

## Protective immunity in fish against protozoan diseases

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**Abstract.** The demand for and costs of producing land-based animal protein continues to escalate as the world population increases. Fish is an excellent protein, but the catch-fishery is stagnant or in decline. Intensive cage culture of fish is a viable option especially in countries with lakes/ivers and/or a long coastline; however, disease outbreaks will likely occur more frequently with cage culture. Hence protective strategies are needed, and one approach is to exploit the piscine immune system. This discussion highlights immunity (innate/natural and adaptive/acquired) in fish against three pathogenic protozoa (*Amyloodinium ocellatum*, *Ichthyophthirius multifiliis* and *Cryptobia salmositica*). Histone-like proteins in the mucus and skin of naturally resistant fish kill trophonts of *A. ocellatum*, and also may cause abnormal development of tomonts. Breeding of *Cryptobia*-resistant brook charrs is possible as resistance is controlled by a dominant Mendelian locus, and the parasite is lysed via the Alternative Pathway of Complement Activation. Production of transgenic *Cryptobia*-tolerant salmon is an option. Recovered fish are protected from the three diseases (acquired immunity). Live *I. multifiliis* theronts injected intraperitoneally into fish elicit protection. Also, a recombinant immobilizing-antigen vaccine against ichthyophthiriosis has been developed but further evaluations are necessary. The live *Cryptobia* vaccine protects salmonids from infections while the DNA-vaccine stimulates production of antibodies to neutralize the disease causing factor (metalloprotease) in cryptobiosis; hence infected fish recover more rapidly.

**Key words:** pathogenic protozoa; innate and adaptive immunity; selective breeding; vaccines, antibodies, cell-mediated cytotoxicity

The demand for animal protein continues to escalate as the world population increases to about 8 billion by 2020. This will exert additional pressures on food production and will also compete with other human activities (e.g. transportation, housing, industry) for the limited usable land. Animal protein contains essential amino acids which are important components of a well-balanced diet. Free ranging land animals are no longer a significant source of protein, and the production costs of farm animals continue to escalate. To increase efficiency and to reduce production costs farms are large and are close to human habitations. However, the large scale breeding of mammals and birds not only further pollute the environment it also increases the risks of disease outbreaks in the animals and the subsequent interspecies transmission of zoonotic diseases (e.g. Nipah virus in pigs, avian influenza H5N1 virus in birds, cryptosporidian parasites in cattle) to humans (Woo, 2006a).

Fish is an excellent protein (e.g. the beneficial polyunsaturated fatty acids in marine fish), however the traditional capture-fishery is stagnant or in decline. In many areas natural fish stocks have been reduced significantly due to over and/or indiscriminate fishing, and the

loss and/or destruction of spawning grounds. Also, industrial wastes (e.g. heavy metals, organophosphates) discharged into the aquatic environment can reduce fish growth, survival and reproduction, and in some areas pollutants have accumulated in fish to the extent they are no longer suitable for human consumption. Aquaculture is a good option as start-up and production costs are lower and it does not have many of the problems associated with the raising of warm-blooded animals. Intensive culture of freshwater and marine fishes in cages is one solution to producing affordable animal protein. This is especially important in countries with limited land but have large numbers of lakes/ivers and or with long coastlines. However, outbreaks of diseases occur more frequently; e.g. intensive aquaculture facilitates transmissions of infectious pathogens. Protective strategies have to be developed, and one approach is to exploit the piscine immune system (Woo, 2006a).

Animals are constantly exposed to infectious organisms, hence they have evolved a very efficient immune system. The system in vertebrates is complex and well-integrated, and it consists of two basic components - innate (natural) and adaptive (acquired) immunity. The chief differences between them are immunologic specificity and memory (anamnesis), and these are only in adaptive immunity (Stites and Terr, 1991). The piscine immune system (Van Muiswinkel and Vervoom-Van Der Wal, 2006) is similar to that in mammals and it also has a comparable set of immunocompetent cells (Ardelli and Woo, 2006). In general, adaptive response is slower to develop in fish than in mammals and this is

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partly due to lower body temperatures; however, their innate immunity is as well developed as in mammals. The present discussion is to highlight the immune responses of fish to three common and well-studied pathogenic protozoa (*Amyloodinium ocellatum* - an ecto-parasitic dinoflagellate; *Ichthyophthirius multifiliis* - a sub-epidermal ciliate; *Cryptobia salmositica* - a blood flagellate). They are selected because they are taxonomically unrelated and infect a wide range of warm-water and cold-water fishes from different geographical localities. Part I is on aspects of the biology of the pathogens relevant to the present discussion while Part II is on protective immunity (innate and adaptive protection) in fish to the three pathogens and the development of protective strategies against the parasites.

### Part I. Biology of Parasitic Protozoa

(a) *Amyloodinium ocellatum* is an important pathogen in warm-water mariculture. This ecto-parasite is not host specific and it infects both elasmobranchs and teleosts fishes. It has a direct and relatively simple life cycle – the trophont (pear-shaped to ovoid) is the parasitic stage and it has rhizoids to attach itself to host epithelia cells. Mature trophonts detach themselves from the fish and they give rise to free-living reproductive tomites in the aquatic environment. Division occurs within encysted tomites to produce large numbers of dinospores that are released into the water column. The free-swimming dinospores are infective to fish and after attachment they start the infection. Fish that have recovered from an infection are presumed to be protected from re-infection (Noga and Levy, 2006). (b) *Ichthyophthirius multifiliis* is the most pathogenic ciliate of freshwater fishes. It is not host specific and has been found in fishes from the tropics to the sub-Artic. This histophagous parasite has a direct life cycle and the ciliated infective stage (theront) in the water column attaches to the epithelium of the fish and penetrates to the basal layer of the epidermis within minutes. It develops into a ciliated trophont and creates a space in the epithelial layer as it feeds. Mature trophonts leave the fish and settle on the substrate as free-living tomites. Divisions occur within tomites to give rise to ciliated tomites. Large numbers of tomites are released into the aquatic environment where they differentiate into infective theronts. Fish that have recovered from ichthyophthiriosis are protected from the pathogen (Dickerson, 2006). (c) *Cryptobia salmositica* is a blood flagellate and it has been reported in all Pacific salmon *Oncorhynchus* spp. along the west coast of North America. The parasite is normally transmitted from fish to fish by the freshwater leech *Piscicola salmositica* in streams and rivers. It multiplies in the crop of the leech, and is inoculated when the vector feeds. Direct transmission

between fish (in the absence of leeches) occurs under certain aquaculture conditions. Severe outbreaks of cryptobiosis have occurred in salmon maintained in sea cages and in freshwater hatcheries. The prevalence of the parasite in juvenile salmon in streams is highly variable (e.g. from 3-21%) and fingerlings have detectable infections in the autumn and winter. Sexually mature salmonids become infected within days of returning to fresh water from the marine environment and many have heavy infections when they spawn or die before spawning. The parasite multiplies rapidly by binary fission in the blood of fish and severity of cryptobiosis (e.g. anaemia) and mortality are directly related to parasitaemias. The virulent factor is a 200 kDa metalloprotease secreted by the pathogen. Fish that have recovered from salmonid cryptobiosis are protected from the pathogen. (Woo, 2003; 2006b).

### II. Protective Immunity Innate (or natural) Immunity

Innate immunity is non-specific response to pathogens. The body surface/integument is the first line of defence and it is an important physical barrier against the attachment of and penetration by parasites. Some skin secretions contain lysozymes that may damage the surface membrane of parasites while others (e.g. mucus) block the adherence of parasites to epithelial cells and trap them to be got rid off later. If a pathogen bypasses one or more of these barriers then it encounters other elements of the innate response, and these include histone-like proteins, complement, phagocytic cells, non-specific cytotoxic cells, and  $\alpha 2$  macroglobulins. A distinction is made here to distinguish between two forms of innate resistance: resistance to disease in infected fish (pathogen-tolerant), and resistance to infection (pathogen-resistant).

#### (a) *Amyloodinium ocellatum*

Although most fish species can be infected there are some that are more resistant to infection, and they include killifish *Fundulus grandis*, American eel *Anguilla rostrata* and molly *Poecilia latipinna*. Resistant-fish generally produce 'thick mucus' and are able to tolerate low oxygen levels; however the mechanism of resistance is not known (Lawler, 1977).

Trophont are highly sensitive to histone-like proteins (HLPs) while dinospores are not. These histone-like proteins are small (13-21 kDa) and they occur in high concentrations in the skin and gills of some fish including the hybrid striped bass (*Morone saxatilis* X *M. chrysops*). Some antibiotic activities of HLPs are in the mucus but most of the killing power of the proteins is in the epidermal compartment of the skin (Noga *et al.*, 2001). HLPs are also known to cause delayed and/or abnormal development of free-living tomites. Normal-looking parasites may die during

development after leaving the host and this type of delayed innate 'defence' against a pathogen is rather unusual (Noga *et al.*, 2002).

(b) *Ichthyophthirius multifiliis*

Little is known about innate immune response. In light infections epithelial cells form a capsule around the parasite, and in heavy or repeated infections there are extensive epithelial proliferations with increase infiltrations of granulocytes, macrophages and lymphocytes (Ventura and Paperna, 1985). It was hypothesized this epithelial proliferation would interfere with subsequent penetration by theronts. Also, Graves *et al.* (1984) found non-specific cytotoxic lymphocytes on the skin of infected fish, and they (Graves *et al.*, 1985a; 1985b) suggested these non-specific lymphocytes leave the blood circulation for the skin where they are involved in killing immobilized theronts. Also, *in vitro* studies indicate the Alternative Pathway of Complement Activation may be involved in immobilization and lysis of theronts (Buchmann *et al.* 2001).

(c) *Cryptobia salmositica*

The vaccine strain (Woo and Li 1990) multiplies in the blood of Atlantic salmon *Salmo salar* and circulating leukocytes are activated at 3-7 weeks post vaccination (wpv). The percentage of activated cells peak at 4 and 5 wpv which corresponds to rising antibody titres, and the percentage of activated leukocytes decline from 5-8 wpv (Chin and Woo, 2005).

(i) Cryptobia-resistant fish: Some naïve non-salmonids (Wehnert and Woo, 1980) and brook charr *Salvelinus fontinalis* (Forward *et al.*, 1995) cannot be infected with the parasite. The resistance to *Cryptobia* infection in charrs is controlled by a dominant Mendelian locus and it is inherited by progeny (Forward *et al.*, 1995). Briefly, the pathogen is lysed in the blood via the Alternative Pathway of Complement Activation in *Cryptobia*-resistant charrs (Forward and Woo, 1996). Little is known about this type of immunity; further studies on the inheritance of natural resistance by progeny would be rewarding as it could be exploited to protect fish against pathogens (Woo, 1998).

(ii) Cryptobia-tolerant fish: Parasitaemias in some *Cryptobia* infected brook charr are as high or higher than those in *Oncorhynchus* spp., but the charrs do not have cryptobiosis (i.e. *Cryptobia*-tolerant). There are no detectable differences in the biology (e.g. growth) and immune responses (humoral and cell-mediated immunity) of *Cryptobia*-tolerant and *Cryptobia*-resistant charrs to antigenic stimulations (Ardelli and Woo, 1995). *Cryptobia*-tolerant charrs do not suffer from cryptobiosis because the metalloprotease secreted by the parasite is neutralized by the natural anti-protease ( $\alpha 2$  macroglobulin) in the blood. The amount of  $\alpha 2$  macroglobulin is higher in charrs than in rainbow trout *Oncorhynchus mykiss* prior to infection and it remains high (about 40%) even during acute infec-

tions; however, in infected rainbow trout it drops to about 12%. Neutralization of the metalloprotease by the  $\alpha 2$  macroglobulin was demonstrated under both *in vivo* and *in vitro* conditions (Zuo and Woo, 1997a; 1997b; 1997c). Since *Cryptobia*-tolerant charrs do not have disease they recover more rapidly from the infection. An option is to produce transgenic *Cryptobia*-tolerant salmon and they like *Cryptobia*-tolerant charrs will be able to maintain a high level of  $\alpha 2$  macroglobulin during infection. This novel approach to manage an infectious disease does not need further human interventions (e.g. vaccination, chemotherapy) once the transgenic animals are produced (Woo, 2001)

### Acquired (Adaptive) Immunity

(a) *Amyloodinium ocellatum*

Fish immunized with dinospores had agglutinating antibodies against live dinospores in their sera, and the sera killed the parasite in cultures (Smith *et al.*, 1993). Also, antibodies were detected in the blood of hybrid striped bass that had recovered from natural infections (Smith *et al.*, 1994). Experimental studies showed that clownfish *Amphiprion frenatus* given weekly sub-lethal exposures to the parasite developed significant immunity to infection in about a month. This protection lasted at least 6 months and it appeared to be directed against trophonts (Cobb *et al.*, 1998a) and the protection was associated with the production of antibodies (Cobb *et al.*, 1998b). It was suggested that local antibody in the skin was more important than serum antibody as protection was still present when serum antibody was not detectable in immune fish.

(b) *Ichthyophthirius multifiliis*

Fish that survived either an infection (e.g. Hines and Spira, 1974; Ling *et al.*, 1993) or treated with drugs during an infection (e.g. Clark *et al.*, 1988; Leff *et al.*, 1994) were protected. The mechanism of protection in immune fish is not well understood. Cross and Matthews (1992) showed that theronts readily penetrated the skin of naïve and immune fish; however, theronts left immune fish within a couple of hours (evasion strategy by the parasite) but stayed on to colonize and produce disease in naïve fish. This was proposed as one mechanism operating in immune fish (Clark and Dickerson, 1997). Other suggestions include humoral and/or cell-mediated immunity as antibodies from immune fish immobilize theronts. Immobilizing antibodies are in the blood and mucus on the body surface of immune carp *Cyprinus carpio* (Hines and Spira, 1974), rainbow trout (Wahli and Meier, 1985), and catfish *Ictalurus punctatus* (Clark *et al.*, 1987). Also, cell-mediated immune response to the parasite is in infected carp (Houghton and Matthews 1986, 1990; 1993; Cross and Matthews, 1993) and in goldfish *Carassius auratus* (Sin *et al.*, 1996).

Immobilizing antigens (i-antigens; 40-70 kDa) are associated with protection and they react with specific antibodies. These surface antigens are also used to serotype parasite isolates. Serotypes are distinguished using monoclonal antibodies that bind to specific i-antigens to immobilize theronts (Dickerson *et al.*, 1993). Five serotypes have been identified, and they include serotype 'A' which has 3 expressed epitopes (56, 46 and 42 kDa) while serotype 'D' has only a single 55 kDa i-antigen (Wang *et al.* 2002). There seem to be differences in virulence between serotypes; e.g. isolate NY1 (serotype 'A') kills 100% of infected catfish while fish mortality is about 49% with isolate G5 which is a serotype 'D' (Swennes *et al.*, 2006). Purified i-antigens of the two serotypes (NY1 and G5) produced high titres of immobilizing antibodies in catfish. The antisera only immobilized homologous parasites but not the heterologous strain. When vaccinated catfish were challenged with the pathogen the survival rates varied from 33-70%. The study supports the suggestion that i-antigens play an important role in protection (Wang *et al.*, 2002). Gaertig *et al.* (1999) successfully expressed the 48 kDa i-antigen on the surface membrane of *Tetrahymena thermophila* and this study has opened a novel approach to using the free living ciliate to produce and deliver recombinant protein vaccines to fish. A recombination i-antigen vaccine has been developed (He *et al.*, 1997) and preliminary results are encouraging. Briefly, a 316 bp gene fragment of the i-antigen was assembled from six oligonucleotides and expressed in *Escherichia coli* with the production of recombinant protein. Specific antibodies were detected in fish after they were inoculated with the protein, and 95% of vaccinated goldfish survived a parasite challenge. More conclusive work needs to be done as 50% of the vaccinated-challenged fish had "heavy" infections and 55% of the controls survived the parasite challenge.

An intraperitoneal injection of live theronts into fish protected them from infection (e.g. Burkart *et al.*, 1990; Sin *et al.*, 1996; Xu *et al.*, 2004). Recent studies confirmed this in catfish, and there was cross protection between serotypes. Also, the efficacy of the theronts as a candidate vaccine varied between serotypes. Catfish vaccinated with live G12 theronts were completely protected against both G12 and G5 while vaccination with G5 only conferred partial protection against both serotypes. Lysates of trophonts did not confer protection when injected into fish (Swennes *et al.*, 2007).

(c) *Cryptobia salmositica*:

Antibody production (e.g. Woo, 1990; Sitja-Bobadilla and Woo, 1994) and cell-mediated immunity (e.g. Thomas and Woo, 1990; Mehta and Woo, 2002) are detectable in salmonids at about 2 weeks after infection. Juvenile and adult fish that have recovered from the infection are protected from infection and their antisera contain agglutinating, neutralizing and complement fix-

ing antibodies (Woo, 2001; 2003).

Two experimental vaccines (a live attenuated *Cryptobia* vaccine and a DNA-vaccine) have been developed. The live vaccine protects (antibodies and cell-mediated cytotoxicity) fish from infection while the DNA-vaccine does not prevent infection. Antibodies produced in the DNA-vaccine fish neutralize the metalloprotease (secreted by the pathogen) and hence reduces the severity of the disease and infected fish recover earlier. (i) Live Vaccine: The attenuated live vaccine infects and produces low parasitaemia in rainbow trout. It does not cause disease, circulates in the blood for at least 6 months, and protects 100% of the vaccinated fish from re-infection (Woo and Li, 1990). In trout partial protection occurs at 2 weeks post vaccination and full protection is at 3-4 weeks post vaccination (Li and Woo, 1995). A single dose of the vaccine protects fish for at least 24 months (Li and Woo, 1997), and it has no detectable bioenergetic cost to juvenile rainbow trout (Beamish *et al.*, 1996). It is used routinely to study the acquisition of protective immunity as the vaccine protects 100% of juvenile and adult salmonids from the pathogen (e.g. Sitja-Bobadilla and Woo, 1994; Li and Woo, 1995; Feng and Woo, 1997a, 1998b; Ardelli and Woo, 2002; Mehta and Woo, 2002; Chin *et al.*, 2004).

Fish vaccinated in fresh water and transferred to sea water were still protected (Li and Woo, 1997). The complement fixing antibody titres (e.g. Li and Woo, 1995; Ardelli and Woo, 1997) and cell-mediated response (Mehta and Woo, 2002) in vaccinated fish rose significantly soon after parasite challenge (a classical secondary response). Protection is through the production of complement fixing antibodies and enhanced cell-mediated cytotoxicity (antibody-independent and antibody-dependent). Also, macrophages from vaccinated fish are much more efficient in engulfing live parasites under *in vitro* conditions, especially with antibodies (Li and Woo, 1995).

(b) DNA Vaccine: A recombinant metalloprotease protein and a recombinant metalloprotease-plasmid vaccine (DNA-vaccine) have been produced (Tan, 2005). Trout injected intramuscularly with the DNA-vaccine had a slight anaemia during the first 3-4 weeks post vaccination (wpv), and had detectable agglutinating antibodies against *Cryptobia* between 5-7 wpv. These antibodies likely react like mAb-001, a monoclonal antibody against a 200 kDa surface membrane glycoprotein on the parasite (Feng and Woo, 1996; 1998). mAb-001 inhibits the enzymatic activity (Zuo *et al.*, 1997) of purified metalloprotease (Zuo and Woo, 1998), and inhibits parasite multiplication and aerobic respiration (Feng and Woo 1996; Hontzas *et al.*, 2001). It is also prophylactic and therapeutic against *Cryptobia* when injected into fish (Feng and Woo, 1997b). Fish injected with the DNA-vaccine had lower

parasitaemia when challenged, delayed peak parasitaemia and faster recovery (Tan, 2005). Antibodies from vaccinated fish also agglutinated an unrelated pathogen (*Spironucleus* sp.) from chinook salmon. This cross-reaction indicates that the *Cryptobia* DNA-vaccine may have the potential to be a broad-spectrum vaccine.

## Conclusions

Although the piscine immune system has been well studied (Van Muiswinkel and Vervoom-Van Der Wal, 2006; Ardelli and Woo, 2006) relatively little is known about protective mechanisms in fish against parasitic organisms. However, what is known is encouraging as it indicates that immunological strategies can be developed to protect fish from pathogenic protozoa once the protective mechanism(s) has been elucidated. Histone-like proteins in the mucus and skin of naturally resistant fish (innate immunity) kill trophonts of *A. ocellatum*, and/or cause abnormal development of tomonts. In *Cryptobia*-resistant fish the parasite is lysed via the Alternative Pathway of Complement Activation. Selective breeding of *Cryptobia*-resistant brook charrs is possible as the resistance to infection is controlled by a dominant Mendelian locus. Also, the production of transgenic *Cryptobia*-tolerant salmon (infected but with no clinical disease) is an option.

In adaptive immunity fish are protected from ichthyophthiriosis when they are injected intraperitoneally with live *I. multifiliis* theronts. Also, a recombinant i-antigen vaccine against *Ichthyophthirius* has been developed but further evaluations are needed. The successful expression of the 48 kDa i-antigen on *Tetrahymena thermophila* has opened a novel approach to using the ciliate to produce and deliver recombinant protein vaccines to fish. There are two experimental vaccines against cryptobiosis – the live *Cryptobia* vaccine readily protects juvenile and adult salmonids from infection for at least 24 months. However, the DNA-vaccine does not prevent infections but it stimulates fish to produce antibodies to neutralize the virulent factor (metalloprotease) in cryptobiosis and hence infected fish recover faster.

Hopefully gaps in our knowledge are obvious in the current review and that they will stimulate further research which will include elucidation of protective mechanisms against parasites with the eventual development of novel strategies against pathogens in fish.

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