Highlights from the integrated research project IMAQUANIM
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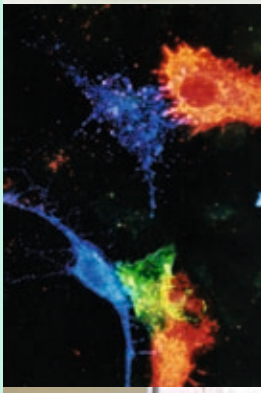
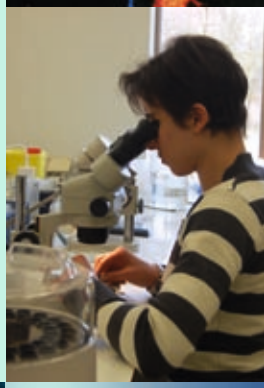
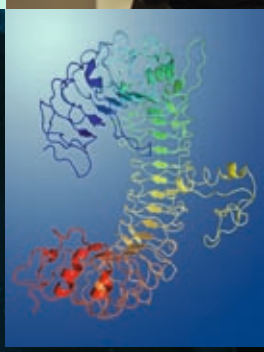


IMAQUANIM



Improved Immunity of Aquacultured Animals



Improved immunity of **aqu**acultured **anim**als. **IMAQUANIM**
Highlights from the integrated research project IMAQUANIM
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SIXTH FRAMEWORK
PROGRAMME

Introduction by Timothy J. Hall

Head of Unit Agriculture, forests, fisheries and aquaculture
DG RTD, European Commission

The European Union´s (EU) involvement in research goes back almost half a century. However, in the 1980s the EU took on a broader responsibility for stimulating and coordinating scientific research in its member states. To carry out this task, the EU set up a series of 'framework programmes' (FPs) which reflect the constantly changing nature of scientific and technological research and the Union's evolving priorities.

The first framework programme covered the period 1984–87. The current one, the seventh (FP7), runs from 2007 until 2013 with an overall budget that exceeds 50 billion Euros. Today, the Union possesses three key funding instruments to support research and innovation: the first is the aforementioned Research Framework Programme, along with the Cohesion policy which is funded through the Structural Funds and Cohesion Fund; and the Competitiveness and Innovation Framework Programme.

The European aquaculture sector is one of the many important socio-economic sectors covered by the framework programme. It has a high potential for innovation and technological development and is supported by a very active and productive research community (driven by public and industrial stakeholders). The European Union supports an integrated approach for aquaculture research aiming at filling the gaps in knowledge, building capacities and critical mass for research, supporting the industry and promoting international cooperation based on the principle of mutual interest and benefit. The EU actions in this field intend to maximise synergies between Member States and Community efforts, to improve the dialogue between the scientific community, industry, policy makers and relevant stakeholders, to stimulate public and private investment in research technological development and innovation (RTDI) and to promote knowledge transfer and innovation.

FPs are implemented through various types of projects or "funding schemes" which, among others, aim at stimulating collaborative research, coordination, networking, dissemination, popularization and improvement of the take up of RTD results by end users. In particular, "Collaborative projects" are focused research projects with clearly defined scientific and technological objectives and specific expected results (such as developing new knowledge or technology to improve European competitiveness). They are carried out by consortia made up of participants from different countries, and from industry and academia.

IMAQUANIM is a collaborative project (Integrated Project, 8 Million € EC contribution for five years, 22 participants from 9 European countries) funded under FP6, with the aim of developing a knowledge technology platform for improved immunity to infectious pathogens in the major fish and shellfish cultured species in Europe (Atlantic salmon, rainbow trout, sea

bream, European sea bass, carp and mussel). This should serve a longer term objective, which is the determination of optimal strategies for disease prophylaxis in aquacultured animals. The IMAQUANIM project has significantly increased our knowledge on fish and shellfish innate and fish adaptive immune systems, as well as, a better understanding of their evolution. In particular, through this large collaborative effort new immune genes have been identified and characterized in different species and their genetic variability has been studied. New methods, tools and assays (including antibodies, gene arrays etc) have been developed, to explore the functioning of immune molecules, to monitor immune responses, immune functions and host-pathogen interactions. Vaccination and infection responses have been monitored and new vaccine models for protective immunity have been developed. Furthermore, the ability of immunostimulants and herd immunity in mitigating disease outbreaks has also been considered. In addition to the long list of scientific achievements (more than 130 scientific articles published so far) the project has also contributed to the training of students and young researchers (29 PhD students) and most importantly has allowed them to become connected to a European network of fish and shellfish immunologists, which will be essential for their future careers.

On behalf of my colleagues working in DG RTD Agriculture, forests, fisheries and aquaculture research unit, I would like to convey to the IMAQUANIM consortium members our compliments for their commitment and the quality of their work, together with our appreciation for their contribution towards strengthening the aquaculture-related European Research Area.

Timothy J. Hall
Head of Unit Agriculture, forests, fisheries and aquaculture
DG RTD
European Commission

Content

Integration of knowledge is the key to healthy fish and shellfish

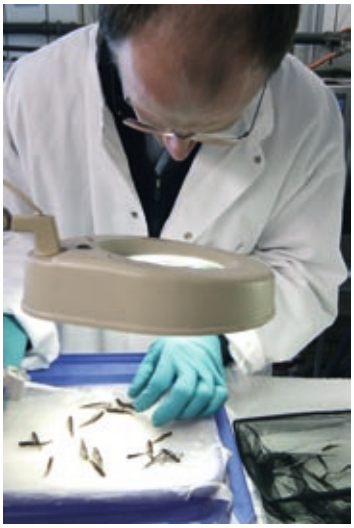
Aquaculture is becoming increasingly important in meeting the worldwide growing demands for healthy aquatic animal food products due to reduced wild fish stocks. Infectious diseases represent a major threat towards expansion of aquaculture production and the current use of antibiotics, drugs, and chemical disinfectants is compromising both consumer safety, as well as the environment. Implementation of a strategy based on prevention rather than cure is therefore crucial for a sustainable expansion of aquaculture production compatible with the environment.

In the late 1980ies development of vaccines against two devastating bacterial diseases in Atlantic salmon started in Norway and Scotland, and the vaccination programmes later implemented both increased production and reduced use of antibiotics, thus demonstrating the great potential of fish vaccination. During the next 20 years although further efforts were devoted to the development of vaccines to other fish diseases, progress was slow as knowledge of the fish immune system was poor and simple trial and error strategies did not prove effective.

Vaccination is based on the capacity of the immune system to recognize and eliminate foreign components such as bacteria and viruses from the body. Whereas the human immune system is relatively well characterised and many tools have been developed to monitor what is going on, *immunological research on fish and shellfish has long suffered from a lack of knowledge about functional details and methods to monitor the response to infections*. Knowledge of the immune system is crucial for developing efficient strategies for disease prevention, e.g. by vaccination but, here fish and shellfish clearly are behind humans.

The IMAQUANIM partners at the project kick off meeting in Wageningen, The Netherlands, April 2005.

In order to resolve the knowledge gap the *Integrated Research Project IMAQUANIM, funded by the European Commission Framework 6 programme, was established in 2005 and brought together 22 partners* including 17 universities and governmental research institutes, as well as five small and medium size enterprises (SMEs). These 22 partners have been working in a joint effort to establish a knowledge and technology platform for the development of vaccines and other tools to improve immunity to infectious diseases of Europe's major aquacultured species. This brochure should give you an impression of the many, scientific and technological advances made by the joint effort of the IMAQUANIM consortium. Infectious diseases will continue to represent a major threat towards aquaculture production, *but the outcomes of IMAQUANIM form a sound basis for development of efficient strategies for minimising disease outbreaks in the future.*



Improved perspectives in human and aquatic animal health

Dr. Niels Lorenzen from the Technical University of Denmark is the co-ordinator of IMAQUANIM, a large integrated project funded by the 6th Framework of the European Community. Here he comments on the aims and the outcomes of the project as well as the future plans.

Q: IMAQUANIM is the acronym for your project. It is quite difficult to pronounce but it triggers your memory once the tongue succeeds. What does it stand for?

A; Niels Lorenzen: IMAQUANIM stands for 'Improved immunity of aquacultured animals' and that is exactly what this large integrated project is about: establishing knowledge that can help us to improve the disease immunity of animals in European aquaculture by, for example, vaccination. We strongly believe that prevention always is better than cure. Simply think of successful eradication of polio and small pox thanks to vaccination programs in humans. Although vaccination is central to a successful protection against many diseases, we also explored other strategies for improving the immune status of aquatic animals including usage of enriched feed and breeding for improved resistance. We have been faced with the fact that, at least compared to humans, there is only limited information on the immune systems of these animals. So a key point of IMAQUANIM has been to develop numerous techniques and tools for a better characterisation of the immune mechanisms that are fundamental to reaching our goals. Furthermore, do not forget that these cold-blooded animals, unlike us humans, depend on the surrounding temperature. This adds an additional challenge to their immune defense.

Q: Why should the European community put so much research effort in studying aquacultured animals?

A; Niels Lorenzen: That is indeed a good question and there are several reasons for this. First, healthy fish and shellfish equals healthy food, prevents heart attacks and thus makes healthy people. In scientific terms: the fatty acids in fish products do not cause dangerous cholesterol levels to rise in your blood as the case is for fatty acids in meat from warm blooded animals, and should be part of any healthy diet. Secondly, and directly related of course, aquaculture is the fastest growing sector for animal food production in the world. Natural resources of shellfish are limited, so raising healthy fish and shellfish using the minimum amount of drugs, such as antibiotics or chemical disinfectants, is crucial for all of us. Third, but not necessarily least important, enhanced immunity against diseases sustains the welfare of the aquacultured animals themselves.

Q: When you refer to 'aquacultured animals', that terminology is a bit vague. What aquacultured animals are you referring to and why did you need 22 participants?

A; Niels Lorenzen: When referring to aquacultured animals, we not only talk about farming fish such as carp, rainbow trout, salmon, sea bass and seabream, but also shellfish e.g. mussels. As fish species like salmon and carp are as different as a mouse is from an elephant, it required a large consortium to bring together the expertise required to cover the most important animals in European Aquaculture. Furthermore, different fish are grown in different systems located in fresh- or marine waters and face different diseases. Last but not least and almost needless to say: mussels are completely different from fish and represent an intriguing bivalve model. For example, and very important

to our project, in contrast to fish, they do not have an immune system that specifically remembers the pathogen once it appears again. Nevertheless mussels rarely suffer from diseases and understanding their immune mechanisms can provide important information about how to combat infections in general.

Q: Not too long ago the human genome was sequenced and, if I am not mistaken, also fish genomes have recently been sequenced. How important has this type of genetic information been for a project like IMAQUANIM?

A; Niels Lorenzen: Indeed the genomes of three fish species used in genetic research have been sequenced and information on the genes that direct the immunity has been extremely important to us. As I already mentioned, we have worked with several fish species and also with shellfish. Although it helps to have information on one fish species, the genome sequences of the aquacultured fishes remain to be fully determined (annotated), and we still have to spend a substantial amount of time to correctly identify genes which show enough similarity to human or mouse genes for aquacultured fishes. Luckily we have been quite successful and for example, new techniques like microarrays will help us understand how the various animal species react to diseases or to vaccination. In addition, the scarcity of genome information on shellfish was overcome by IMAQUANIM that has provided the first complete information on active genes in mussels. Overall, this knowledge and these tools will be instrumental for the development of efficient disease prevention strategies for fish and shellfish.

Q: So what about the future? Is it now possible to vaccinate both, fish and shellfish against all diseases?

A; Niels Lorenzen: *Smiling*. I wish it was true. But simply consider the effort that has gone into studying HIV and still no vaccine is available. How can we then expect to have solved all problems for fish and shellfish? The number of cultured fish species and related disease problems is high and ever increasing. We have gained so much on the knowledge of the immune system of fish that we now start to comprehend that it is as difficult as it is for humans to develop effective vaccines. Having said this, we are getting closer in determining what essential elements are needed for a good fish vaccine. As I mentioned earlier, mussels lack the immune memory so crucial for vaccination. Nevertheless, although somewhat simplified, they certainly do have an immune system special in many ways. This knowledge will also help us understand the innate defences of vertebrate animals such as fish. We see a great future for exploiting the so called anti-microbial peptides and their variability for making bivalve populations more resistant to diseases.

IMAQUANIM coordinator Niels Lorenzen: Healthy fish is a prerequisite for healthy food.

Atlantic salmon is one of the major fish species in aquaculture in Europe.

Aquaculture – present and future challenges

There is no discussion: fish and shellfish are healthy food and the consumption of these tasty fish food products is increasing. However, natural stocks of fish, crustaceans and bivalves cannot sustain the ever increasing demands. The solution, therefore, has to come from an expansion of aquaculture. Here, Dr. Kurt Buchmann from the University of Copenhagen, Denmark explains some of Europe's challenges, both solved and unsolved by IMAQUANIM, for a wide variety of aquaculture systems.

Tuna fish is highly appreciated by many consumers, but most of the supplies still depend on the decreasing wild stocks.

Due to its robust nature carp is one of the preferred farmed fish species in central Europe.

In Northern Europe and in mountain regions the cold climate provides optimal conditions for rainbow trout culture.

Ever increasing demands

Aquatic food products are tasty, of high nutritional value, and contain many elements that promote human health. These are the reasons for an annual increase in the intake of aquatic products all over the world which, at present, is some 17 kg per person per year. Natural stocks of fish, crustaceans and bivalves, however, do not have the capacity to cover the ever increasing demand for fish food set by a world population which has exceeded 6.6 billion people and which may reach more than 9 billions by the year 2050. Capture fisheries worldwide amounts to 92 million tonnes; a huge amount of aquatic food products. However, despite an increasing fishing effort this catch cannot be further increased due to over-exploited fish stocks. *The only solution to the problem, it seems, is the expansion of aquaculture.*

Growth of aquaculture production

In contrast to capture fisheries the world aquaculture production, which at present amounts to some 52 million tonnes per year, increases 8.7 % per year. As a result, the Food and Agricultural Organisation (FAO) expects the world fish market to be dominated by aquaculture products within a few years only. The continued growth of the aquaculture sector, however, is faced with among others, animal health problems. The expected expansion of aquaculture requires a well-thought-through action plan based on a philosophy of sustainability aided by environmental-friendly technology. From a health control perspective the future goal must be to control or reduce the use of antibiotics, anthelminthics, disinfectants and other chemical substances. *IMAQUANIM has paved the way to achieve this goal by pin-pointing parts of the immune system that can be stimulated to provide fish and shellfish with the urgently-needed protection against diseases caused by bacteria, viruses and parasites.*

Unity in variety

Aquaculture production systems deal with a multitude of different species, ranging from fish species as different as salmon, eel, sea bass, seabream, trout, carp and catfish to shellfish including mussels and oysters. Further, the technologies applied to culture all these different species show an equal range of variety. *The challenge is to implement the results of IMAQUANIM in the diversity of existing as well as future aquaculture production systems.* At present, the systems in practice range from ancient low-technology pond systems with a high outlet of waste material to modern high-technology and environment-friendly recirculation systems.

One-by-one

The question to solve is how to implement our improved knowledge of immune defense mechanisms of fish and shellfish into practical immuno-prophylactic measures in aquaculture systems as diverse as: 1) Freshwater fish ponds fed by natural water contaminated with pathogens, 2) Recirculated

freshwater farms, 3) Land-based mariculture systems, 4) Recirculated seawater farms, 5) In-shore mariculture systems, 6) Off-shore mariculture net-pens, 7) Intensive line production of mussels and 8) Oyster beds. The implications of these diverse aquaculture systems for animal health are listed below.

1. Classical pond-culture systems are fed by natural water sources (rivers, lakes), subjecting fish to a variety of natural fluctuations. Of these fluctuations temperature is of particular relevance because of the cold-blooded nature of fish and shellfish. Further, natural water sources are largely uncontrolled and can introduce a range of pathogens from wild fauna.
2. Recirculated freshwater fish-farms are based on re-use and cleaning of water after mechanical and biological filtering and sustain a more stable temperature. Still, also in these systems the infection pressure may be high due to recirculation of pathogens and optimal temperature regimes for pathogens.
3. Raceways or fish tanks ashore are fed by sea water pumped from the sea into the landbased system. The pathogens may freely propagate dependent on stocking density and flow-through rate of the seawater.
4. Recirculated seawater fish-farms use water that, although cleaned by mechanical and biological filtering may develop high numbers of recirculating pathogens.
5. In-shore mariculture systems are known to be exposed to several pathogens which may propagate intensely due to the high stocking densities typical of these aquaculture systems. Furthermore, high temperatures during summer periods may support excessive multiplication of pathogenic bacteria.
6. Off-shore mariculture production units may have a relatively low infection pressure due to a high rate of water exchange and high water depth below the cages. On the other hand, migrating wild stocks might transmit infectious diseases to and from such units.
7. The modern production of bivalves is based on an intensification of production methods. The location of animals in the upper water-column may represent both challenges and assets. Although confronted with many pathogens, the oxygen conditions are better which may improve their immune status.
8. Oyster production systems suffer from a high infection pressure associated with a high population density in shallow water.

Bivalve culturing preferably takes place in calm costal zones not exposed to open sea.

To optimise the rearing and health conditions, fish farmers have started to grow younger fish stages in enclosed recirculated systems.

The future challenge

True for all of the above-described aquaculture systems, a combination of improved management, improved breeding status (improved genetics), improved nutrition and last-but-not-least improved immuno-prophylactic strategies should lead to the development of a healthy aquaculture of the 21st century. IMAQUANIM has explored the biological background for effective immuno-prophylaxis, studying a wide range of fish and shellfish species in a variety of aquaculture systems. The precise description of genes and proteins important for protective immune responses has lead to the development of invaluable knowledge and tools for future aquaculture research world-wide. The results will fuel the development of more sustainable aquaculture systems. Implementation at fish-farm level will benefit European aquaculture. *Independently of the fish or shellfish species cultured and independent of the system applied to culture these animals, immuno-stimulation and vaccination will help sustain the task of aquaculture to feed the ever-growing human population.*

Oyster is an appreciate dish in many countries, but infectious diseases often cause serious problems.

The immune system

Bacteria and viruses make you ill by finding a way into your body through physical contact. Nobody likes being sick. Luckily, we can live healthy and longer lives by being vaccinated. Here, Dr. Maria Forlenza from Wageningen University, The Netherlands explains the biological background to the successful vaccination of millions of people and husbandry animals including fish every year.

Special cells of the immune system can recognise and remove foreign agents like viruses and bacteria. This important 'cleaning' process is called the phagocytosis or 'eating' process.

1. Recognition of pathogen and phagocytosis

2. Secretion of free-radicals and pathogen digestion

3. Release of pathogen components

All of us have developed recognition mechanisms to discriminate 'non-self' (e.g. parasites, bacteria and viruses) from 'self' and protective mechanisms to defend ourselves from pathogens. This defence is called the 'immune system'. The complexity of the immune system increases with the complexity of the organism (we like to think that humans are the most complex organisms in the world). For example, unicellular organisms (e.g. baker's yeast) rely exclusively on the production and secretion of (numerous) molecules with anti-microbial activity that can directly kill disease-causing micro-organisms. Multicellular organisms (fish, mammals, birds etc.) have a more sophisticated defence mechanism where specialised cells have the ability to recognise, phagocytose (eat), and kill such pathogens.

Fact box: Pathogens, viruses, bacteria, and parasites
Pathogens *are viruses, bacteria and parasites that cause disease in animals and man.* ***Viruses*** *cause disease by growing within and destroying the cells of the infected animal. In contrast, most* ***bacteria*** *grow outside the animal cells and can cause disease by being toxic.* ***Parasites*** *are small animals themselves specialised for living inside other animals and often disturbing tissue function.*

Innate immunity

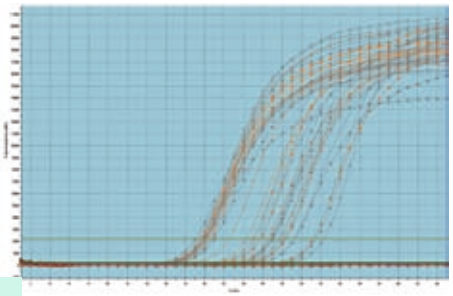
In general, the immune system can be divided in two branches: the innate and the adaptive immune system. The innate immune system is the first line of defence, since it is the first one to be activated upon entry of a pathogen. *Phagocytic cells and cytotoxic cells are part of the innate immune system. Phagocytic cells have the useful ability to neutralise pathogens by simply 'eating' them.*

Innate immune cells: these cells send an alarm signal to the adaptive immune system that alerts of the presence of a dangerous pathogen. What the innate immune system cannot do is remember having seen this pathogen. Once a phagocytic cell has done its job it dies, leaving the innate immune system without any 'memory' of the pathogen.

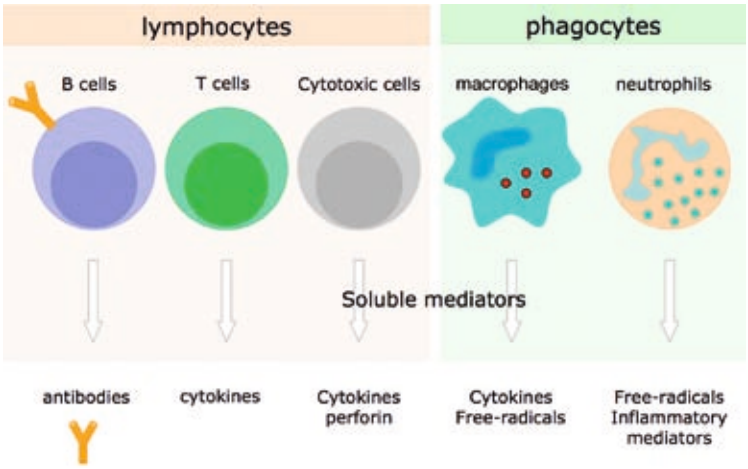
Adaptive immunity

Highly specialised cells called lymphocytes (B or T lymphocytes) belong to the adaptive immune system. Each individual cell possesses unique B or T cell receptors which are able to recognise different pathogens. Upon recognition these cells begin to multiply and the B cells then produce proteins called antibodies.

The activity of immune genes can be measured by a technique called PCR in which individual genes are monitored one by one.



Antibodies are soluble proteins circulating in the blood that can directly neutralise a pathogen but can also help the phagocytic cells of the innate immune system to eat the pathogens more efficiently. T cells in their turn, of which we also have many, produce signalling molecules called cytokines, specifically designed to help B cells to multiply and produce even more antibodies. Some T cells known as cytotoxic T cells are able to recognise and kill virus-infected cells.



White blood cells or leucocytes represent an essential element in our immune defence. The different cell types are specialized to have different functions such as making antibodies, eating or killing microbes.

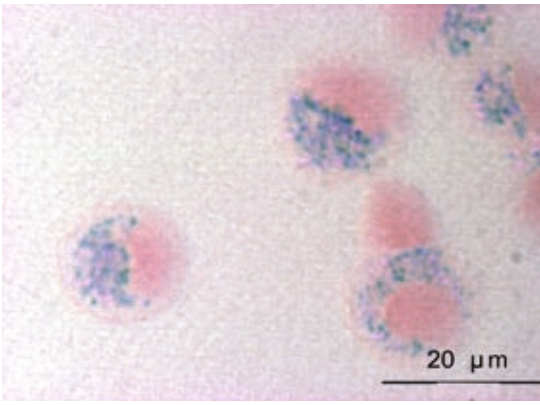
The immunological basis of vaccination

The adaptive immune system differs from the innate immune system in that it can remember having seen a pathogen. That is possible due to the formation of memory B cells and memory T cells. When B and T cells begin to multiply, a fraction of them are long lived, remaining present in case the same pathogen enters the body again. This renders the adaptive immune system highly effective because memory cells react faster and stronger upon second activation by the same pathogen.

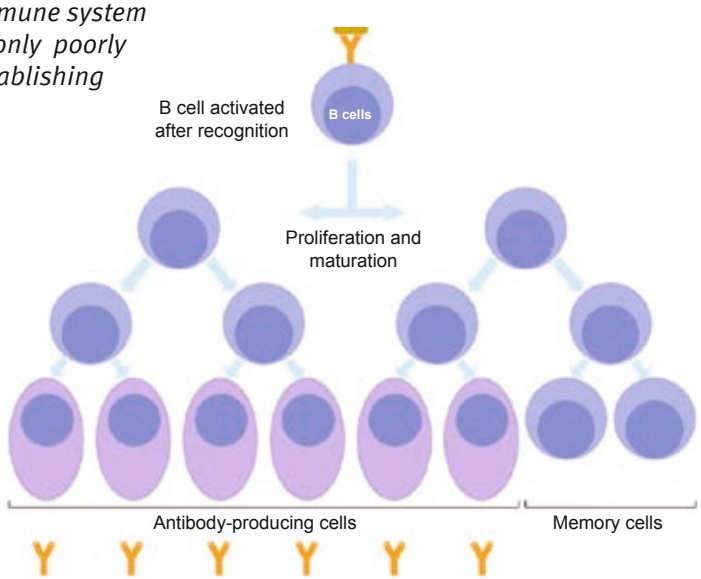
Specificity and memory are essential for successful vaccination. When we get vaccinated, the pathogen is often provided in a safe, killed form e.g. inactivated influenza virus or tetanus toxoid. In this way we do no more than trigger our adaptive immune system to produce antibodies, specifically directed against that particular pathogen and at the same time induce the formation of memory cells. If ever we then encounter the real, live pathogen we will be protected against the disease: i.e. we are 'immune'.

Immunostimulation

Invertebrates, animals with no spine and without a jaw (e.g. insects, worms, molluscs and mussels), but also plants, rely completely on the innate immune system to protect themselves from pathogens. Vertebrates, animals with a backbone and with jaws (e.g. humans, birds, amphibians, reptiles but also fish), possess both, an innate and an adaptive immune system. Mussels have a short memory, since they do not have an adaptive immune system. But before the IMAQUANIM project little was known about their ability to handle infectious micro-organisms. New findings now reveal that mussels posses a high capacity to generate so called anti-microbial peptides which may be useful to combat diseases. Fish were the first animals to develop the adaptive immune system 600 million years ago. *Despite being ancient, the fish immune system still has high complexity and many mechanisms are only poorly understood. This was one of the main reasons for establishing IMAQUANIM.*



Purified white blood cells (neutrophilic granulocytes) from a fish. In blue are the numerous granules present in the cytoplasm of these cells which contain enzymes that can remove intruding agents.



When white blood cells like B- and T-cells meet a foreign agent (like virus, bacteria, parasites) in the body, they divide to produce more cells ready to fight the infection as well as to produce long-lived memory cells that makes the body immune to re-infections.

Towards the perfect vaccine

We vaccinate our children at a young age to protect them from several nasty diseases, for example the pandemic flu. We would like to do the same for farmed fish if possible, without the painful pinprick. Here, Dr. Bjørn Brudeseth from PHARMAQ, Norway explains why fish vaccines should be cheap, easy to administer and effective.

Although vaccination by injection requires handling of the fish, it is still the most efficient way to get a protective immune response.

Classical vaccination is based on injection of biological material (= antigen) which activates specific immune memory cells that at a later time point remember the antigen and thereby can eliminate a pathogen before it causes disease. *Disease prevention by vaccination is for ethical and economical reasons more appropriate than treatment of disease not only for humans, but also for fish.*

Vaccines may consist of weakened but live pathogens. These may cause clinical symptoms, but only on a limited scale, triggering the immune system to build a protective response. However, the risk of re-gaining virulence limits the use of such vaccines, especially for fish in their aquatic environment. As a result there is a growing need for vaccines based on killed or inactivated pathogens.

Fact box: Vaccination
*The word **'vaccination'** is derived from 'vacca', the latin name for cow. Back in the 1790's the English doctor Edward Jenner discovered that he could protect people against small pox by exposing them to cowpox – a related infection not causing disease, but inducing immunity. 'Vaccination' thus refers to its origins from the cow.*

With a little help

Inactivated pathogens are unable to replicate, unable to revert to virulence and therefore do not cause disease. Unfortunately, these antigens show little or no immunogenicity. Therefore, most inactivated vaccines contain adjuvants. An immunological adjuvant (the word 'adjuvant' comes from the Latin word adjuvare, meaning to help or aid) is defined as any substance that acts to accelerate, prolong or enhance antigen-specific immune responses.

An efficient 'black box'

Often adjuvants are effective in inducing protective antibodies against micro-organisms living outside the cells of the animal, but they are not always effective in controlling infections where the microorganism exist inside host cells. Strangely enough, we know little about how the immune system responds to vaccine adjuvant. In fact, adjuvants have been referred to as 'the immunologist's dirty little secret'. It may be clear that identifying the factors that determine the induction, magnitude, persistence and characteristics of immune responses will significantly contribute to the rational design of fish vaccines.

Manual vaccination under field conditions.

Inducing memory

The immunogenic components of most registered bacterial vaccines for fish consist of whole inactivated bacteria. Although highly efficient, there is a clear trend to move away from inactivated whole microbes to highly-purified antigens. These subunit vaccines can be more specifically targeted by the immune system than whole cells. However, highly purified antigens cannot mimic the whole immune process typical of an infection. Vaccines, like

pathogens, must induce innate and adaptive immune responses in order to mediate good protection. While innate responses are not specifically directed against a single pathogen, and are usually only transiently protective, their components are indispensable for an effective adaptive immune response. An efficient adjuvant should thus provide additional signals for the immune system to trigger every branch of the immune system.

Fish vaccines

The introduction of highly efficient oil-based vaccines against bacterial diseases of cultured fish has been a key facilitator for reaching the present high levels of production in aquaculture. Fish vaccines for salmonids usually contain several different antigens (i.e. different inactivated bacteria and viruses), resulting in lifelong protection against various diseases by a single injection. Unfortunately, the oil based vaccines also causes side-effects, although this has been significantly reduced compared to the first generation emulsion vaccines. In IMAQUANIM, the commercial vaccine partner supplied both licensed and experimental vaccines to the research labs doing vaccination and infection trials. *Protective immunity was demonstrated also for some of the experimental vaccines, thereby establishing ground knowledge for development of new vaccines.*

Vaccines against viral diseases of fish have proven difficult to develop. One important reason for the low efficacy of experimental antiviral vaccines for fish may be the lack of adequate adjuvants. Due to the intracellular replication of viruses, inactivated viruses or their components are ineffective in terms of protective immune responses if traditional adjuvants such as oil are used. However, recently *highly efficient experimental DNA-vaccines against certain serious viral diseases in salmonids have been developed and further optimised in the IMAQUANIM project, and need only to be clinically tested before licensing* (see also “Naked DNA for vaccination”, page 14).

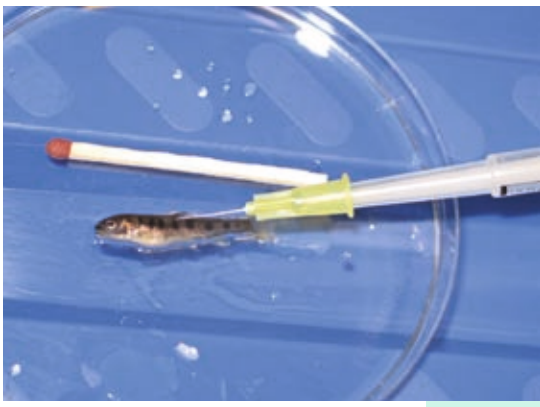
In addition, experimental vaccines against pathogenic bacteria, where at present no commercial vaccines exist, were tested in the IMAQUANIM project in the relevant fish species. Also commercial vaccines from PHARMAQ have been included in some of the studies in IMAQUANIM.

A bath would be preferable

There are several different ways of vaccine administration to fish; oral administration through feed, bath treatment within fish tanks, immersion treatment and injection with use of netpen collection. In general, both efficacy and labour increase stepwise from oral to injection administration. It remains a major challenge to the vaccine industry to develop cheap, efficient vaccines which induce lifelong protection by a route of administration that employs minimal labour per fish.

Mobile machines for mass vaccination at fish farms facilitates the work.

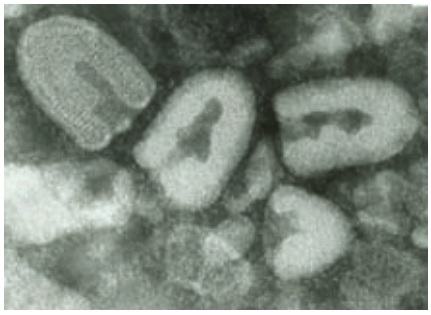
Vaccination of fish early in life is often desirable, but here injection is impractical. Alternatives are vaccination by bath or via the feed, but more research is needed to determine how good protection can be obtained by these methods.



Naked DNA for vaccination

Dr. Niels Lorenzen from the Technical University of Denmark, explains the pros and cons of DNA vaccination, a method proven highly successful for protection of farmed fish, such as rainbow trout and salmon against deadly viruses, but still under debate within the European Community.

During springtime, carp often get infected by a virus known as SVC virus.



Spring Viraemia of Carp (SVC) particles are very small and can only be seen by electron microscopy.

Vaccination experiments in IMAQUANIM in Dr. T. Vesely's lab in Brno, Czech Republic, demonstrated the protective effect of a DNA vaccine against SVC virus.

Vaccination aims at stimulation of immune memory to infectious micro-organisms. Traditional vaccines against viruses are based on killed or weakened live virus and thus contain the same elements or antigens as the virus, but without ability to cause disease. Well-known vaccines for viral diseases in humans are those against hepatitis, rabies, polio and smallpox. For fish, similar well-known vaccines exist against diseases called vibriosis and enteric red mouth, both caused by bacteria. But as in humans where we did not (yet) manage to develop a good vaccine against HIV, we lack effective vaccines against many viral diseases in fish. New strategies such as DNA vaccination may represent an alternative.

Fact box: How to make vaccines

In the laboratory, viruses require cultured animal cells to grow, while bacteria can grow by themselves in a simple substrate. Making large amount of killed bacteria for a vaccine is therefore much simpler and cheaper than for viruses. Parasites are complex and often impossible to grow in the laboratory. No vaccines exist against fish parasites.

What is a DNA vaccine?

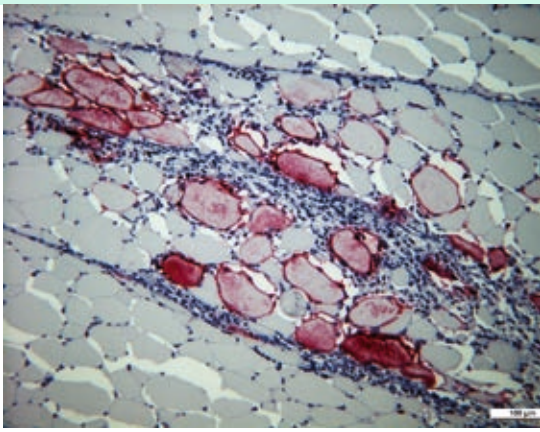
A DNA vaccine is based on a piece of genetic material from the pathogen which upon injection into muscle drives some of the muscle cells to produce the protein it encodes i.e. the protective vaccine is actually produced in the fish. The advantages are several: *high safety, easy production and handling, high stability and, very efficient activation of the immune system*. The backbone in a DNA vaccine is a small circular DNA molecule called a plasmid. Plasmids are known from bacteria such as *E. coli* in which they can be produced in high amounts. By gene cloning, a plasmid can be designed to carry a virus gene which will be translated into protein when the plasmid is injected into the muscle of an animal (see p. 15, top figure).

Why does it work so well?

There is a certain group of dangerous viral diseases in fish for which DNA vaccination works extremely well. It may appear strange that injection of a small amount of plasmid DNA can trigger such a powerful protection, but analysis during the IMAQUANIM project suggests the explanation is that *the vaccine triggers an immune response very similar to that induced by a natural infection* but, importantly, without involvement of the virus itself. New data obtained in IMAQUANIM suggest that vaccine efficacy can be even further improved by including immune signalling molecules.

DNA vaccines for fish are almost ready to be applied, but...

In IMAQUANIM we have been exploiting DNA vaccines against viral diseases in both trout and carp and the results are very promising. The vaccines do not only induce protective immunity, but also work across a range of temperatures. Basically, commercial use of these vaccines only awaits a vaccine company to take them to the market stage and get them licensed. This has already happened in Canada. But *in Europe we are more cautious*



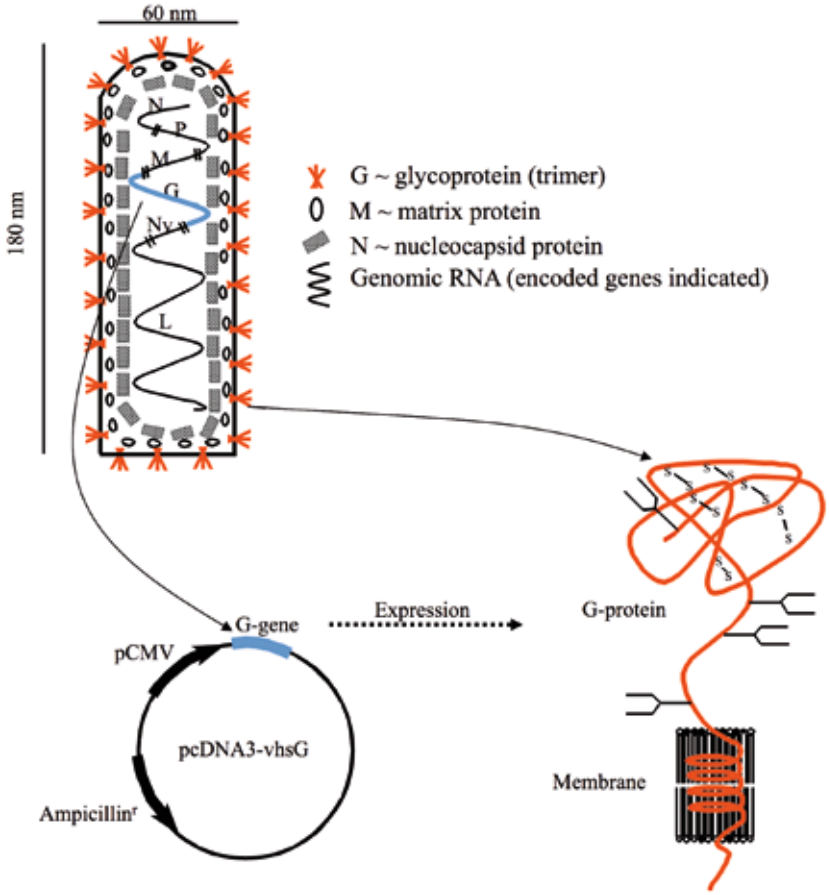
How does DNA vaccination work? Muscle cells producing vaccine protein (red staining) after DNA vaccination activate immune cells (blue staining) to establish protection.

about introducing new concepts in food production, particularly when this involves gene technology like DNA vaccines. This indeed is a pity, as all current data demonstrate that DNA vaccines are safer than traditional vaccines. One future challenge is thus to inform consumers and politicians properly about the advantages of the new technology.

How to take full practical advantage of this new technology?

It is very important that scientists dissect the molecular mechanisms behind the efficacy of the DNA vaccines to be able to expand the concept to other important fish diseases – and possibly to human diseases. So far, DNA vaccines have proven much more efficient in fish than in humans, so here a *basic understanding of how the fish vaccines work might even provide important information to clinical research in humans*. In terms of practical application in aquaculture and the use of DNA vaccines in very small fish, more research into how DNA vaccines can be delivered is required as vaccination by injection is impractical. Very recent data suggest that oral delivery could be a realistic option for the future.

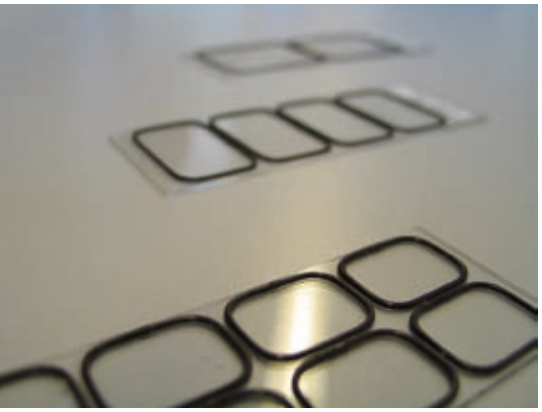
Vaccination by injection is the most efficient vaccination method, but it is not applicable for protection of farmed small fish. Development of alternative efficient delivery methods is therefore needed.



A DNA vaccine is based on a circular DNA element called a plasmid in which a gene from the virus is inserted. Once the plasmid is injected into the fish muscle, some muscle cells will take it up and start to produce virus protein. The DNA vaccine disapears after a few months and does not integrate in the fish host DNA. The vaccinated fish is therefore not a genetically modified organism, i.e. not GMO.

Powerful DNA chips for health profiling

DNA chips, also known as genearrays or microarrays, are small glass plates that carry tens of thousands of genes. They are used in modern life science laboratories, for example, to detect genes responsible for controlling the outcome of diseases. Here, Dr. Sam Martin from the Scottish Fish Immunology Research Centre, University of Aberdeen, Scotland, explains how IMAQUANIM has successfully developed DNA chips to analyse the immune responses of fish and shellfish.



DNA chips: By robot technology, thousands of genes can be spotted on glass slides in so called gene arrays.

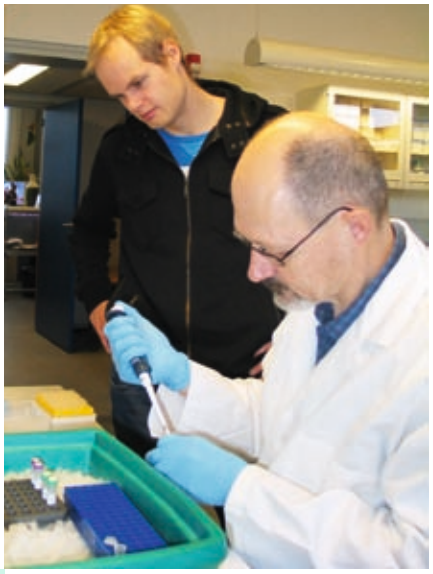
To gain a comprehensive view of the genes that control the immune response, new tools are being continually developed. In the last 5 years there has been an unprecedented increase in the numbers of gene sequences available for Atlantic salmon and rainbow trout, as well as several other species of farmed aquatic animals. At present there are markers for more than 30,000 different salmon and trout genes. Many of these genes have direct functions in the immune system, but at present the gene sets available for farmed fish are poorly characterised. *Within IMAQUANIM many new immune genes involved in the defence against viruses and bacteria have been identified.*

Glass slides that carry thousands of genes

DNA chips are extremely powerful tools that allow the activity of many genes to be examined in parallel. This is possible because 1000's or 10,000's of genes are printed onto a single glass slide (normally a microscope sized slide). The genes are printed in an ordered grid and information, which includes the gene sequence, its identity and location on the slide, is recorded on a computer file. Within the IMAQUANIM project we have developed and enhanced DNA chips for salmon, rainbow trout and mussels, and used these to monitor the genes involved in immune response. Current DNA chips have up to 4 x 44,000 genes printed on a single slide.

Fact box: Gene activity is measured as amount of RNA
When a gene is active, copies in the form of messenger RNA (mRNA) are produced and translated into proteins, the main molecules in our bodies. Levels of gene activity can therefore be measured as the levels of mRNA.

In IMAQUANIM, DNA chips designed to monitor the immune response in trout and mussel have been developed.



Results glow in the dark

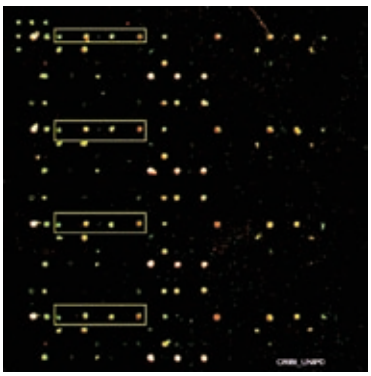
Fluorescent dyes help visualise the results of DNA chips. For example, to examine how the activity of genes is altered following infection or vaccination, RNA is extracted from both an 'experimental' and 'control' group. The two samples are labelled with two different fluorescent dyes (e.g. red and green). In order to determine which genes have changed in activity, the two labelled samples are mixed and added to the DNA chip. Under specific conditions, the sample genes will bind to their corresponding gene spotted on the slide.

The slide is then scanned at high resolution and analysed to determine whether the activity of any of the genes is altered. If the activity of a gene is increased in the experimental group, there will be more red- labelled RNA compared to the green control and the colour of the spot will be dominantly red. Using statistical analysis several 100's of genes can be found regulated (either up or down) and the key biological processes that change within the cell or tissue in response to infection or vaccination can be determined. Thus, DNA chips establish activity patterns of large groups of genes that alter in parallel and help to interpret complex reactions such as the immune response to infection or to vaccination.

IMAQUANIM has produced DNA chips for monitoring immune response in fish and mussels

During the IMAQUANIM project we have identified a large number of genes involved in the immune response in trout and salmon, as well as in mussels. Much new information about these genes, and in some cases also the proteins they encode, has been obtained. Especially for mussels, only a limited number of genes had been previously characterised, but thanks to the project work, more than 7,000 genes have now been identified. For rainbow trout, a DNA chip with more than 43,000 gene fragments has been extended with genes important for the response to infections.

Prototype DNA chips focusing on immune genes for fish and mussels, have been developed and found to work well for analysing the immune responses to important infections. It is expected that these chips will become commercially available after the end of the project, and be a valuable tool for future monitoring of immune status.

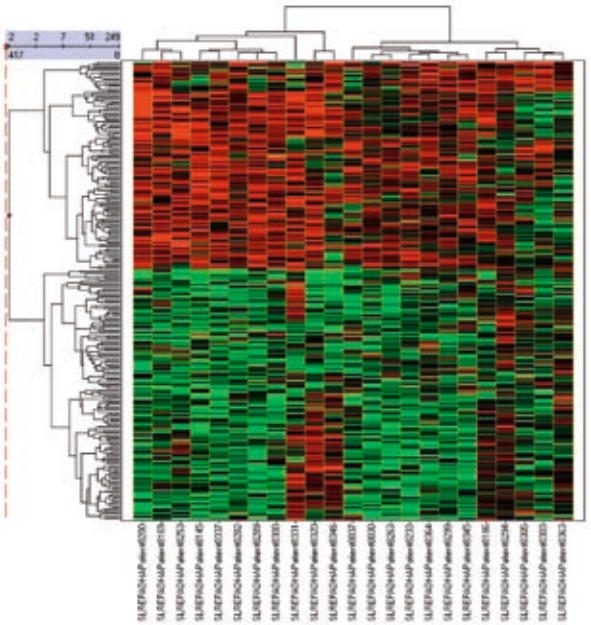


The fluorescent dyes visualise the results of DNA chips and hereby the gene activity in the sample.

Still some way to go before gene activities are fully understood

For all animals, the immune response is highly complex and varies under influence of specific infections. The DNA chips developed during IMAQUANIM are expected to be very useful in describing gene activity profiles in fish and mussel, but *there is still a gap in knowledge to fully understand the functional implications of what happens at the gene level.* Scientists thus still have challenges ahead, in terms of illuminating the black box between gene activity and animal health.

By implementing dedicated software programmes, the readout from DNA chips can be ordered according to individual genes and their activity level expressed as colour and intensity.



Fishing for genes in shellfish

Dr. Paola Venier from the University of Padova, Italy explains how the meticulous characterisation of thousands of mussel DNA pieces has allowed the establishment of a so called 'transcriptome' which has revealed numerous novel characteristics of the immune system of mussels.

Seeding of ropes and nets for mussel culturing is often based on spawning of wild populations, but considerable maintenance work is required.

Nearly 810,000 tons of shellfish produced per year along the Atlantic and Mediterranean coasts testify the importance of mussels, oysters and clams for the European economy. In the water column or sediments, bivalves are exposed to a variety of micro-organisms and other environmental factors. In critical periods, these factors can overwhelm the immune defences of the shellfish, possibly leading to mass mortalities, with unacceptable economic losses and significant weakening, and shortening, of the natural stocks. Interestingly, a specific group of mussels appear to be notably resilient to most of these external factors. *Results from the IMAQUANIM project contribute to addressing the burning question "why are mussels so resilient" and to the application of these important findings.*

The secret of mussels

Mussels can rapidly close their shell and wait for hours before restarting contact with their water environment. Thereby they are resistant, for example, to tidal-related changes and save valuable energy for growth. From the perspective of the mussel, dealing with exposure to toxic compounds or potential infections may also cost energy, which otherwise would have been used for growth and reproduction. A frequent observed example is when infection of mussels lead to reduced attachment strength and a slowing down of physiological processes resulting in irreversible weakening and the onset of mortality.

At the beginning there was ...

Initially the IMAQUANIM project faced a fragmented dataset on the basic mechanisms of bivalve immunity. Development of a new knowledge platform using up-to-date biotechnological approaches was therefore one of the main objectives of the project. With the aim of improving European shellfish production and quality, IMAQUANIM brought together a number of European institutions with expertise on bivalve diseases, mussel physiology and innate immune reactions, DNA sequencing and gene activity analysis.

Despite their simple body organisation, mussels have developed an apparently efficient immune defence.

Fact box: Transcriptome and anti-microbial peptides
*The **transcriptome** is the set of RNA molecules present at any given time and reflects the genes that are active. The profile varies with external environmental conditions e.g. temperature or infection. **Innate responses** are immune mechanisms developed early during evolution (see also section "The immune system", page 10). **Anti-microbial peptides (AMPs)** are small proteins that are able to kill bacteria and viruses.*

MytiBase: an interactive database of bivalve genes

Groups of mussels were treated with different bacteria and various tissues from each treated or untreated 'control' animal was then collected. Subsequently, libraries of the active gene profiles were generated thereby enabling identification of differences between the profiles of treated and untreated groups. Based on the comprehensive experimental material, it was possible to obtain and characterise more than 18,500 DNA pieces

from mussels. To organise and structure this remarkable amount of data, *a new interactive database called MytiBase was established and made freely available to the scientific community (http://mussel.cribi.unipd.it).*

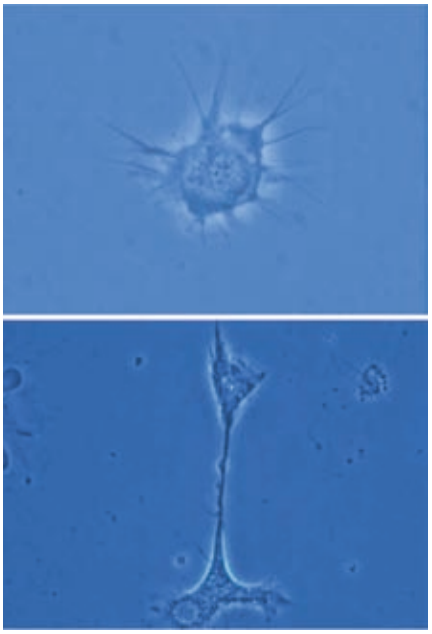
The large amount of data in the database emphasises the great variety of functions and molecular variation between mussels, as well as their ability to handle different environmental changes.

Gene activation profiles

The production of a DNA chip for monitoring the immune response in mussels is an important outcome of the IMAQUANIM project (see also section "Powerful DNA chips for health profiling", page 16). This has enabled the analysis of mussels from different geographical locations, as well as exposed to various different stresses, diseases or other environmental conditions. The long-term perspective of these analyses is identification of factors that influences the health status of mussels, and subsequent optimisation of growth site location, as well as selection of the genetically superior animals.

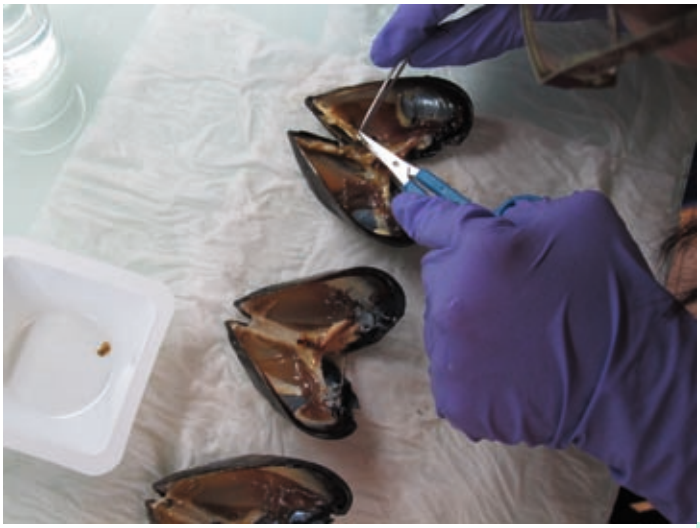
The importance of gene discovery during IMAQUANIM

The large set of independent gene transcripts forming Mytibase emphasises the great variety of cellular functions and molecular variation for (immune stimulated) mussels. One of the most interesting findings was that 'white blood cells' (haemocytes) of mussels are highly specialised when activated by bacterial or viral infections. As much as 40% of the whole haemocyte gene activity profile represents so called anti-microbial peptides (AMPs) (see also section "Anti-microbial peptides: ancient invention with modern applications", page 20). Through the IMAQUANIM project we have identified much higher variation of AMPs in mussels than previously described which can probably explain why mussels, who are considered to have a simple immune system, nevertheless are able to overcome very diverse environmental influences. *An interesting future application of AMPs is their potential use as antibiotics in husbandry animals and humans.*



IMAQUANIM research suggests that the usual good health of mussel populations is due to a powerful immune response based on small variable proteins called anti-microbial peptides (AMPs) produced by cells called haemocytes in the mussel body fluids.

Gene activity profiles as well identification of new mussel genes are based on material collected from mussels treated differently or originating from various geographic regions.

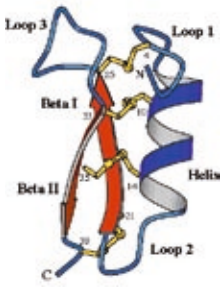


Anti-microbial peptides: an ancient invention with modern applications

Here, Dr. Philippe Roch from the University of Montpellier, France, explains that mussels have an extraordinary capacity to fight invasion with micro-organisms, such as bacteria, by using a large number of very small proteins as part of their defence mechanism. These anti-microbial peptides (AMPs) kill or inhibit a broad range of micro-organisms. AMPs also exist in humans and may have future applications in fighting antibiotic-resistant bacteria.

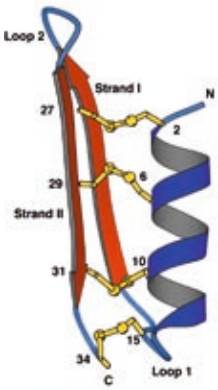
Fact box: Anti-microbial peptides (AMPs) and their application
Proteins *(also known as polypeptides) are made of amino acids arranged in a linear chain and folded into a globular form.* ***Anti-microbial peptides*** *(AMPs) are short proteins consisting of 12 to 50 amino acids with the ability to kill or inhibit the growth of micro-organisms.*

Defensin



Different groups of anti-microbial peptides (AMPs) have been found in mussels and have been further characterised in IMAQUANIM.

Mytilin



Anti-microbial peptides (AMPs) are among the key players of the innate immune response. These peptides are universally present throughout evolution and are found in bacteria, plants, animals and man. AMPs hereby represent a simple ancestral immune system without specialised cells and molecules, such as white blood cells (leucocytes) and antibodies otherwise used for disease prevention by the adaptive immune system.

AMPs can have the ability to kill different bacteria, viruses, fungi and even cancerous cells. Unlike the majority of usual antibiotics it appears that anti-microbial peptides may also have the ability to enhance immunity. As such, *AMPs are not only important in defending the animals themselves but can be considered as potent and broad antibiotics with potential to become future therapeutic agents for humans.*

Anti-microbial peptides of mussels

The IMAQUANIM project focused on characterising the gene activity of AMPs in mussels using a special DNA chip designed within the project (see also section “Fishing for genes in shellfish”, page 18). Several small mussel proteins have also been purified. Based on amino acid sequence similarity analyses, the peptides have been grouped into four AMP families. Differences in amino acids have allowed characterisation into sub-families. Interestingly, the different AMP families, and in some cases the sub-families, possess different biological activities, including anti-bacterial, anti-viral and anti-parasite activities, suggesting the presence of specificity of this ancestral innate defence system.

Differences between mussels and oysters

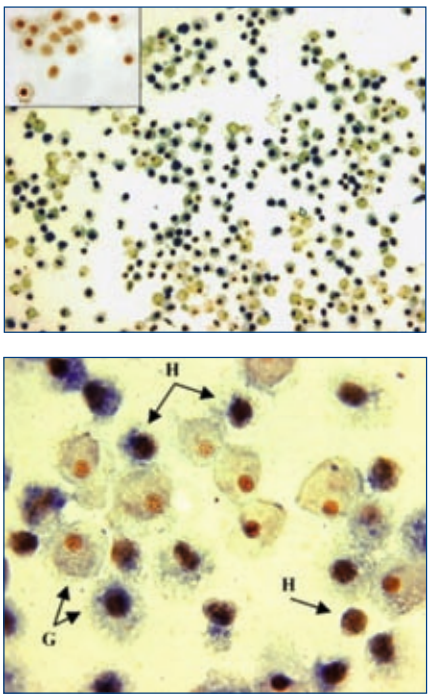
Mussels colonise many places and appear highly resistant to environmental changes, whereas oysters appear very sensitive. Therefore, it is likely of importance to clarify and compare the AMP repertoire of mussels and oysters and its involvement in fighting infections. In some mussels, different families of AMPs are present in different quantities. Whether similar differences between AMP gene activities are present in oysters still needs to be clarified. Furthermore, the complexity is increased since IMAQUANIM has revealed that AMP gene activity levels are differentially regulated depending on the type of bacteria the mussels were exposed to.

IMAQUANIM may have identified a novel class of antibiotics

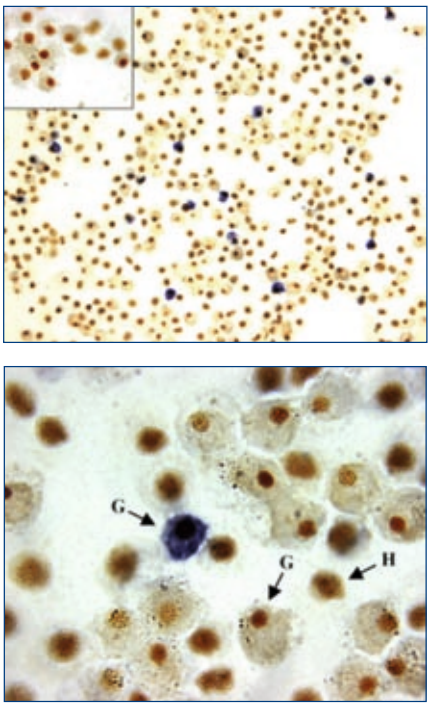
The construction of a DNA chip focusing on mussel immune genes combined with information acquired by comprehensive gene sequencing has extended our view on mussel innate immunity, and given a comprehensive view on the role of AMPs in the immune defence system as well as their possible application in disease prevention in other animals. *Benefits of these findings are thus both a better ability to select the best bivalves (mussel, oyster or others) for a given location and the potential to develop a new class of antibiotics.*

While oysters are highly appreciated as food, their immune defence is less efficient than that of mussels, possibly due to a more limited AMP repertoire. Searching for oyster variants with an improved AMP profile might be a fruitful future strategy.

Defensin



Mytilin B



Instead of the white blood cells in fish and mammals, mussels and other bivalves have hemocytes in their blood or body fluid. By using coloured gene-probes developed in IMAQUANIM it was shown that hemocytes produce anti microbial peptides (AMPs).

What parasites can teach us

Parasites can hide from the immune response and the immune response can kill the infected animal (or host) itself. Here, Dr. Geert Wiegertjes from the Cell Biology and Immunology Group in Wageningen University, The Netherlands, tells us how beautiful parasites can be and what we can learn about the fish immune system from studying host-parasite interactions.

As many other animals, common carp can get infected by viruses, bacteria and parasites. Research efforts to prevent these fatal incidences are therefore crucial for the aquaculture industry.

Although not always appreciated, important parts of what we know about the human immune system comes from studies of interactions between hosts and their parasites. For example, human trypanosomes are parasites of the size of a single cell which move around using flagella, just like sperm cells. Each year, approximately half a million people, spread over some 36 sub-Saharan African countries suffer from African trypanosomiasis caused by different forms of these parasites. Adaptation and strategies for evading host immunity have allowed 'tryps' to be found in almost all animal groups including species of fish. Indeed carp infected with the parasite *Trypanoplasma borreli* show many of the disease signs typical of human African trypanosomiasis.

Fact box: Parasites and their effect on the host

Trypanoplasma borreli *parasites live in the bloodstream of carp and are transmitted through the bite of leeches. Infections are widespread in farmed carp and in some European fish farms the percent of juvenile carps infected with 'tryps' may range between 75 and 100. Typical of carp 'tryps' infections is the production of extremely high levels of nitric oxide (NO), a highly reactive 'radical' molecule which causes inflammation and tissue damage.*

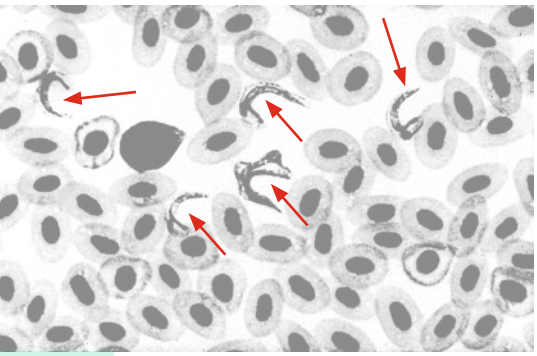
Tricks and puzzles

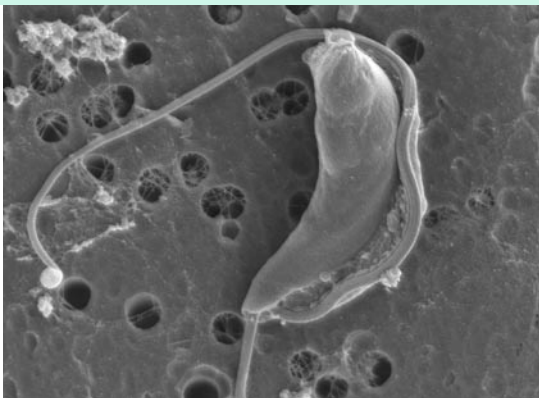
Trypanosomes have acquired many 'tricks' to avoid the immune system of their host. These include continuously changing their protein coat in order to escape recognition by the host as well as 'intracellular hiding' where the parasite hides inside cells of the host thereby becoming invisible to its immune system.

Balancing the rope

Host reactions aiming at the removal of infectious micro-organisms, sometimes progress irrespectively of the negative side-effects they may cause to the host animal itself. For this reason maintenance of a physiological balance between host and parasite are essential elements of their close relationship. Infections of carp with trypanosomes provide an excellent example of such a relationship. On the one hand, nitric oxide (NO) produced by the host slows down the parasite, thereby reducing the parasite defence against host recognition. On the other hand, too much NO has suppressive effects on the blood cells of the host immune system – which are among other things important for the production of antibodies and which will eventually contribute to the final clearance of these parasites. In other words the host and parasite have to find a balance in order to be able to live together.

Blood smear of Trypanoplasma borreli-infected carp. The parasites (half moon shape) are indicated by arrows and are near red blood cells (round cells).



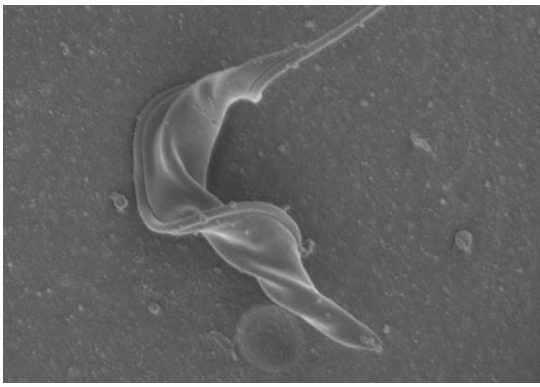


High resolution microscopy picture of Trypanoplasma borreli. Note the presence of two flagellas protruding from the upper and lower side of the parasite

Fish can get immune to parasites

The 'tryps'-model has been studied by the Cell Biology and Immunology Group during the last 5–10 years and was adopted by IMAQUANIM for studies on host-parasite interactions in fish. The experiments included challenge of in- and outbread carp with *Trypanoplasma borreli* and subsequent characterisation of the immune gene activity patterns of infected opposed to untreated control animals.

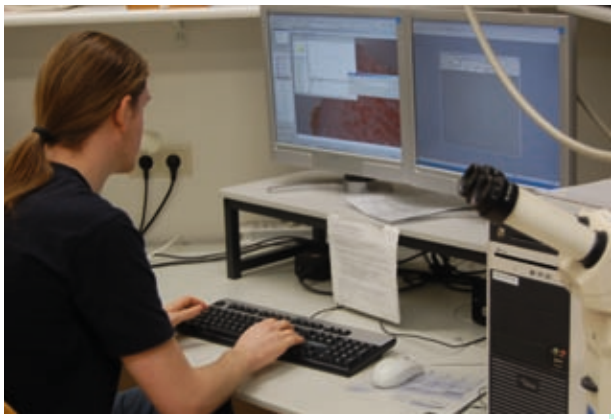
An anti-trypanosome drug (Arsobal, Melarsoprol) normally used to cure humans from acute cases of African trypanosomiasis, was proven extremely effective in curing carp from fish trypanosomes. After treatment, 100% of infected fish were cleared from these parasites and proved to be fully protected against re-infection. *This finding during IMAQUANIM shows for the first time, the possibility to treat and/or vaccinate fish against these parasites.*



High resolution microscopy picture of Trypanosoma carassii. Note the presence of only one flagellum protruding from one side of the parasite.

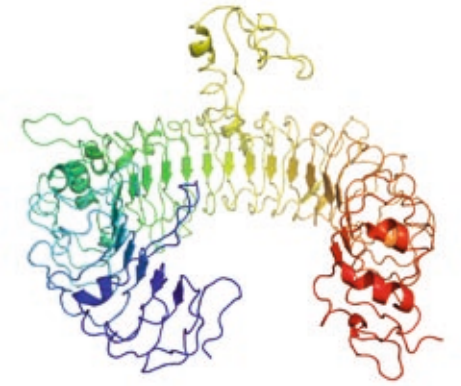
The number of trypanosomes in blood are counted with the help of a microscope.

Microscope pictures can be digitalized for publication.



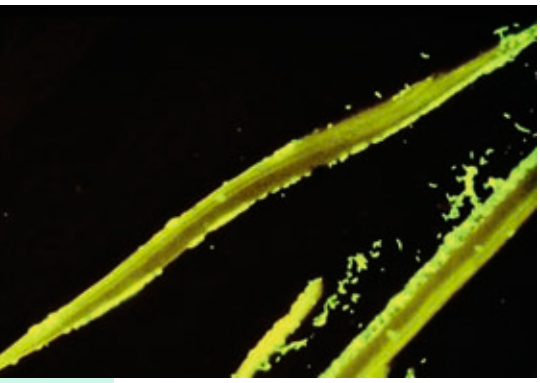
What fish have that humans don't have

As in humans many of the immunological processes in fish are performed by specialised cells with diverse functions. Some of these cells signal 'danger' the moment they encounter micro-organisms that could cause disease. Others cells react upon these danger signals and get involved by clearing the host from infection. A large set of cell proteins, called receptors are involved in recognising surface molecules of bacteria and viruses. Fish share many of these receptors with humans but also have several receptors which are only found in fish. Here, Dr. Roy A. Dalmø from the University of Tromsø in Norway explains the progress in understanding the role played by these fish-specific receptors. Niels Hjermitslev from BioMar Ltd, Denmark explains how to incorporate promising new stimulants of these receptors in fish feed in order to artificially stimulate the immune defence system of the fish.



Computer drawing of a TLR receptor: the ability to see the difference between self and foreign molecules and cells is central in immune defence. Proteins called toll like receptors or TLRs are the first to see foreign elements like intruding virus or bacteria in the body. Recently, it has been found that fish have more TLRs than mammals.

Rainbow trout fin with infecting bacteria (green dots). Bacteria called Flavobacterium cause high mortality in small rainbow rout. Trials in IMAQUANIM showed that immunity can be induced, but no vaccine is yet available. Knowledge of how TLRs recognize the bacteria would be very useful in vaccine development.



Despite overall similarity, many immunological differences exist between mammals and fish, and to complicate things more, there may even be significant inherited differences from one fish species to another. The fact that a group of fish species have, through their evolution, experienced rounds of copying of their genes means that many proteins with immunological function can be found in duplicate in fish. Therefore several immunological receptors and effectors in fish are found which have identical or nearly identical function. *There are nearly 22,000 different fish species, and most of them have their own 'immune peculiarities'.*

Receptors designed to recognise micro-organisms

Pattern recognition receptors on the surfaces of cells are responsible for recognising bacteria and viruses that can cause disease. They do so by 'looking' for special patterns in molecules on the infectious microorganism. These patterns are more or less specific 'fingerprints' of the microorganism in question. Upon binding to such molecules, the receptors trigger an immune response to the infectious micro-organisms.

One important family of receptors are the 'Toll-like receptors' (TLRs). Interestingly, the number of TLR receptors has increased dramatically during the evolution of fish. Accordingly, fish as a group, show a much more diverse repertoire of these receptors compared to warm-blooded vertebrates. Whereas mammalian species can have up to 11 different TLRs, some fish can possess up to 23 of these.

Fact box: Toll-Like-Receptors (TRLs) and their targets
Toll-Like-Receptors (TLRs) *are proteins found on, and inside, many animal cells. Some TLRs recognise viruses, others recognise bacteria or parasites. All TLRs trigger innate immune reactions which help the animal to control the infection.*

Practical implications

The IMAQUANIM project aimed at describing the regulation of tissue levels of selected TLRs during bacterial or viral infections in fish. A further objective was the production of antibodies for specific detection of TLRs. Molecules from micro-organisms that bind TLRs may be useful as helper agent molecules in vaccines or as immune stimulating additives in fish feed.

Production of feed containing immunostimulants

Fish feed usually undergoes a number of treatments that may affect its functional ingredients including immunostimulants which have been added. First, a milling process beats the raw mix until it is small enough to pass through a screen with holes smaller than one millimetre in size. The raw material then is pre-conditioned by hot steam to moisturise the mix, allowing starch to swell. After this process, pellets are shaped and cut to millimetre sizes. A fast drop in pressure and temperature physically opens up the pellet structure at which time additional oil is added. Coating is performed under vacuum to ensure that the oil enters the centre of the pellets. This step can be used for incorporating ingredients, such as immunostimulants that can be dissolved or dispersed in oil.

The ideal way to stimulate the immune system in fish is via the feed. This results in both easy delivery and minimal stressing of the fish. Apart from putting vaccines in the feed, use of feed immunostimulants with a more general effect is a progressing field. Immunostimulants initially tested in IMAQUANIM are now used commercially.

Search for new immunostimulants

Much research has been conducted on the use of new immunostimulants for improved animal health in aquaculture systems. *Collaboration between the fish feed company BioMar and fish disease research institutes/universities in IMAQUANIM made it possible to identify promising new immunostimulants.* Some of these are now being incorporated into commercial fish feed.

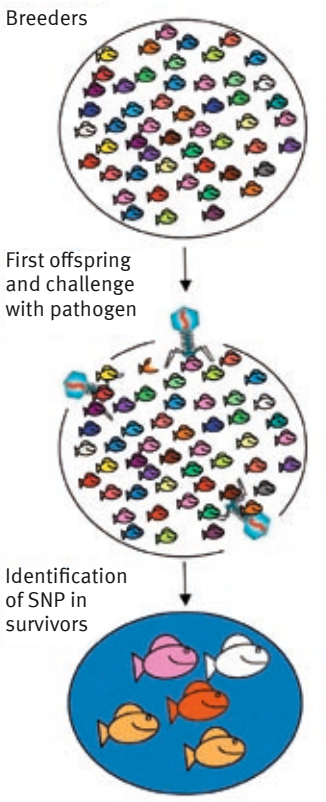
Breeding for survival of the fittest

Prevention is better than cure - this is something everybody can agree on. Most often our mind then goes to vaccination as the best known prevention strategy. However, selective breeding of the 'fittest' animals can also be an attractive way to diminish disease outbreaks in aquaculture. Here Dr. Maria Carmen Alvarez, from Málaga University, Spain, explains how cross-breeding fish with an inborn resistance against particular diseases, may help to prevent future disease problems, reduce the use of antibiotics and contribute to a sustainable aquaculture.

Selective breeding, intended at crossing the best-performing individuals so that the progeny succeed in having the desired trait, is no different from taking the most out of natural variation. Charles Darwin, in his book Origin of Species, already discussed how naturally occurring selection successfully produced changes over time. *Often, selective breeding is aimed at improving productivity but it can also be aimed at improving disease resistance.*

Breeding for the best

In general, animal breeding begins with a breeding stock: a group of animals used for the purpose of planned breeding. The aim is to identify valuable traits of interest that can be established and steadily maintained in animals of the next generations. By 'breeding the best to the best', accepting a certain degree of inbreeding, and by selecting for 'superior' qualities, the result is the development of a new breed superior to the original breeding stock.



Each individual fish, as any living creature on this earth, has developed biological responses to effectively deal with its environment, including life-threatening infections. These biological responses are driven by a large number of different genes, sometimes expressed in multiple alternative forms. Also variants of genes exist and sometimes these variants show different degrees of protection against infections. Identification of the best gene variants present in the breeding stock is an important step that underscores an effective breeding programme.

Fact box: Single-nucleotide polymorphism (SNP)
Identification of candidate genes and their allelic variants (an alteration in the normal sequence of a gene) has become much easier through the straightforward identification of so called SNPs by DNA sequencing. A single-nucleotide polymorphism (SNP, pronounced snip) is a small but distinct variation between inividuals in the genomic DNA sequence.

SNPs as genetic markers in fish

Identification of candidate genes and their variants has become much easier through the straightforward identification of very small changes (called SNP markers) in genes by use of the newest DNA sequencing methods. These changes may either affect the amino acids that form proteins, or the overall gene. The new condition may be advantageous and have a positive effect on disease resistance.

Background: Fish species of interest

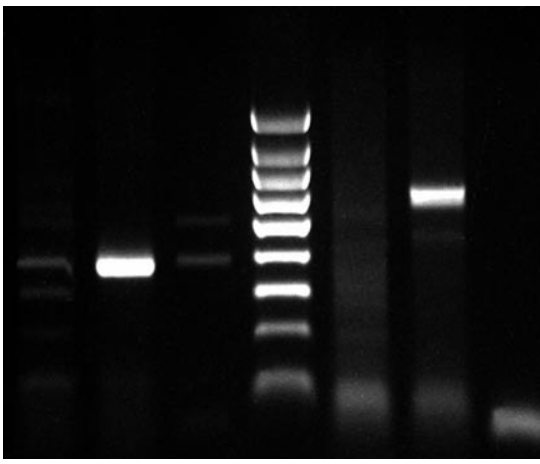
IMAQUANIM has directed its search for genetic markers towards two related aquacultured Mediterranean marine fish species: gilthead sea bream *(Sparus aurata)* and sea bass *(Dicentrarchus labrax)*. Progeny from genetically tagged broodstock fish were exposed to bacterial and viral infections, providing information on genetic disease resistance genes. Interestingly, the two fish species reacted quite differently to these experimental infections; whereas all sea bream proved highly resistant, a variable response to infection was noted for sea bass.

Further selective breeding

Major genes of interest are those responsible for economically important characteristics including disease resistance. To evaluate inheritance patterns, it is essential to assign beneficial gene variants to the parents in the breeding stock. Genotyping of the fish can be achieved by looking for SNPs linked to differences in disease resistance of fish after viral or bacterial infection. This work is in progress but to take optimal advantage of the observed disease resistance trends in further breeding, the broodstock fish families will be transferred to a commercial fish farm.

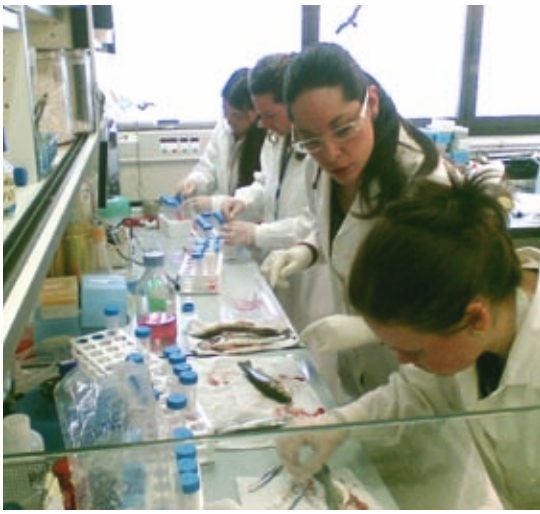
Immune genes of interest

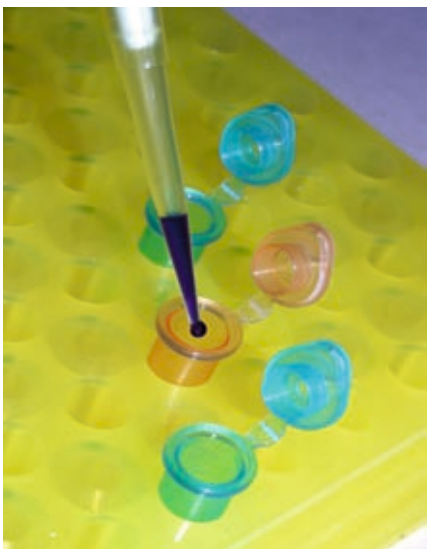
Among the numerous genes that could be considered 'as major' for disease resistance, IMAQUANIM focused on a particular gene encoding an antiviral protein (named Mx) that accumulate in infected cells to prevent virus replication. The gene sequence was cloned for both sea bream and sea bass and proved very different for the two fish species. At present, it remains to be shown whether these differences could be related to the naturally present genetic differences in disease resistance between the two fish species. However, the availability of a large number of highly informative DNA samples from sea bream and sea bass provided by the IMAQUANIM project opens possibilities for further searches, including additional genes important for resistance against viral infections. Future research should address the design of selective breeding programmes based on marker-assisted selection for disease resistance, enhancing a sustainable domestication of these fish species in the Mediterranean area.



Cloning and comparing Mx genes: Some proteins are able to inhibit virus growth within cells. This includes the so-called Mx proteins, which were cloned and shown to inhibit fish viruses. Variability in Mx genes might explain differences in disease susceptibility between and within fish species. This is a challenge for future research to resolve.

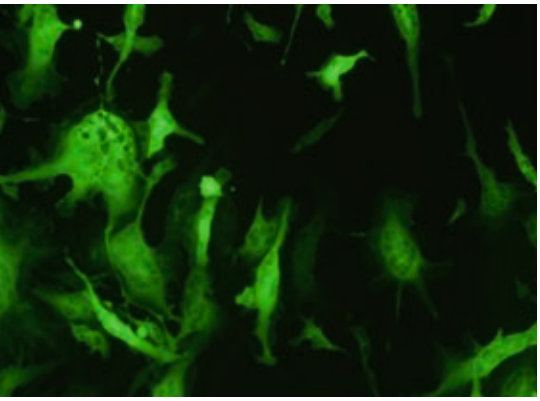
Different tissue- and organ samples are collected for subsequent gene activity analysis.





Laboratory tests with cells

The use of experimental animals is an issue under continuous debate. IMAQUANIM made an effort to lower the number of experimental animals in fish disease research by developing animal-free tests to determine the immune status of fish. Here, Dr. Bertrand Collet from Marine Scotland, Aberdeen, explains the work of his group on reporter tests using fish cells in the laboratory that can be used to measure the response of fish to infection by viruses.



Fish cells growing on a plastic dish expressing a reporter gene, the Green Fluorescent Protein (GFP) after transfection.

A shortcut to measure the function of a specific immune gene can be accomplished by use of a reporter test where the direct detection of a specific immune function is replaced with measurement of a characteristic molecule, called a reporter, which is very easy to detect. Luciferase is one of the most frequently used reporter molecules. It is the enzyme responsible for light emission in firefly insects. Changes in this light intensity can easily be measured with high sensitivity and high accuracy.

Fact box: Indirect detection of immune status
*A **promoter** is a region of DNA that controls the activity of a particular gene located close to it.*
*A **reporter** is a gene that researchers attach to a promoter of another gene of interest. The reporter gene product can easily be measured, and is therefore a measure for the activity of the gene of interest.*

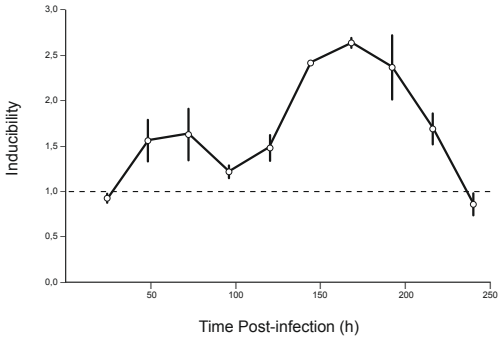
Promoting light

The general method to develop a reporter test in the laboratory is to construct an artificial circular DNA molecule called a plasmid. Two elements are then inserted into the plasmid: A specific part of an immune gene that regulates the activity (called a promoter) and the gene for the reporter molecule (e.g. luciferase). In this expanded plasmid construct, the production of the reporter molecule is strictly dependent on the activity of the promoter it is linked to. When the plasmid is inserted into a fish cell line, the cells will only produce the reporter molecules when the promotor is activated. The amount of reporter molecule produced is a direct measure of immune gene activity.

Cell lines

Cell lines are cells originally isolated from animal tissue. When placed under optimal laboratory conditions they grow indefinitely. The reporter construct described in the previous section can be integrated into the fish cell lines. Such a cell line produces the reporter molecule at a rate that depends entirely on the activity of the selected promotor which can easily be monitored under different experimental conditions.

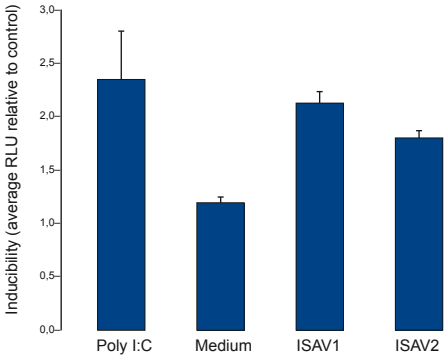
A good example is the cell line that was constructed from a rainbow trout gonad (RTG) cell line by integrating a plasmid containing the luciferase gene linked to the promoter of an anti-viral protein. The promoter is activated by interferon and the cell line can thus be used to measure interferon as outlined below.



Luciferase activity measure in RTG-P1 cells after infection with the Infectious Salmon Anaemia Virus (ISAV).

Important anti-viral genes

When a fish is infected by a virus, molecules called interferons (IFN) are produced by the first cells infected and released in the bloodstream. Most of the cells in the fish that come in contact with interferon molecules will respond by producing a large number of antiviral molecules including the Mx protein. In infected cells Mx prevents the ability of viruses to grow. The protein also reduces entry of the virus into neighbouring uninfected cells. Thus activation of the gene results in an increase in quantity of the protein and the establishment of an antiviral state. Using the reporter test described above it now is possible to measure functional IFN activity simply by growing RTG-P1 cells with a small amount of serum taken from a virus-infected fish.



By adding virus to reporter-cells the ability of the virus to control gene activity in the fish can be analysed. ISA virus is deadly to Atlantic salmon – this might be due to the ability of the virus to suppress activation of interferon and Mx.

Novel reporters

Many viruses have ways to block the host immune response. The RTG-P1 reporter cell line can be used to measure the extent of this inhibition and in turn, can help to predict the ability of a virus strain to induce disease on a fish farm. During the IMAQUANIM project, other reporter cell lines were generated by inserting the newly identified promotors of different important immune genes. Once established, cellular reporter tests can be used to compare different strains of viruses for their ability to evade or block the immune system. Such knowledge is crucial to understand which component of the host immune system should be enhanced to improve disease resistance.

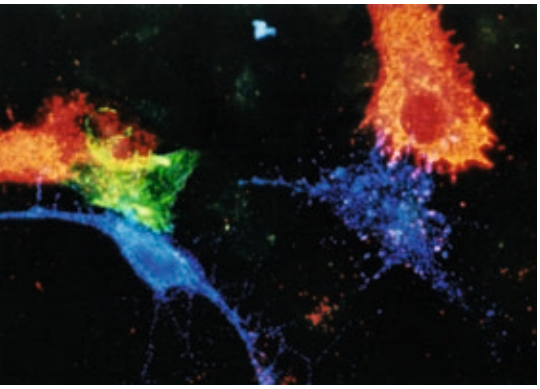
Animal-free tests

Another advantage of using reporter tests is that immune parameters can now be measured from small serum samples taken from a small number of live fish. This turns the reporter test into a non-lethal, animal friendly alternative for analysis of numerous samples. *The test developed during IMAQUANIM is therefore animal friendly and provides the unique possibility of following the immune response within the same individual over time.*

Seen from below: Atlantic salmon actively competing for feed – a sign of good animal health.

Antibodies: a colourful tool box

The ability of the animal immune system to produce specific antibodies to foreign matter can be used to develop tools for monitoring infection and immunity, as well as diagnostics. Here, Dr. Uwe Fischer from the Friedrich-Löffler-Institute in Germany, explains the efforts made by IMAQUANIM to produce antibodies against cells of the immune system of fish and Dr. Kim Thompson from Aquatic Diagnostics Ltd. in Scotland explains how antibodies are being made available to the wider aquaculture community through commercialisation.

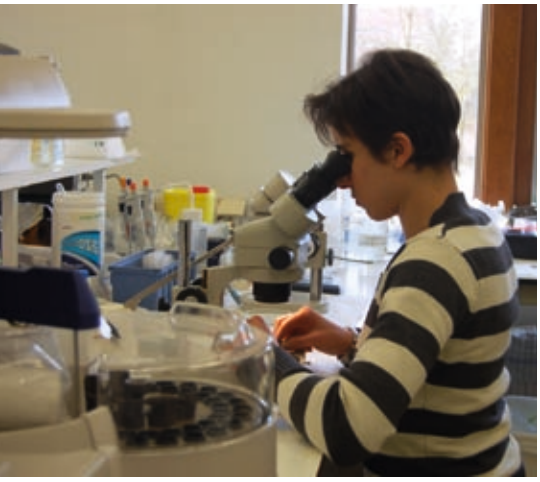


Fish cells infected by three different viruses. The use of specific antibody-staining allows powerful visualisation in fluorescence microscopy.

Despite having similar functions, the cells and proteins in different animals species are diverse, like the animals themselves. When cells and proteins from one animal species are injected into another species, the latter will produce specific antibodies to the foreign agents. Such cross-species antibodies, typically made in mice, rats or rabbits are very valuable tools for studying infections and the immune response in fish.

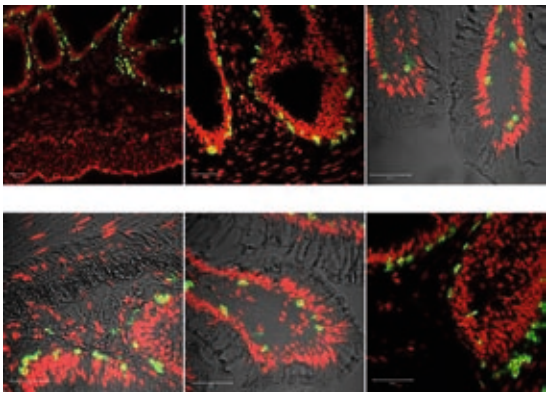
Fact box: Detection tests using antibodies
Depending on the type of test, the infectious microbe or immune cell is detected by using a specific primary antibody. If the primary antibody has been labelled with a colour, the primary antibody directly detects binding to the infectious organism. However, if the primary antibody is unlabelled, then a secondary labelled antibody is required to recognise the primary antibody. Few labelled antibodies are available commercially in comparison to unlabelled antibodies and so the majority of applied tests require labelled secondary antibodies.

Antibodies to antibodies and antibodies to cells

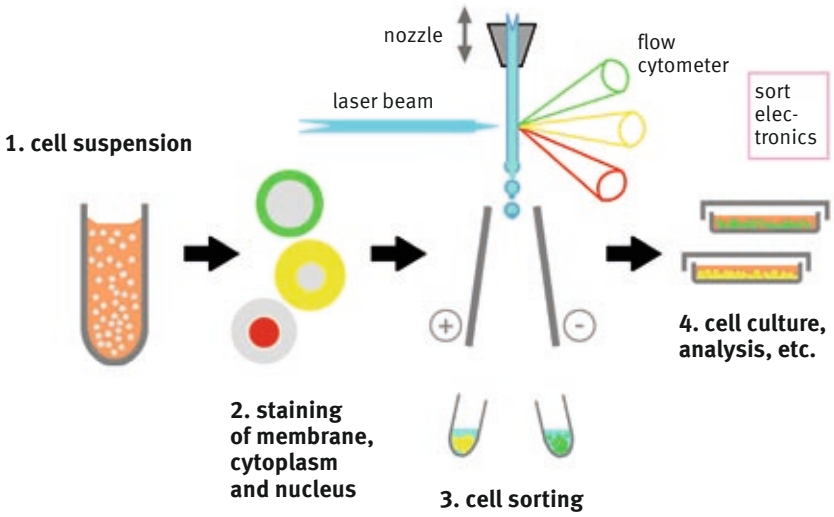


The detection of specific antibodies against infectious microbes in the blood of host animals is recognised as a useful indicator of previous exposure to infections and is regularly used in both human and veterinary medicine. However, such methods are underused in aquaculture. The primary antibody in this test recognises fish antibodies bound to the virus or bacteria that have infected the fish. This requires antibodies against fish antibodies for several species. *Such anti-fish antibodies were made available to the research labs in IMAQUANIM by the commercial diagnostic partner Aquatic Diagnostics Ltd.*

Several new antibodies against other components of the fish immune system were developed during the IMAQUANIM project. This includes antibodies to surface molecules on white blood cells that can be used as markers to identify subpopulations of white blood cells as well as antibodies to innate receptors like TLRs (see section “What fish have that humans don’t have”,



T-cells (green) are detected in the intestine of rainbow trout using an antibody produced during IMAQUANIM.

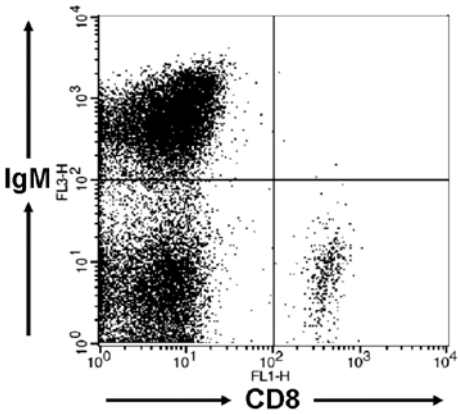


Cell sorting: by using antibodies labelled with different colours it is possible to separate white blood cells into B and T cells and hereby increase our insight to how these cells are involved in immunity.

page 24). Aquatic Diagnostics Ltd. was an SME partner in the IMAQUANIM project whose role was to provide reagents (antibodies against micro-organisms infecting fish and fish antibodies) and services to IMAQUANIM partners, as to make the antibodies generated during the project available to the wider aquaculture community through commercialisation.

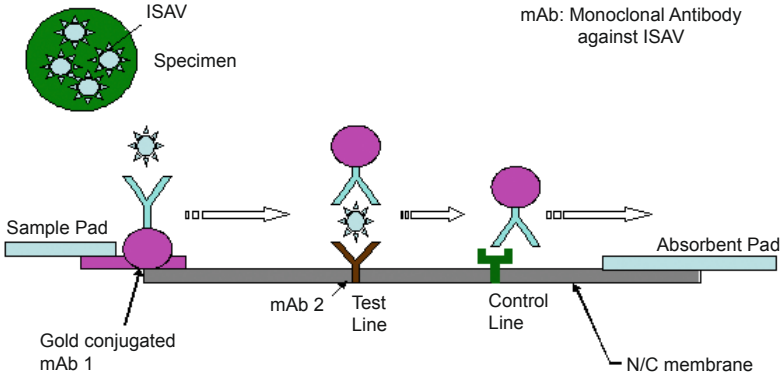
New technologies

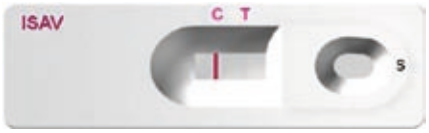
New, highly sensitive, rapid immunodiagnostic methods with potential use in aquaculture include Bluespot technology, and Lateral Flow Kits, but also more advanced methods such as Luminex Technology, nano-technology and antibody microarrays are receiving growing attention among fish researchers and diagnosticians.



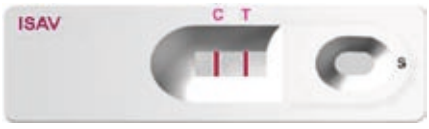
Cell sorting using antibodies to trout B and T cells demonstrate presence of two distinct cell types in fish blood. More cell types are currently defined using newly developed antibodies.

By combining bound and free antibodies to the same antigen (e.g. virus), ADL develops rapid tests for health check of fish.





Negative result



Positive result

Training and dissemination for the future

In IMAQUANIM, training and dissemination have been important activities throughout the project. Training means integrating PhD students into the project as well as organising an open workshop for researchers and others with interest in the field. Dissemination; telling others about what has been done, has mainly been done at the scientific level, but it has also included producers and industry. Consumers and others are addressed with this brochure. Here, Dr. Geert Wiegertjes and Dr. Maria Forlenza from the Cell Biology and Immunology Group in Wageningen, The Netherlands, tell us about the Fish and Shellfish Immunology workshops organised by them in the context of IMAQUANIM.

Participants of the workshop preparing for the lecture and discussing new information.

IMAQUANIM has not only been about integration of knowledge of the immune system of aquacultured animals at European research level, but also about transferring this knowledge to the next generation of scientists, company researchers and management staff in aquaculture. The Fish and Shellfish Immunology workshops organised as part of the project's training effort, welcomed young PhD students and others with interest in the field worldwide.

Fact box: The Fish and Shellfish Immunology workshops in Wageningen
These workshops have been organised on an annual basis since 1998 and attract more than half coming from outside the IMAQUANIM network. Initiated by the Cell Biology and Immunology Group of the Wageningen University, the workshop is the collaborative result of people from this group, together with invited lecturers from other universities and research institutes. The objective of the workshop is to provide participants with advanced knowledge, both theoretical and practical, on the immune system of fish and shellfish.

PC-based tutorials during the workshop on recent advances in molecular biology such as microarray analysis.

Both theory and practice

IMAQUANIM allowed for a renewed set-up of the workshop, now including several shellfish experts from the network as invited lecturers. The new workshop combined hands-on practical afternoons with PC-based tutorials on the most recent technical advances in molecular biology, with a choice between these two. The new programme also included two evenings of poster sessions to give each young scientist the chance to 'sell' his/her story to the other scientists present.

Concentration on the faces of workshop participants when listening to complicated new information from teacher.

Workshop manual with on the background poster presentations of the participants.

Evolution of the immune system

Immunology is a form of biomedical science that covers the study of all aspects of the immune system in all organisms. It deals with the physiological functioning of the immune system in states of both health and disease. There are several very good textbooks that deal with immunology, but they are less useful for understanding the details of the immune system of (shell) fish. During the workshops the latest insights in the evolution of the fish and shellfish immune system as well as related issues such as (experimental) animal welfare were discussed. Typically, the presentations took as a starting point the immunology found in textbooks but progressed into the most recent and detailed immunological knowledge of both fish and shellfish, as well as practical implications of this knowledge in aquaculture.

Associated with the International Society for Fish and Shellfish Immunology

The (Shell)Fish Immunology workshops have been very well attended with some 30–50 participants each time, thereby having trained ›200 young scientists during the period of IMAQUANIM. The project brought together a large group of younger scientists as well as experienced supervisors who taught at the workshops. In total, *29 PhD students have been fully or partly financed by IMAQUANIM* and many more have been trained as a part of the teaching activities. The Fish and Shellfish Immunology workshops have become a training platform here to stay. In the near future, they will be associated with the International Society for Fish and Shellfish Immunology and as such be guaranteed a lifespan beyond IMAQUANIM. It is likely that this training platform will continue to train hundreds of more young scientists in (shell)fish immunology over the coming years. Thereby, the knowledge acquired during IMAQUANIM will find continued dissemination for a long time to come.

Participants from the 2010 workshop in front of the main teaching building of Wageningen University

Management of IMAQUANIM

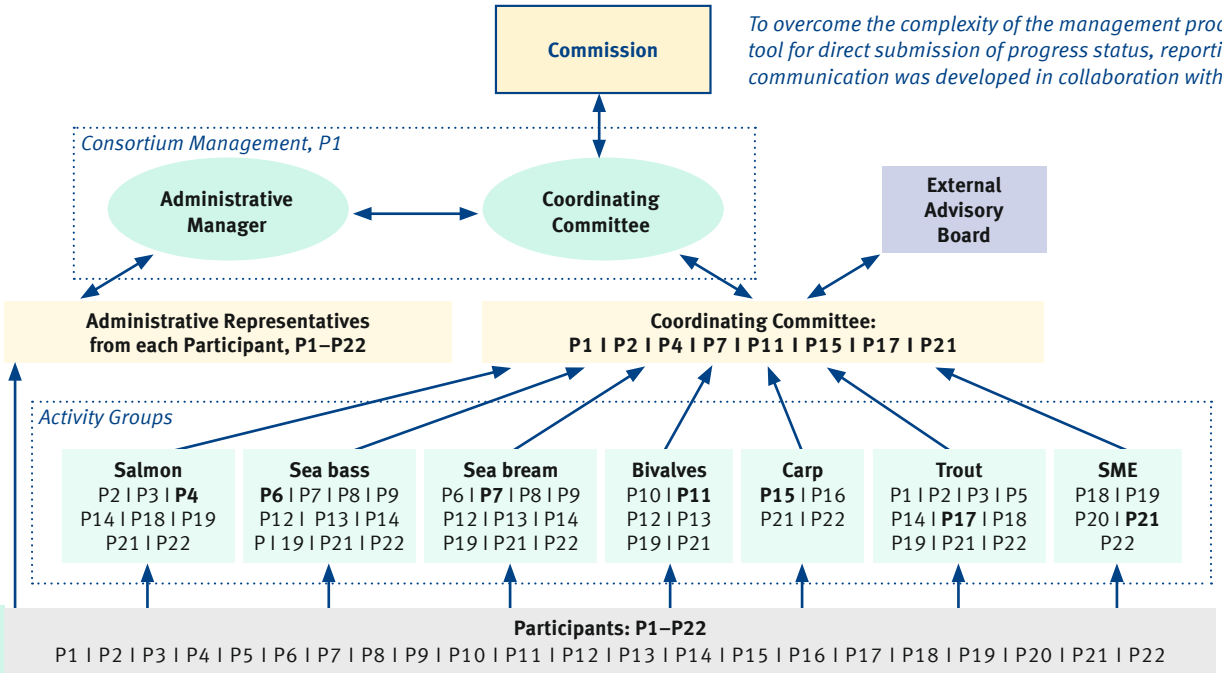
Large integrated projects like IMAQUANIM require a very efficient technical- and scientific management to keep ongoing. Throughout the project, the coordinating team at P1 was assisted by a coordinating committee and stayed in continuous dialog with the external advisory board consisting of three internationally recognised experts within fish and shellfish immunology.

Fact box: IMAQUANIM in numbers
Partners: 22 hereof 17 RTDs and 5 SMEs
Duration: 66 months, initiated April 2005
Project cost: 10.54 million euro
EC grant: 8.02 million euro
Scientific plan: 27 work packages resulted in 1186 deliverables
Project meetings: Annual, contribution from 1–3 representatives from each partners and external advisors
Coordinating committee meetings: Twice a year, each member being responsible for a group of 3–6 participants working on the same animal species.
Scientific publications: ›125 in peer reviewed journals. Many additional manuscripts are in preparation.
PhD students: 29 PhD students have been fully or partly financed by IMAQUANIM.

Three representatives from the EC supported the successful initiation of IMAQUANIM during the kick off meeting in Wageningen, The Netherlands, April 2005.

Fruitful scientific discussions and close interactive collaboration between partners were key components of the IMAQUANIM project.

To overcome the complexity of the management procedures, a website tool for direct submission of progress status, reporting and project communication was developed in collaboration with ***www.minadmin.eu.***



Pathogens, viruses, bacteria, and parasites
Pathogens are viruses, bacteria and parasites that cause disease in animals and man. ***Viruses*** *cause disease by growing within and destroying the cells of the infected animal. In contrast, most* ***bacteria*** *grow outside the animal cells and can cause disease by being toxic.* ***Parasites*** *are small animals themselves specialised for living inside other animals and often disturbing tissue function.*

Vaccination
The word ***'vaccination'*** *is derived from 'vacca', the latin name for cow. Back in the 1790's the English doctor Edward Jenner discovered that he could protect people against small pox by exposing them to cowpox – a related infection not causing disease, but inducing immunity. 'Vaccination' thus refers to its origins from the cow.*

How to make vaccines
In the laboratory, viruses require cultured animal cells to grow, while bacteria can grow by themselves in a simple substrate. Making large amount of killed bacteria for a vaccine is therefore much simpler and cheaper than for viruses. Parasites are complex and often impossible to grow in the laboratory. No vaccines exist against fish parasites.

Gene activity is measured as amount of RNA
When a gene is active, copies in the form of messenger RNA (mRNA) are produced and translated into proteins, the main molecules in our bodies. Levels of gene activity can therefore be measured as the levels of mRNA.

Transcriptome and anti-microbial peptides
The ***transcriptome*** *is the set of RNA molecules present at any given time and reflects the genes that are active. The profile varies with external environmental conditions e.g. temperature or infection.* ***Innate responses*** *are immune mechanisms developed early during evolution (see also section "The immune system", page 10).* ***Anti-microbial peptides (AMPs)*** *are small proteins that are able to kill bacteria and viruses.*

Anti-microbial peptides (AMPS) and their application
Proteins *(also known as polypeptides) are made of amino acids arranged in a linear chain and folded into a globular form.* ***Anti-microbial peptides*** *(AMPs) are short proteins consisting of 12 to 50 amino acids with the ability to kill or inhibit the growth of micro-organisms.*

Parasites and their effect on the host
Trypanoplasma (T.) borreli *parasites live in the bloodstream of carp and are transmitted through the bite of leeches. Infections are widespread in farmed carp and in some European fish farms the percent of juvenile carps infected with 'tryps' may range between 75 and 100. Typical of carp 'tryps' infections is the production of extremely high levels of nitric oxide (NO), a highly reactive 'radical' molecule which causes inflammation and tissue damage.*

Toll-Like-Receptors (TRLs) and their targets
Toll-Like-Receptors (TLR) *are proteins found on, and inside, many animal cells. Some TLRs recognise viruses, others recognise bacteria or parasites. All TLRs trigger innate immune reactions which help the animal to control the infection.*

Single-nucleotide polymorphism (SNP)
Identification of candidate genes and their allelic variants (an alteration in the normal sequence of a gene) has become much easier through the straightforward identification of so called SNPs by DNA sequencing. A single-nucleotide polymorphism (SNP, pronounced snip) is a small but distinct variation between inividuals in the genomic DNA sequence.

Indirect detection of immune status
A ***promoter*** *is a region of DNA that controls the activity of a particular gene located close to it.*
A ***reporter*** *is a gene that researchers attach to a promoter of another gene of interest. The reporter gene product can easily be measured, and is therefore a measure for the activity of the gene of interest.*

Detection tests using antibodies
Depending on the type of test, the infectious microbe or immune cell is detected by using a specific primary antibody. If the primary antibody has been labelled with a colour, the primary antibody directly detects binding to the infectious organism. However, if the primary antibody is unlabelled, then a secondary labelled antibody is required to recognise the primary antibody. Few labelled antibodies are available commercially in comparison to unlabelled antibodies and so the majority of applied tests require labelled secondary antibodies.

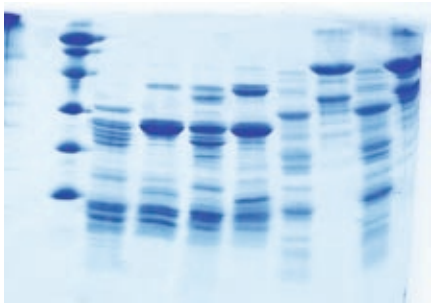
The Fish and Shellfish Immunology workshops in Wageningen
These workshops have been organised on an annual basis since 1998 and attract more than half coming from outside the IMAQUANIM network. Initiated by the Cell Biology and Immunology Group of the Wageningen University, the workshop is the collaborative result of people from this group, together with invited lecturers from other universities and research institutes. The objective of the workshop is to provide participants with advanced knowledge, both theoretical and practical, on the immune system of fish and shellfish.

What is still missing in fish and shellfish immunology research?

An insiders point of view. **Here Professor Chris Secombes from the University of Aberdeen, UK, in his capacity as Editor of Fish & Shellfish Immunology, considers what scientific challenges will remain after the IMAQUANIM programme finishes.**

It is clear to me that the last decade has seen a tremendous increase in fish and shellfish immunology research, coupled with an equally impressive increase in the number of (shell)fish immunologists! This is evidenced by the number of papers being published and the citation rate of these papers, and represents a very good sign for a comparatively young field. Without doubt the stimulus has been the need to improve the health of aquacultured animals, where significant increases in production have been seen globally in an effort to meet the demand for marine produce that has not been met by traditional fisheries. However, as we have researched the immune systems of these cultured species it has become apparent that we can learn a lot about immune system evolution, and that there are many wonderful solutions to staying alive in an aquatic environment. So fundamental studies on the expansion of particular gene families in some animal types, or the importance of unique tissues as immune sites (such as the gill of fish), give an insight into adaptive physiology and the evolution of traits that give a selective advantage to surviving in different environments, and potentially contributing to species radiation. *Nevertheless, as we learn more about what defence mechanisms exist, we are in a better position to manipulate these responses for the benefit of aquaculture.*

Challenges



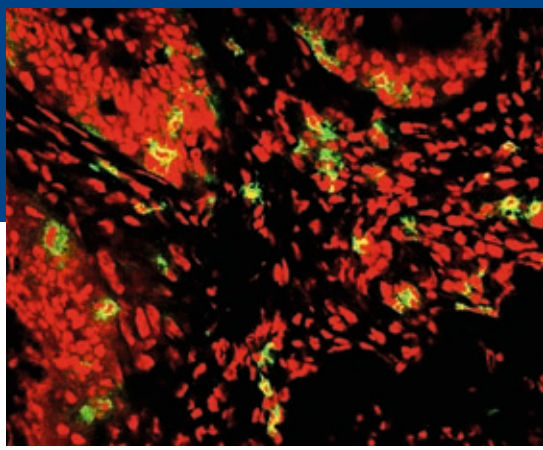
The new knowledge about fish genes allows us to produce recombinant proteins that can stimulate growth of immune cells.

Fish cell cultures are an important research link between genes and live animals.

Fish immunologists face a number of issues going forwards in a relatively new field. Firstly, there is the classical problem of dilution of effort, by studying multiple species. It is difficult to see a way to resolve this issue, since the diversity of fish, especially teleost fish, means that many unique solutions to stay alive in a particularly environment with a particular pathogen load are likely to have arisen within individual fish lineages. If we only consider the main species cultured around the world, *still most of the main fish groups need to be studied* (ie cyprinids, salmonids, gadoids, perciformes, anguilliformes, etc). Whilst studies of Atlantic salmon will have some value for research of say Atlantic cod, nevertheless there will be differences in their immune systems which may be crucial in terms of the best way to manipulate these defences to increase survival in aquaculture situations. Having said this, it seems *there is an appetite to generate a model fish species* to be used by many groups across the globe, akin to the use of the mouse in mammalian immunology. The features that are desirable include a short life cycle, high fecundity, availability of mutants, ease of genetic manipulation, etc, etc. It is clear that the *zebrafish fits the bill and will allow many in vivo studies of gene function* almost impossible to achieve in most of the commercially important species. Knowledge gained in this way will be unique to zebrafish but will highlight genes and pathways that should be studied in the other species.

3R's (replacement, refinement, reduction)

Another challenge for fish immunologists is to become less dependent on the use of live fish for basic and applied studies. Development of *in vitro* correlates of disease resistance/ immune competence would help reduce the need for disease challenge experiments, but will require both optimisation and validation. The generation of more fish cell lines is also desirable and



Antibodies to fish immune cells allow studies of their behaviour in tissues. An antibody developed by Dr. Uwe Fischer shows trout T-cells in the intestine. While hundreds of antibodies are available for human immunology research, we still need to develop more for fish and shellfish.

would help study of particular cell types as well as reducing the numbers of experimental fish. Whilst this has been difficult to achieve in most fish species to date, the vast increase in *discovery of white blood cell growth factors means that these molecules can be used to help drive the growth and maintenance of white blood cells in culture*. The production of the recombinant proteins (i.e. growth factors) may also be achieved through the generation of fish cell lines carrying the relevant genes.

Tools – advances and bottlenecks

Finally, gene research has flourished in recent years, and has driven the amazing increase in gene discovery and our ability to study the activity of immune genes. Indeed, there is the potential to soon have methods able to analyse the entire gene activity of fish relatively cheaply, whether focussed at the cell or tissue level. However, such information is limited in terms of the unknown relationship between gene and protein activities, with the latter generally requiring specific antibody reagents for detection. *The number of antibodies available for analysis of fish immune molecules has expanded thanks to IMAQUANIM and related projects, but the limited number is still a severe bottleneck and without doubt a major limiting factor currently.* Mostly these need to be developed from scratch, although a few of the molecules of interest will have conserved domains (eg transcription factors) that may allow pre-existing antibodies to mammalian molecules to be used for fish, especially where small peptides have been used for immunisation.

Ways forward

So what will the future bring us? I would predict an increased use of zebrafish as a model fish species for immunological studies, to enable maximal exchange of reagents and in-depth study of a single fish species. We need to put further effort into antibody production to fish immune molecules and further development of fish cell lines, as an adjunct to *in vivo* studies. Development of cell culture methods that can be used as a proxy of disease resistance, whether to study innate defences or those induced by vaccination, will be challenging but would have many rewards, not least the potential for large scale screening of pilot vaccines or putative immunostimulants. Still, it is clear that functional studies of genes, proteins and cells in live fish exposed to infection or vaccination will be crucial for our ability to fully understand host-pathogen interactions and to develop the perfect vaccines.

Zebrafish is an ideal experimental fish species for future basic research.

What will the future bring us?

The IMAQUANIM coordinator's point of view. **IMAQUANIM has had a great impact on the advancement of our knowledge on the fish and shellfish immune system. Here, Dr. Niels Lorenzen from the Danish Technical University summarizes the challenges for Europe that remain to be faced.**

Food products derived from fish and shellfish are an excellent source of high quality protein, which in combination with a favourable fatty acid composition, give a nutritional profile superior to animal meat products from terrestrial animals. Given the fact that fish and shellfish are healthy food, the demand for meat products derived from aquaculture and fisheries will only increase. However, as wildlife fisheries resources have been exhausted it is aquaculture that is expected to fill the gap in the world's future fish supplies.

Infectious diseases represent one of the major threats towards a continued successful expansion of aquaculture production world wide, and implementation of the EU animal health strategy 2007-13: "Prevention is better than cure" is an essential element here. The successful implementation of vaccination strategies for some of the important diseases in Atlantic salmon in Norway and Scotland has demonstrated that this is the way ahead. In some cases, vaccine development based on traditional trial and error strategies has worked well. However, for the majority of diseases in fish and shellfish an in-depth understanding of the underlying immune reactions leading to protective immunity and how to activate these is required.

Today's knowledge of the mechanisms underpinning protective immunity and vaccination of fish and shellfish is still insufficient. Yet, there is progress. IMAQUANIM and related EC-supported research projects have started up knowledge- and technology-platforms for functional research on the immune system. Now it is time to harvest and proceed with a trans-European joint research effort addressing a number of points with applied perspectives, such as

- How can we deliver vaccines to small fish?
- How early can fish be vaccinated?
- How can we make vaccines to viral fish disease?
- How can we improve immunostimulants and fish vaccine adjuvants?
- Can the mussel anti-microbial peptides be used for obtaining more robust bivalve populations and possibly also for development of new antibiotics?

As outlined below, the benefits of uniting the research efforts within this field are multiple. This will not only strengthen the aquaculture industry but European society as a whole.

Discussion of future research strategies at the final IMAQUANIM meeting in Viterbo, Italy, 2010.

Industrial impact

An extensive knowledge- and technology-platform would strongly support the optimisation of current aquaculture production with respect to sustainable and environment-friendly approaches. The platform would also ensure that the foreseen expansion of the industry can take place without major animal health problems. From a more global perspective, the action would be supportive for the ability of the European aquaculture industry to compensate for the decreasing natural fisheries resources in a competitive manner. Furthermore, companies indirectly involved in aquatic animal health, such as producers of fish vaccines and producers of analytical kits will be able to expand their range of products and improve their current products (e.g. adjuvanted vaccines), hereby increasing their international competitiveness. Since aquaculture is usually most intensive in remote areas, a sustainable expansion of the industry will be beneficial for the occupation and life quality, in general, at remote locations.

Impact on human welfare

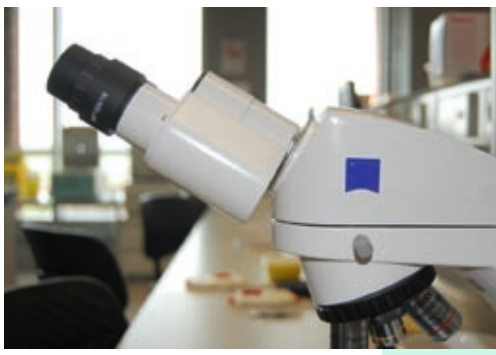
Increasing animal health will result in improved food quality and safety and hereby reduce risks to human health. In many European countries, consumers prefer food products derived from warm-blooded animals (red meat). Decreasing wildlife fisheries resources will push the consumers' diet towards red meat, whereas an extended range of high quality products derived from aquatic animals most likely will increase consumption of fish food. Increased consumption of fish will have a positive impact on human health by lowering the frequency of coronary heart diseases and other common circulatory diseases related to the inexpedient fatty acid composition of consumers' current food preferences.

Impact on animal welfare

Implementation of new and improved vaccines and other prophylactic measures will reduce the numbers of animals suffering from and succumbing due to infectious diseases. Improved vaccine delivery strategies including less aggressive adjuvants will minimize stress and negative side effects related to current prophylactic practice.

Environmental impact

By minimizing the losses caused by infectious diseases, the release of waste products into the environment will be kept to a minimum. Equally important, efficient implementation of prophylactic measures will reduce the use and release of antibiotics, disinfectants and other polluting compounds.



Scientific impact

A united European research effort within aquatic animal health and prophylactic measures in particular, including in depth understanding of immunity to infectious diseases, is expected to bring Europe to the scientific forefront within this field. Industrial exploitation and implementation of results will simultaneously be facilitated.

More functional understanding is still needed

Three internationally recognized external experts have been associated with IMAQUANIM and have participated in project meetings from the beginning of the project in 2005 until the end in 2010. These experts, namely Professor Anthony Ellis at Marine Scotland, Aberdeen, UK, Dr. Scott LaPatra at Clear Springs Foods Inc., Idaho, USA and Dr. Kenneth Söderhall at University of Uppsala, Sweden have consistently provided invaluable advise and comments to the project work and strategies.

Dr. Scott E. LaPatra is director of research and farm services at Clear Springs Foods Inc., Idaho, USA. Clear Springs Foods is one of the major rainbow trout producers in the world. In his function, Scott LaPatra is faced on a daily basis with the gaps in immunological knowledge so important for successful vaccination of fish.

As one of the major freshwater rainbow trout food fish producers in the world we take an integrated approach to aquatic animal health management. Our use of drugs and chemicals is very minimal because of their cost, limited utility, environmental concerns and consumer perception issues. We emphasize fish husbandry, selective breeding and the use of vaccines. Use of vaccines is a powerful approach but without an adequate understanding of the immune system and the types of functional immune responses that are best to elicit in rainbow trout for optimum protection this type of strategy can be compromised. *The type of immunological research that has been undertaken in the IMAQUANIM project is critical to us in attaining this goal of understanding the immune response and actual function.*

Difficult to mimic industry conditions, but IMAQUANIM has provided remarkable results

High quality work has been produced within IMAQUANIM, specifically the research on development of challenge models that mimic the natural routes of infection of aquacultured animals. Experimental set-ups have their limitation of only looking at a limited number of hosts, and it is therefore always important to keep in mind the fish size and age, differences in the season of the year, temperature effects as well as differences between strains of fish and families within a strain. From a producer's perspective it is of course important to mimic the production conditions used in the industry as closely as possible. However, given all of these limitations an impressive number of good wet-lab studies have been successfully completed during the 5½ year project period.

Studying how different farmed fish and shellfish defend themselves against infections in real life is essential for our ability to improve their immunity.

What we can learn from experimental set-ups

Tests for qualitative and quantitative monitoring of key elements of the innate and adaptive immune system at genetic and functional levels have also been established during IMAQUANIM and these tools have enabled determination of response profiles which correlate with protective immunity. For all species, infection trials with various types of infections, including the re-exposure of survivors, was used to determine reference response profiles of untreated and vaccinated animals. For finfish, vaccination trials with efficiently working commercial and experimental vaccines and corresponding control reagents were used to identify critical response elements/profiles for vaccine efficacy. Variability both in terms of gene variation and occurrence of different forms of the same protein among the immunological key elements and their regulation was related to the functional response observed when using laboratory tests as well as during experiments including live animals in terms of disease susceptibility of untreated and vaccinated animals. Development of tests for direct determination of gene functions was also accomplished.

What Europe has but the USA would like to have

A major impact of the project has been to unite some of the major immunology research laboratories in the EU into a joint research activity that minimizes duplication and maximizes the use of collaborative expertise. To my knowledge, similar fish immunology focused consortiums have never been set-up in the USA, and I doubt they ever will. The European Union should therefore be extraordinarily pleased with how well the coordinator of this project managed to establish this unique knowledge platform, and if possible, build on this success by prioritising further research within this important area for improving the immunological status of aquacultured animals. *The type of research performed within IMAQUANIM must be continued so that we can have a functional understanding of how to produce and use vaccines and how to utilize immune-stimulating or disease-inhibiting agents for the maintenance of optimal fish health.*

The activities of the 22 participants in the integrated project were synchronised by establishing a coordinating committee with each member taking responsibility for a group of 3–6 participants working on the same animal species.

Contractors involved

1 Technical University of Denmark, DTU Veterinary, National Veterinary Institute, Division of Poultry, Fish and Fur Animals, Aarhus, Denmark

2 Norwegian School of Veterinary Science, Department of Basic Sciences and Aquatic Medicine, Oslo, Norway

3 Norwegian College of Fishery Science, Faculty of Biosciences, Fisheries and Economics, University of Tromsø, Tromsø, Norway

4 The Scottish Ministers acting through Marine Scotland, Marine Laboratory, Aberdeen, UK

5 Friedrich-Löffler-Institute, Federal Research Institute for Animal Health, Institute of Infectiology, Greifswald - Insel Riems, Germany

6 Tuscia University, Department of Environment Sciences, Laboratory of Animal Biotechnology, Viterbo, Italy

7 University of Malaga, Department of Cellular Biology, Genetics and Physiology, Faculty of Science, Malaga, Spain

8 University of Murcia, Department of Cell Biology and Histology, Faculty of Biology, Murcia, Spain

9 Universitat Autonoma de Barcelona, Department of Cell Biology, Physiology and Immunology, Bellaterra, Barcelona, Spain

10 Institute for Marine Research, Spanish National Research Council (CSIC), Vigo, Spain

11 Montpellier 2 University, National Centre of Scientific Research, Montpellier, France

12 University of Padua, Department of Biology, Padua, Italy

13 Istituto Zooprofilattico Sperimentale Delle Venezie, Fish Pathology Department, Legnaro (PD), Italy

14 University of Aberdeen, Scottish Fish Immunology Research Centre, Aberdeen, UK

15 Wageningen University, Department of Animal Sciences, Cell Biology and Immunology Group, Wageningen, The Netherlands

16 Veterinary Research Institute, Department of Virology, Laboratory of fish viral diseases, Brno, Czech Republic

17 University of Copenhagen, Faculty of Life Sciences, Department of Veterinary Pathobiology, Laboratory of Fish Diseases, Copenhagen, Denmark

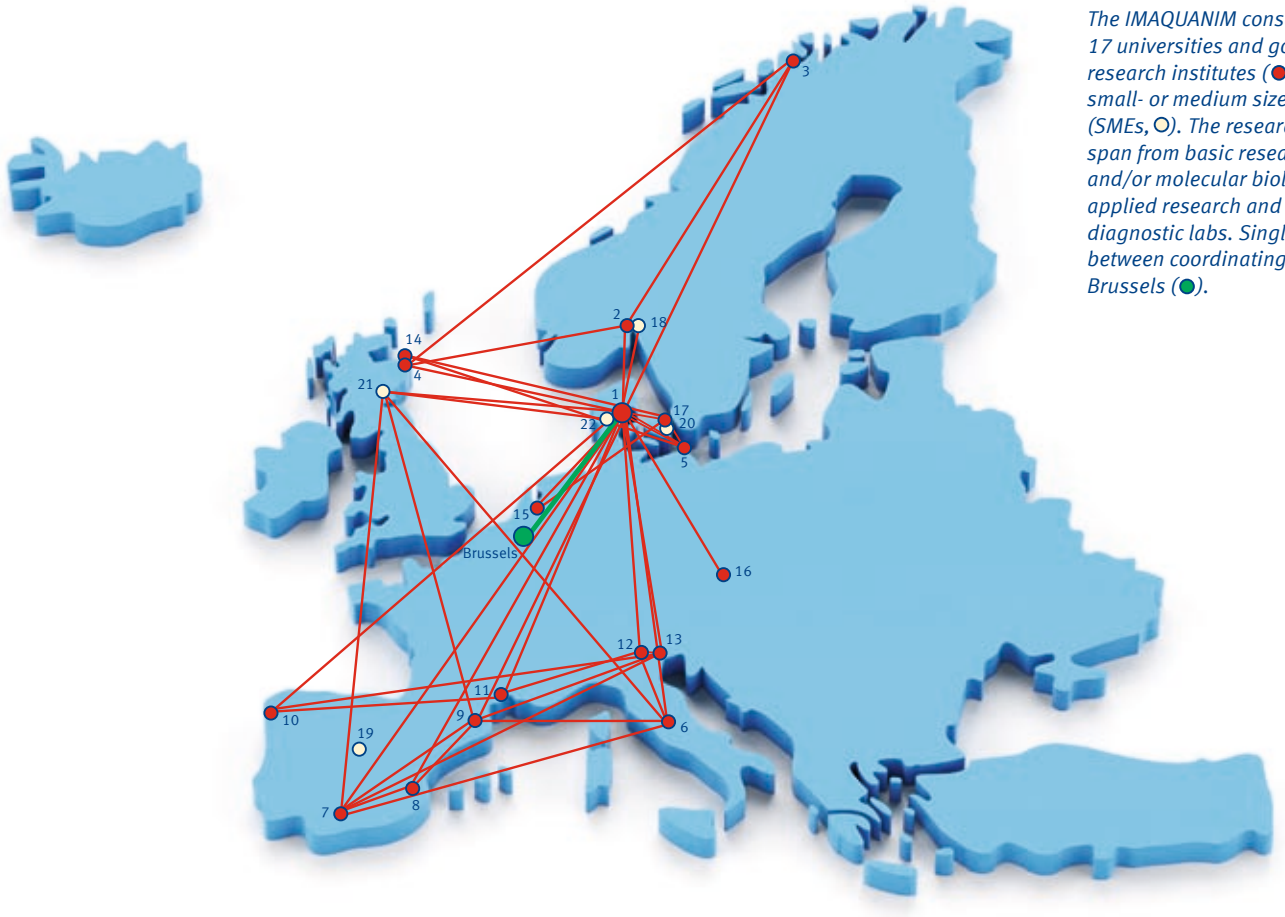
18 PHARMAQ AS, Oslo, Norway

19 Bionostra Aplicaciones Biotechnológicas S.L., Genetic Identification, Madrid, Spain

20 GeneCare, Copenhagen, Denmark

21 Aquatic Diagnostics Ltd, Stirling, UK

22 BioMar A/S, Brande, Denmark



The IMAQUANIM consortium includes 17 universities and governmental research institutes (●) as well as 5 small- or medium sized enterprises (SMEs, ○). The research laboratories span from basic research immunology- and/or molecular biology units to applied research and veterinary diagnostic labs. Single point of contact between coordinating partner 1 and Brussels (●).

Improved Immunity of **Aqu**acultured **Anim**als. IMAQUANIM

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