistake? This acceptance is probably not as benign as it may seem because it raises the question of virulence seen as one of the essential physiological systems at the interface of the pathogen and its environment e.g. the host itself! This was also the definition given above for immune functions of the host.

Conclusion

Mollusc farming has been affected by several major epizootics. Numerous mass mortality outbreaks are examples of pathogen transfers via transfers of live molluscs. In this prospect, it is of a central importance to establish effective programs to prevent transfers of infected stocks in disease free areas. However, in endemic zones, the manichean picture of pathogens is to be considered along with the existence of host populations which appear to be less susceptible to a given pathogen. The defence system of molluscs, although efficient given the long-standing existence of these species, could be illustrated by the evengelic sentence «peace in the Earth to pathogens of good willingness». Tolerance rather than resistance appears as a key word of this immune system. Investigating such a system shows that complexity of interactions is to be considered not only at ecosystem level but also at cellular and molecular levels. This complexity cannot be reduced to linear models that dominated biology over the past decades. This pleads for an ecological approach of immunology avoiding a mechanistic interpretation of host-pathogen relationships. More is waited from knowledge of molluscs defence system as an intimate prospect of host-pathogen relationships and as a way to reduce the impact of infectious diseases in endemic

zones. Molecular biology is to play a pivotal role in this approach of immunology directly connected to the tremendous motion of genomics and sister disciplines such as proteomics and metabolome.

References

- Berthe F., Boudry P., 1999. Pister les huîtres et leurs pathogènes. *Biofutur*, 195, 38-42.
- Berthe F., Burreson E., Hine M., 1999. Use of molecular tools for mollusc disease diagnosis. *Bull. Eur. Ass. Fish Pathol.*, 19(6), 277-278.
- Burreson, E.M., Stokes, N.A., Friedman, C.S., 2000. Increased virulence in an introduced pathogen: *Haplosporidium nelsoni* (MSX) in the eastern oyster *Crassostrea virginica*. *J. Aquat. Anim. Health* 12: 1-8.
- Bushek, D., Allen, S.K., 1996. Host-parasite interactions among broadly distributed populations of the eastern oyster *Crassostrea virginica* and the protozoan *Perkinsus marinus*. *Mar. Ecol. Prog. Ser.* 139: 127-141.
- Chapman, R., 2001. EcoGenomics - a consilience for comparative immunology? *Developmental and Comparative Immunology*, 25 : 549-551.
- Cigarria, J., and Elston, R., 1997. Independent introduction of *Bonamia ostreae*, a parasite of *Ostrea edulis*, to Spain. *Dis. aquat. Org.* 29, 157-158.
- Grizel H., 1996. Quelques exemples d'introduction de coquillages. *Scientific and Technical Review, Office International des Epizooties* 15(2) : 401-408.
- Le Roux F., G. Lorenzo, P. Peyret, C. Audemard, A. Figueras, C.P. Vivarès, M. Gouy and Berthe F.C.J., 2001. Molecular evidence for existence of two species of *Marteilia* in Europe. *Journal of Eukaryotic Microbiology*, 47(3), 288-293.
- Le Roux, F., Gay, M., Waechter, M., Poubalanne, S., Lambert, C., Chollet, B., Nicolas, J.-L. & Berthe F. (2002). Comparative analysis of *Vibrio splendidus* related strains isolated during *Crassostrea gigas* mortality outbreaks. *Aquatic Living Resources*. In press.
- Martin A.G., Gerard A., Cochennec N., Langlade A., 1993. Selecting flat oysters, *Ostrea edulis*, for survival against the parasite *Bonamia ostreae* : assessment of the resistance of a first selected generation. p 545-554. In : *Production, Environment and Quality*. Bordeaux Aquaculture 92. Barnabé and Kestemont (Eds). EAS Special Publication No. 18. Gent, Belgium.
- Mialhe E., Bachere E., Boulo V., Cadoret J.P., Rousseau C., Cedeno V., Saraiva E., Carrera L., Calderon J. & Colwell R.R., 1995. Future of biotechnology-based control of disease in marine invertebrates. *Molec. Mar. Biol. Biotechnol.*, 4 (4), 275-283.
- Montagnani C., Le Roux F., Berthe F., Escoubas J.M., 2001. Cg-TIMP, an inducible tissue inhibitor of metalloproteinase from the Pacific oyster *Crassostrea gigas* with potential role in wound healing and defense mechanisms. *Federation of European Biochemical Societies, FEBS Letters* 500 (2001) : 64-70.
- Norqvist, A., Norrman, B., Wolf-Watz, H., 1990. Identification and characterization of a zinc metalloprotease associated with invasion by the fish pathogen *Vibrio anguillarum*. *Infect. Immun.* 58 (11) : 3731-3736.
- Roch, P., 1999. Defense mechanisms and disease prevention in farmed marine invertebrates. *Aquaculture*, 172: 125-145.
- Wedde, M., Weise, C., Kopacek, P., Franke, P. and Vilcinskas, A., 1998. *Eur. J. Biochem*, 255 : 535-543.